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International Journal of Secondary Metabolite (IJSM) purposes the publication of articles related to secondary metabolites of plant and allied organisms (algae, fungi, and lichens). IJSM is open-access, peer-reviewed academic journal published electronically and quarterly. The journal is the goal to improve the research culture and help knowledge spread rapidly in the academic world by providing a common academic platform in the scope. The journal is published in English.

IJSM is published 4 issues per year (March, June, September, December), and accepting manuscripts related to secondary metabolites of plant and allied organisms (algae, fungi, and lichens). Research areas covered in the journal are phytochemistry, biochemistry, biotechnology, ethnopharmacology, biological and pharmacological activities (antimicrobial activity, antioxidant activity, antiulcer activity, anti-convulsant activity, anti-anxiety activity, antidiabetic activity, anti-gout activity, antiprotozoal activity, anti-inflammatory activity, antispasmodic activity, antiparasitic activity, anti-mutagenic activity, anticholinesterase activity, antidepressant activity, hepatoprotective activity, anti-anxiety activity, anti-convulsant activity, anti-spasmolytic activity, anticancer activity). IJSM welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Authors are required to frame their research questions and discuss their results in terms of major questions in plant biology. Contribution is open to researchers of all nationalities. The following types of articles will be considered:

1. Research articles: Original research in various fields of plant and allied organisms (algae, fungi, and lichens) will be evaluated as research articles.

2. Research notes: These include articles such as preliminary notes on a study or manuscripts new records on secondary metabolites.

3. Reviews: Reviews of recent developments, improvements, discoveries, and ideas in various fields of plant and allied organisms (algae, fungi, and lichens) will be requested by the editor or advisory board.

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Research Article

Phytochemical constituents from corn silk and antimicrobial activity of the isolates

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Abstract: Corn silk (*Stigma maydis*) is one of the traditional medicines for treating many microbial infections. However, there is little literature on the bioactive compounds responsible for these activities. This study was designed to investigate the phytochemical constituents present in corn silk and to screen the isolated compounds for antimicrobial activity. The pulverized plant sample of 1.14 kg was extracted with 3.6 L of methanol by cold maceration for 3 days. The extract was screened for phytochemicals, followed by isolation of constituent phytochemicals, characterization, and identification of isolated compounds. The isolates were screened for antibacterial and antifungal activities. The phytochemical screening revealed the presence of alkaloids, flavonoids, coumarins, tannins, reducing sugars, saponins, terpenoids, sterols, and cardiac glycosides. Further phytochemical investigation of the chloroformic subfraction of the methanolic extract of the silk led to the isolation of behenic acid and stigmasterol after running column chromatography as well as other chromatographic methods. The identity of the isolated compounds was established based on extensive spectroscopic analyses of their IR, 1D, 2D NMR data and comparing the data to the reported literature. Stigmasterol was active against *Staphylococcus aureus* and the fungal strain *Candida albicans* at 25 µg/mL while the mean minimum inhibitory concentration of behenic acid against *Staphylococcus aureus was* 100 µg/mL, *Tinea corporis* and *Klebsiella pneumoniae* were susceptible at 25 µg/mL. The study showed that plant secondary metabolites might be responsible for the reported biological activities of corn silk. It is the first report of behenic acid isolated from corn silk.

1. INTRODUCTION

In Ghana, maize (*Zea mays* L.,) is a dominant cereal in terms of cultivation and use. It is cultivated in all the agro-ecological zones in Ghana (Ragasa *et al*., 2014). The maize plant belongs to the family Poaceae. It contains husk, corn, and silk. Corn silk (*Stigma maydis*) is the shiny, soft thread-like, weak fibers about 10-20 cm long, which grow as part of the ears of corn and are enclosed in the husk. It is dark brown when dry (Fazilatun *et al*., 2012). Corn silk is the

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stigma of the plant, a waste from corn production, and it is very abundant (Maksimovic *et al*., 2004).

Medicinally, corn silk has been documented to possess the following activities: antioxidant (Eman, 2011; Ebrahimzadeh *et al*., 2008; Liu *et al*., 2011), antiprostatitis (Buhner, 2007), anti-diabetic (Guo *et al*. 2009), antiplasmodial (Tordzagla *et al*., 2022), antitumor (Habtemariam, 1998) and antiproliferative activities (Abirami *et al*., 2021). Moreover, corn silk has been reported for treating infection and cystitis (Steenkamp, 2003). In addition, it has been reported as a drug for treating kidney stones and other renal illnesses (Ribeiro *et al*., 1988; Tahraoui *et al*., 2007). Furthermore, hepatitis, tumor, hyperglycemia, and hypertension are treated traditionally using dry corn silk in China and some African countries (Li *et al*., 1995; Lukitaningtyas *et al*., 2020; Ma and Gao, 1998). It also possesses diuretic activity and immune-enhancing effects (Tang & Ding, 1995). Clinically, it is very potent against cystitis, prostatitis, urethritis, nephritis, and urinary infections (Yesilada *et al*., 1995; Grases *et al*., 1993). The medicinal properties of plants reside in the natural bioactive constituents in the plants. Natural bioactive constituents (phytochemicals) found in plants help to sustain plant life by acting as defense systems against diseases (Krishnaiah *et al*., 2007). These phytochemicals are namely primary and secondary phyto-constituents. Primary phyto-constituents are amino acids, proteins, sugars, and chlorophyll while examples of secondary ones are flavonoids, alkaloids, and terpenoids (Krishnaiah *et al*., 2007). Pharmacologically, flavonoids have antioxidant properties (Hollman, 2001) and a wide spectrum of antimicrobial activities, (Hanasaki *et al*., 1994). Isolated alkaloid compounds have *in vitro* and *in vivo* antiplasmodial activities (Nogueira & Lopes, 2011) while terpenoids are used to treat *Candida albicans* infection (Zore *et al*., 2011).

The silk of *Zea mays* was chosen for this study because it has been used extensively in traditional medicine and it is readily available. However, there is scanty literature on the bioactive compounds responsible for their reported medicinal activities, hence, the need for a phytochemical investigation of corn silk, also to screen the isolated compounds for antimicrobial activity.

2. MATERIAL and METHODS

2.1. Instruments/Equipment

The following equipment was employed; Rotavapor (Buchi, R-210) with V-710V(vacuum pump); water bath (Buchi, B-491); melting point apparatus (R000105350) from Stuart, UK/; balance Kern Sohn GmbH capacity =1000 g, D-72336 (Balingen, Germany). Jeol ECA, 500 MHz FT NMR spectrometer(NM/103508-10), Japan incorporating a NM50TH5T/FG2 probe; PerkinElmer ATR-FTIR spectrometer(A/0626/15) Waltham, USA; gravity column L 25 mm x 51 cm, Sigma-Aldrich, Merck Germany; pre-coated TLC plate (Macherey-Nagel, Germany)

2.2. Chemicals

The chemicals used included the following: methanol (BDH Chemical, UK), ethanol (Fischer Scientific, UK), chloroform (BDH Chemicals, UK), ethyl acetate (BDH Chemicals, UK), acetic acid (Needham Market Suffolk, UK), petroleum ether (VWR Chemicals, U.S.A.), hexane (BDH Chemicals, UK) and silica gel 70:230 mesh size (Merck, Germany).

2.3. Collection and Identification of Plant Material

Stigmas of *Zea mays* (corn silk) were collected at Ejisu in the Ashanti Region of Ghana in August 2016 then identified and authenticated by Mr. Clifford Osafo Asare, the horticulturist with the Department of Herbal Medicine, KNUST and a specimen was placed in the herbarium of the Department with a voucher number KNUST/HM/2016/001.

2.4. Extraction of Plant Sample

The dry corn silk was powdered by an electronic grinding mill and was extracted using methanol (CH_3OH). A 1.14 kg of the milled sample was cold macerated with 3.6 L of CH_3OH

for 3 days. After 72 hours, a whatman filter paper was used to separate the residue from the filtrate. The filtrate was concentrated to dryness using rotavapor and water bath at 40 ^{o}C.

Next, 10 g of the dry extract was first dissolved in 300 mL of distilled water, then poured into a separatory funnel and partitioned successively with 300 mL x 3 volumes of chloroform and ethyl acetate, respectively. The two organic fractions were also concentrated to dryness using the rotavapor. A freeze dryer was, however, used to dry the aqueous fraction. The concentrated chloroform fraction was used for the isolation of phytochemical constituents. Before the fractionation, phytochemical screening was done on the methanolic extract.

2.5. Phytochemical Screening

The methanolic extract was screened for possible phytochemicals by employing standard methods (Sofowora, 1993; Evans, 2002; Harborne, 1998).

2.5.1. *Test for flavonoids*

A preliminary test was done using a strip of filter paper. The paper was dipped in the liquid extract, subsequently dried, and exposed to a 2 M ammonia solution. A deep yellow color was formed, and the color disappeared after the filter paper was exposed to fumes of concentrated HCl, showing the presence of flavonoids.

For the confirmatory test, 2 mL of ethanol was used to dissolve about 0.4 g of the extract and 5 drops of concentrated HCl and magnesium turnings were added. A pink color developed, which confirmed the presence of flavonoids (Evans, 2002).

2.5.2. *Test for alkaloids*

The extract was treated with ammoniacal alcohol (ammonia: 95 % ethanol in the ratio of 1: 9 respectively) and filtered. The filtrate was evaporated, and 1% sulphuric acid was added to the residue to convert alkaloids to soluble salt forms. After filtration, the resulting solution was made alkaline with dilute ammonia partitioned in a separating funnel with chloroform, and then shaken for a few minutes. The chloroform layer was separated, and the filtrate evaporated, followed by the addition of 1% H_2SO_4 to the respective residue. Dragendroff's and Mayer's tests were carried out on the acidified residue (Evans, 2002).

2.5.2.1. Dragendroff's test. A solution of potassium bismuth iodide was added to the filtrate. The formation of red precipitate indicated the presence of alkaloids (Evans, 2002).

2.5.2.2. Mayer's test. The filtrate was treated with a solution of potassium mercuric iodide. The presence of alkaloids was confirmed by the appearance of a cream precipitate (Evans, 2002).

2.5.3. *Test for saponins*

About 2 mg of the extract was dissolved in 2 mL of distilled water and the mixture was shaken. Formation of a foam column not less than 1cm in height that persisted for at least 15 min, confirmed the presence of saponins (Evans, 2002).

2.5.4. *Tannins*

About 5 drops of iron (III) chloride ($FeCl_3$) solution were added to 2 mL of the extract. The appearance of blue-black color indicated the presence of tannins (Evans, 2002).

2.5.5. *Test for reducing sugars*

The methanolic extract was hydrolyzed with dilute HCl and then treated with 20 % NaOH. Finally heated with Fehling's solutions A and B. The appearance of brick-red precipitate confirmed the presence of reducing sugars (Sofowora, 1993; Evans, 2002).

2.5.6. *Test for coumarins*

The extract was first treated with chloroform and filtered. The filtrate was evaporated to dryness and hot distilled water was used to dissolve the residue and allowed to cool. The resulting solution was treated with 0.8 mL of 10 % ammonia and observed under UV light at 365 nm. The formation of a deep blue-green fluorescence under UV light indicated the presence of coumarins (Sofowora, 1993; Evans, 2002).

2.5.7. *Test for triterpenes/ terpenoids (Salkowski's test)*

A sample was extracted with chloroform and filtered. About 5 drops of concentrated H_2SO_4 were added to the filtrate and mixed thoroughly after shaking for a few minutes and allowed to stand. A brownish-red coloration showed the presence of triterpenes or terpenoids (Sofowora, 1993; Evans, 2002).

2.5.8. *Test for sterols (Libermann Burchard's test)*

Chloroform was added to a sample and filtered. The filtrate was then treated with about six drops of $(CH_3CO)_2O$ and boiled, then allowed to cool. A few drops of concentrated H_2SO_4 were then added and reacted to form a bluish-green coloration at the interface, which confirmed the formation of a steroidal ring (Evans, 2002; Harborne, 1998).

2.5.9. *Cyanogenetic glycosides*

About 0.2 g of the extract was placed in a dry test tube and a strip of sodium picrate paper was suspended using a cork in the neck of the test tube. The test tube and its contents were heated in a water bath. The color of the test paper changed to brownish red to indicate the presence of cyanogenetic glycosides because of the release of hydrocyanic acid (Sofowora, 1993; Evans, 2002).

2.5.10. *Cardiac glycosides*

A sample of the extract was treated with 70 % alcohol and filtered. About 10 mL of alcoholic filtrate was added to 2 mL of anhydrous CH_3COOH with 5 drops of iron (III) chloride solution. Concentrated H_2SO_4 was gently added by pouring it down the sides of the test tube using a dropping pipette. A reddish-brown coloration at the interface because of the presence of aglycone was observed. Hence, the presence of cardiac glycoside was confirmed (Evans, 2002).

2.6. Isolation of Compounds from Chloroform Fraction of the Methanol Extract of Corn Silk

The dry chloroform fraction of mass 10.0 g was dissolved in about 30 mL of chloroform with a small amount of the silica gel gradually added and stirred continuously to dryness. The homogeneous mixture was loaded onto the packed column ensuring uniform packing by gently tapping the side of the column. To prevent the mobile phase from disturbing the surface during solvent addition, a wad of cotton was placed on top of the packed sample in the column. The gradient elution was employed in developing the column using petroleum ether, ethyl acetate, and methanol. Elution was first started with 100 % petroleum ether. This was followed by petroleum ether/ethyl acetate, 100 % ethyl acetate, and ethyl acetate/ methanol. Various sub-fractions were collected and sub-fractions with similar TLC profiles were bulked and concentrated. A total of four distinct sub-fractions (C1-C4) were obtained.

Fraction C2 was eluted with petroleum ether /ethyl acetate (95:5) and precipitated as white-to-cream crystals after decanting. The solid obtained was thoroughly washed with 100 % petroleum ether to give colorless crystals labeled as compound **1** (38 mg).

Fraction C3 was obtained by eluting the column with petroleum ether /ethyl acetate solvent system (85:15) and precipitated as a colorless powdered solid after decanting off the green mother liquor. The colorless powder was thoroughly washed with pure petroleum ether, followed by 100 % methanol to obtain compound **2** (87 mg).

2.7. Identification and Structural Elucidation of the Isolates

The purity and identity of the isolates were confirmed using melting point determination, qualitative test, infrared spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, and mass spectroscopy.

2.8. Melting Point Determination

Melting point data were obtained using a melting point apparatus with a capillary tube. The melting point range of the isolated compound was read as degrees centigrade (^{0}C). The reading was done thrice.

2.9. Chemical Tests

Investigations such as tests for alcohol and steroid were carried out on compound **2**.

2.9.1. *Test for alcohol*

About 2 g of $(NH_4)_2Ce(NO_3)_6$ was dissolved in a boiling tube containing 5 mL of 2 M nitric acid and then heated gently. A small quantity, about 5 mg of isolated compound **2**, was then dissolved in 1 mL of 1,4-dioxane. The resulting solution was added to 1 mL of $(NH_4)_2Ce(NO_3)_6$ and swirled. A yellow to red color developed indicating the presence of an alcohol functional group (Harborne, 1998).

2.9.2. *Test for steroid (Salkowski reaction)*

A little quantity of about 7 mg of the isolated compound **2** was dissolved in $CHCl_3$ and five drops of concentrated tetraoxosulphate (Vl) acid were added to the resulting solution and a reddish color was formed in the upper $CHCl_3$ layer (Evans, 2002).

2.9.3. *Liebermann-Burchard reaction*

A small quantity of about 7 mg of compound **2** was dissolved in 5 mL of $CHCl_3$ and about five drops of concentrated H_2SO_4 were added to it. Also, about three drops of $(CH_3CO)_2O$ were added. The steroidal ring formation was confirmed as a result of the development of a bluish-green coloration at the interface (Evans, 2002).

2.10. Infrared Spectroscopy

A Perkin-Elmer Fourier Transform Infrared (FTIR) Spectrophotometer (Waltham, USA/A/0626/15) which utilizes attenuated total reflectance with internal calibration was used in the infrared spectrophotometric analysis. About 2 mg of each isolate was put on top of the diamond crystal on the attenuated total reflectance plate and the probe was used to compress it. The infrared (IR) was measured over the wavelength range of 4000 to 400 cm^{-1}. It facilitated the identification of the functional groups in the isolated compounds.

2.11. Nuclear Magnetic Resonance (NMR) Spectroscopy

About 10 mg of each isolate was used in the NMR analysis for 1D and 2D spectra. The samples were dissolved in deuterated chloroform ($CDCl_3$) in a sample vial. The sample was vortexed and filtered into the NMR spectrophotometer tube and ran. Tetramethylsilane (TMS) was used as the internal standard. The ^{1}H NMR and ^{13}C NMR spectra were recorded at 500 MHz and 125 MHz, respectively.

2.12. Test Organisms for Antibacterial and Antifungal Assays

The organisms for the assays were obtained from the Department of Biological Sciences, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana. They included Gram-negative, Gram-positive, and fungal strains.

2.13. Inoculum Preparation of Test Microorganisms

The test bacteria and fungi strains were streaked on 20 mL sterile nutrient agar and Sabouraud dextrose agar plates, respectively. They were incubated at a temperature of 37 ^{o}C and their colonies were fished and suspended in sterile water of 10 mL in test tubes. The turbidity was

compared to 1.0 × 1 10^8 cells/mL, which is the same as 0.5 McFarland standard, and read with the eye.

2.14. Minimum Inhibitory Concentration (MIC) Determination for the Isolates

Minimum inhibitory concentrations of the test compounds were determined by the microdilution method using the method described by Andrews, (2001). Different concentrations of the test solutions of the compounds were prepared ranging from 12.5 to 400 µg/mL. The double strength-nutrient broth of 125 µL was used to fill 96-well (micro-titre plates). A microbial inoculum size of 25 µL was standardized with 0.5 McFarland turbidity solution added to each well. The antimicrobial activity of the standard drugs, as well as the test compounds, was determined against test organisms after incubating at 37 °C. After incubation for 24 h, the minimum inhibitory concentration was determined. The lowest concentration that inhibited the growth of microbes was observed after the addition of 20 µL of 1.25 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide to the medium and then incubating for 30 min, at 37 °C (Andrews, 2001). Ciprofloxacin and clotrimazole were used as the standard or reference drugs for bacteria and fungi, respectively and concentrations ranging from 0.625 to 20.0 µg/mL were used. The results were validated by performing all the tests in triplicates.

3. RESULTS and DISCUSSION

3.1 Phytochemical Screening

The summary of the results from phytochemical screening of methanolic extract from corn silk is presented in Table 1.

Table 1. Phytochemical constituents of corn silk.

Test	Methanolic extract
Flavonoids	+
Alkaloids	
a. Dragendroff's test	+
b. Mayer's test	+
Tannins/phenolic compounds	+
Reducing sugar	+
Coumarins	+
Triterpenes/terpenoids	+
Sterols	+
Cyanogenetic glycosides	-
Cardiac glycosides	+

Key: (+) present and (-) absent

The phytochemical screening of the methanolic extract from corn silk showed the presence of flavonoids, alkaloids, coumarins, tannins, and triterpenes in the extract. Other phytochemicals detected were reducing sugars, sterols and cardiac glycosides. Many studies have established that thousands of isolated secondary plant metabolites possess excellent physiological properties, hence, they have been used as medicines (Bibiso & Anza, 2022; Iwu, 2014; Newman, 2008; Oluduro, 2012). The presence of these phytochemical constituents in corn silk may be responsible for the acclaimed traditional medicinal uses of this part of the plant.

3.2 Phytochemical Investigations of the Isolated Compounds

3.2.1 *Characterization of isolated compound 1*

Compound **1** was isolated as a colorless crystal with a melting point of 78-80 °C and R_f value of 0.32 (Pet. Ether/EtOAc: 9:1). The FTIR spectrum of the isolate showed a very strong peak at 1731 cm^{-1} indicating the presence of C=O (Figure 1) and quite intense bands at 1122 cm^{-1}

and 647 cm^{-1}, were attributed to hydroxyl group (OH), even though the normal broad band around 3200 to 3600 cm^{-1} was missing and this could mean that the (OH) was not free. The very strong peak at 2922 cm^{-1} was attributed to the stretching and bending vibrations of methyl groups. Bands at 2853 cm^{-1} and 1463 cm^{-1} were the vibrations of methylene groups. Also, a peak at 1217 cm^{-1} showed the presence of C-O functional group. In addition, a band at 1072 cm^{-1} represented C-C vibration. The assignments were in exact agreement with those values reported in the literature (Cruz-Castaneda *et al*., 2018).

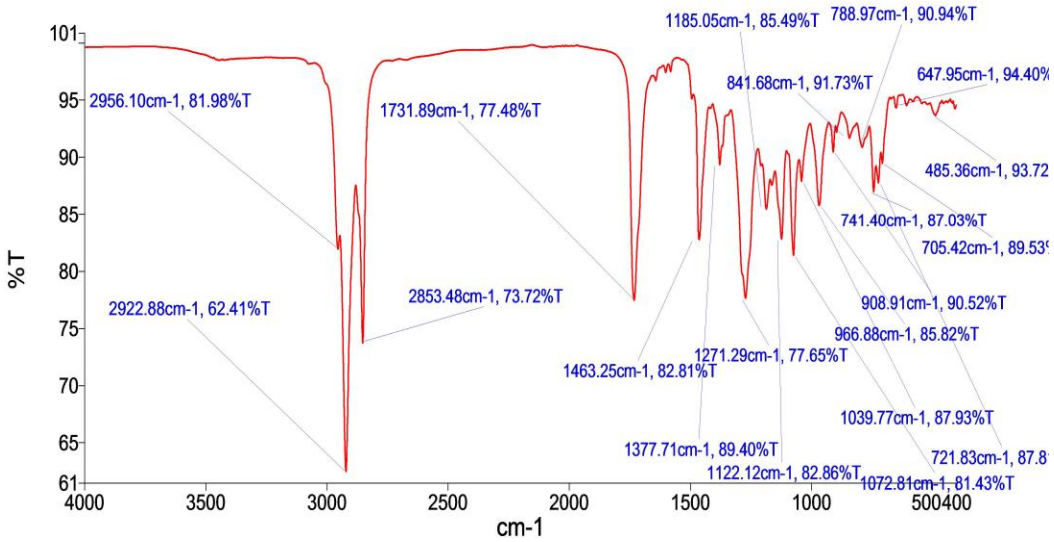

Figure 1. IR spectrum of compound **1** (behenic acid).

The proton NMR spectrum of saturated fatty acids has four distinct signals corresponding to protons on α-CH_2, β-CH_2, ω-CH_3 and the rest are the overlapping methylene (-CH_2) groups (Pietro *et al*., 2020). Signals at δ_H 2.26, 1.57, 0.84 and 1.21 are indicative of protons on α-CH_2, β-CH_2, ω-CH_3 and the rest of the methylene -CH_2 groups, respectively and these chemical shift values were compared with reported literature values for saturated fatty acid (Magritek, 2018). For ^{13}C NMR spectrum, the prominent signals at δ_C 176.7, 34.0 and 24.8 could be assigned to C-1, C-2 and C-3, respectively. Moreover, δc values at 31.8, 22.6 and 14.0 indicated the presence of C-20, C-21 and C-22, respectively. The carbonyl carbon atoms of the free fatty acids have distinct chemical shifts of 175.0-185.0 ppm. The rest of the carbon atoms which are sixteen methylene CH_2 groups appear in similar chemical environments with their chemical shift values close to one another and could be designated to the signals between δ_C 28 and 30 (Pietro *et al*., 2020). Based on detailed analysis of the 1D and 2D NMR spectra in Figures 2, 3, 4, 5, 6, and 7. These spectra were compared to reported data (Pietro *et al*., 2020; Magritek, 2018) and the structure of compound **1** was elucidated as behenic acid (Figure 8).

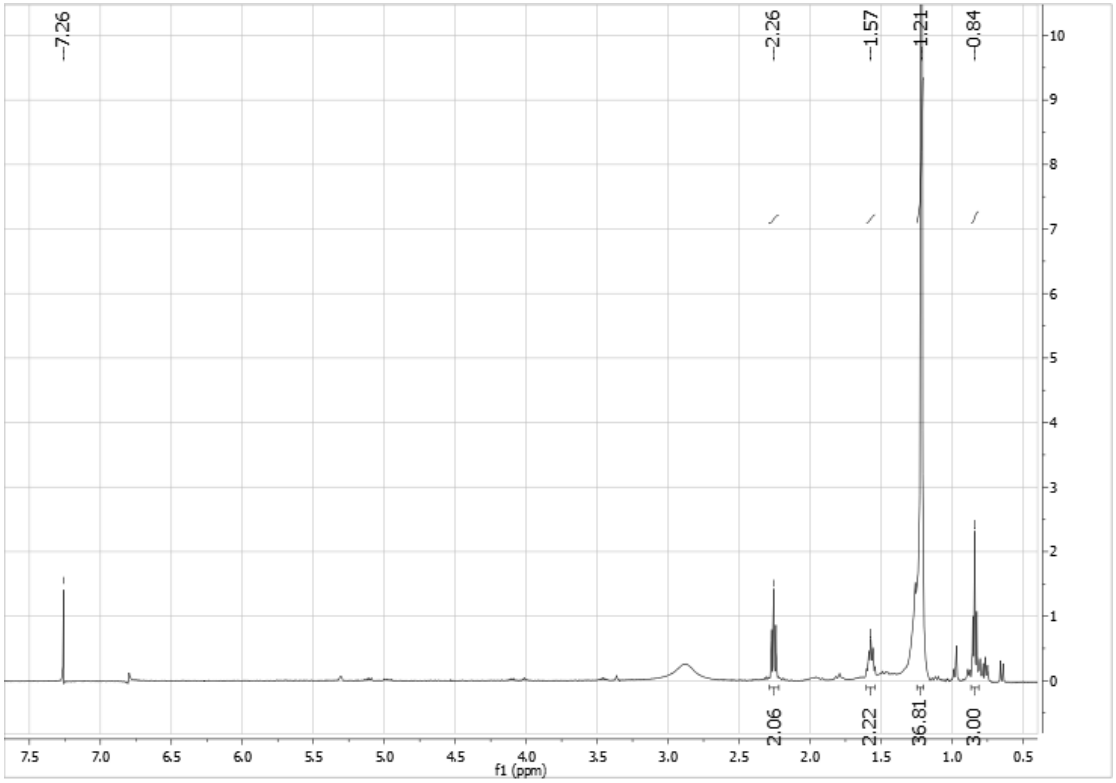

Figure 2. ^{1}H NMR spectrum of compound **1** (behenic acid) in $CDCI_3$ at 500 MHz.

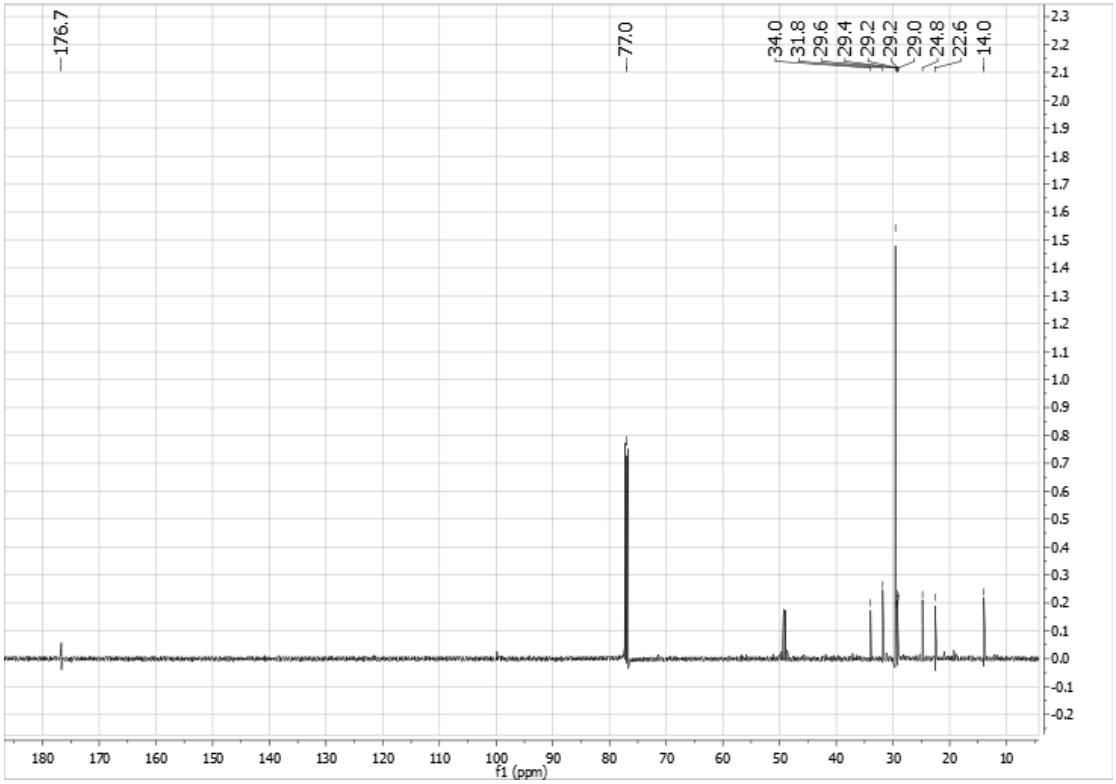

Figure 3. ^{13}C NMR spectrum of compound**1** (behenic acid) in $CDCI_3$ at 125 MHz.

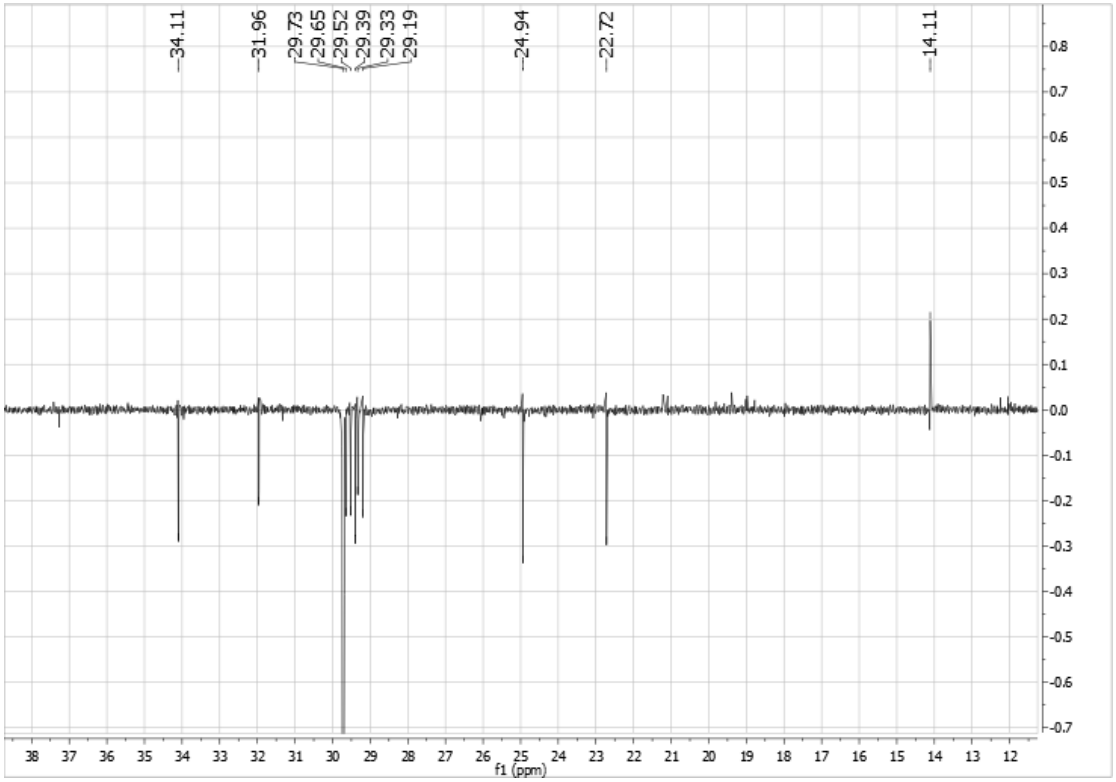

Figure 4. DEPT-135 spectrum of compound**1** (behenic acid) in $CDCI_3$ at 500 MHz.

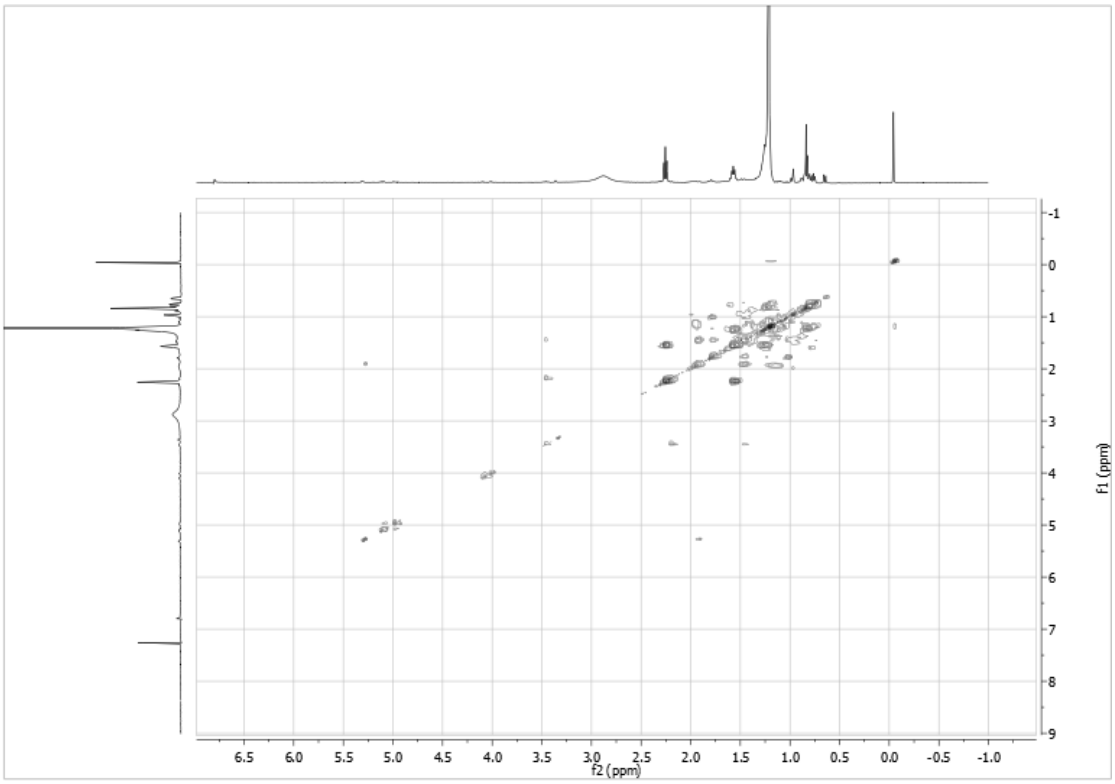

Figure 5. COSY spectrum of compound**1** (behenic acid) in $CDCI_3$ at 500 MHz.

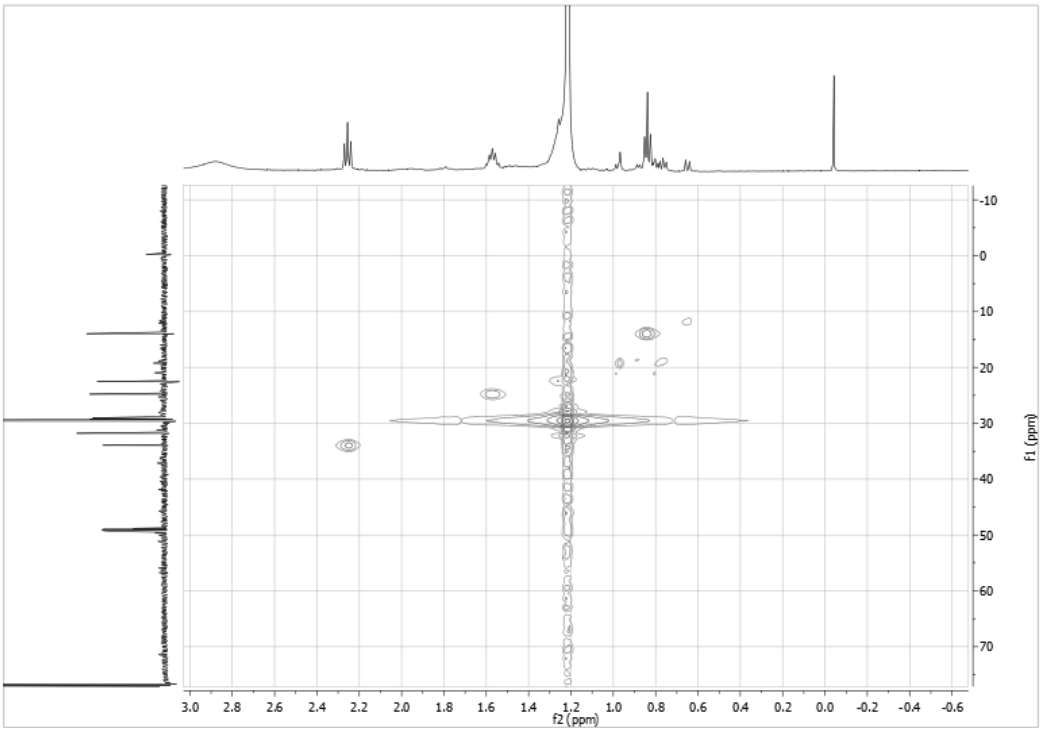

Figure 6. HSQC spectrum of compound**1** (behenic acid) in $CDCI_3$ at 500 MHz.

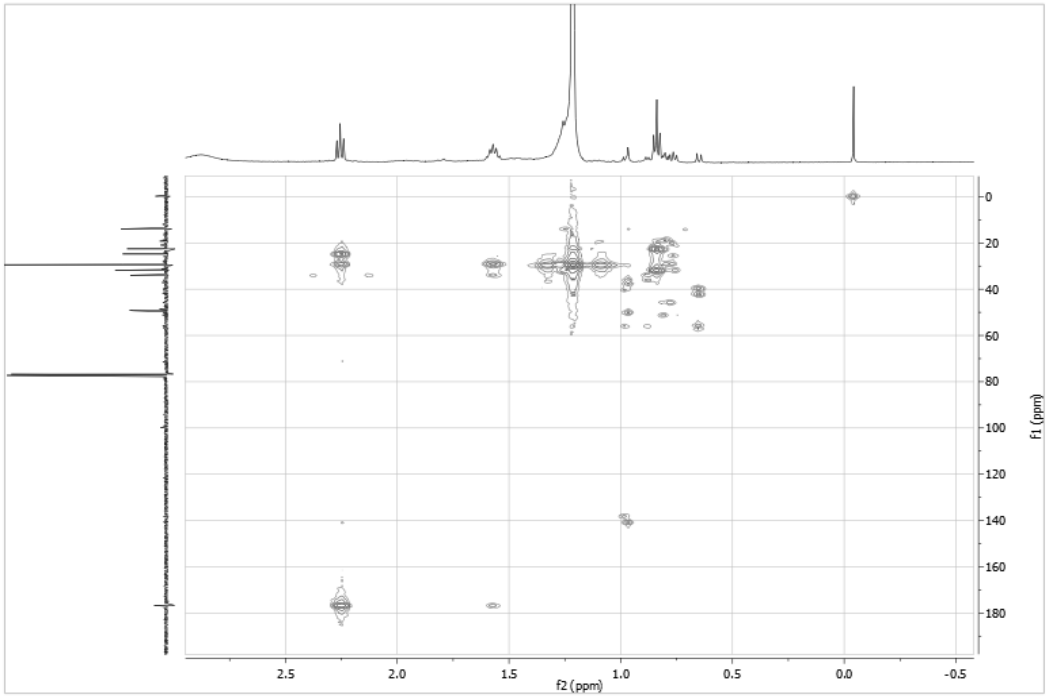

Figure 7. HMBC spectrum of compound**1** (behenic acid) in $CDCI_3$ at 500 MHz.

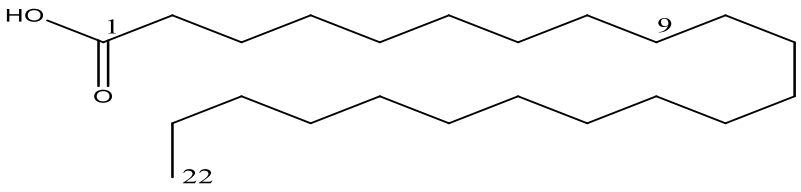

Figure 8. Chemical structure of behenic acid.

3.2.2. *Characterization of compound 2*

Compound **2** was isolated as a colorless powder, and it gave positive tests for steroids and alcohol. The melting point was determined to be 169-171 ^{0}C and the R_f value of 0.52 (Pet. Ether/EtOAc: 6:2). The FTIR spectrum of the compound depicted a broad band at 3361 cm^{-1} and quite intense bands at 1192 and 626 cm^{-1} were ascribed to hydroxyl functional group. A weak band at 1702 cm^{-1} was attributed to C=C stretch of alkenes. This particular band is different from C=O band, in that the carbonyl band has a very intense peak. The out-of-plane C-H vibration of the unsaturated portion was shown at 838 cm^{-1}. The strong band at 2933 cm^{-1} was ascribed to stretching and bending vibrations of the methyl groups. Bands at 2849 cm^{-1} and 1463 were the vibrations of the methylene groups. Also, the vibrational carbon-to-carbon sigma bond was recorded at 1042 cm^{-1}.

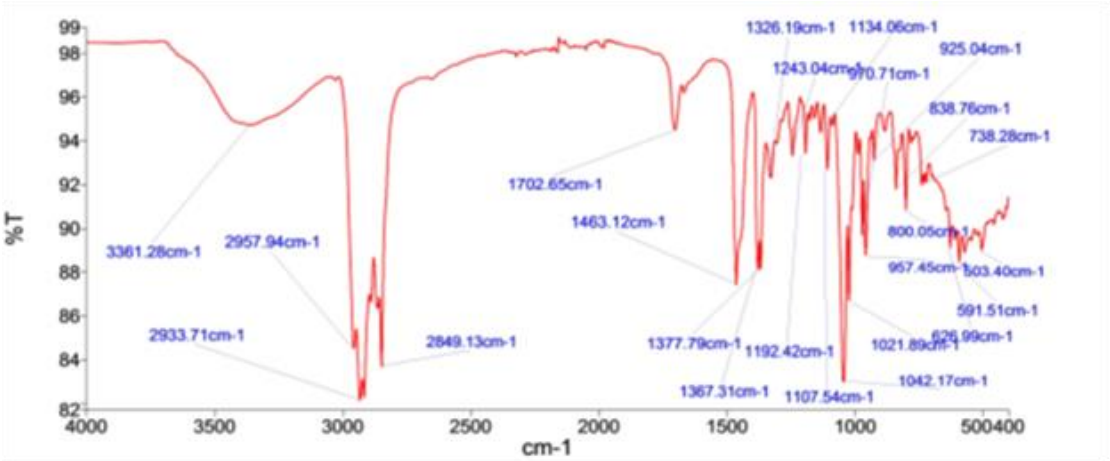

Figure 9. IR spectrum of compound **2** (stigmasterol).

The ^{1}H NMR spectrum ($CDCI_3$, 500 MHz) signals at δ_H 0.69, 0.72, 0.80, 0.85, 1.00, and 1.15 indicated the presence of protons of six -CH_3 groups. Also, the signal at δ_H 3.51 was indicative of a hydroxyl (-OH) group attached to carbon while chemical shift values at δ_H 5.04, 5.14, and 5.34 confirmed the presence of olefinic protons. The NMR spectrum for carbon-13 gave 29 signals including three quaternary carbons. Information from DEPT-135 was useful in distinguishing between carbon-13 signals. DEPT-135 gave twenty-six signals and could be assigned to eleven methine, nine methylene, and six methyl groups. Prominent among carbon-13 NMR signals included δc values at 140.8 and 121.6; 138.4 and 129.4 that could be designated to the double bonds of C-5 and C-6; C-22 and C-23 respectively. In addition, the melting point recorded for compound **2** was compared with the literature values of 169-171 ^{0}C (Woldeyes *et al*., 2012). The ^{1}H NMR and ^{13}C NMR values were designated as a result of HSQC, HMBC, and COSY spectra interpretations (Figures 10, 11, 12, 13, 14, and 15). Figure 16 shows COSY and HMBC correlations of stigmasterol. Compound **2** was identical to stigmasterol (Figure 17).

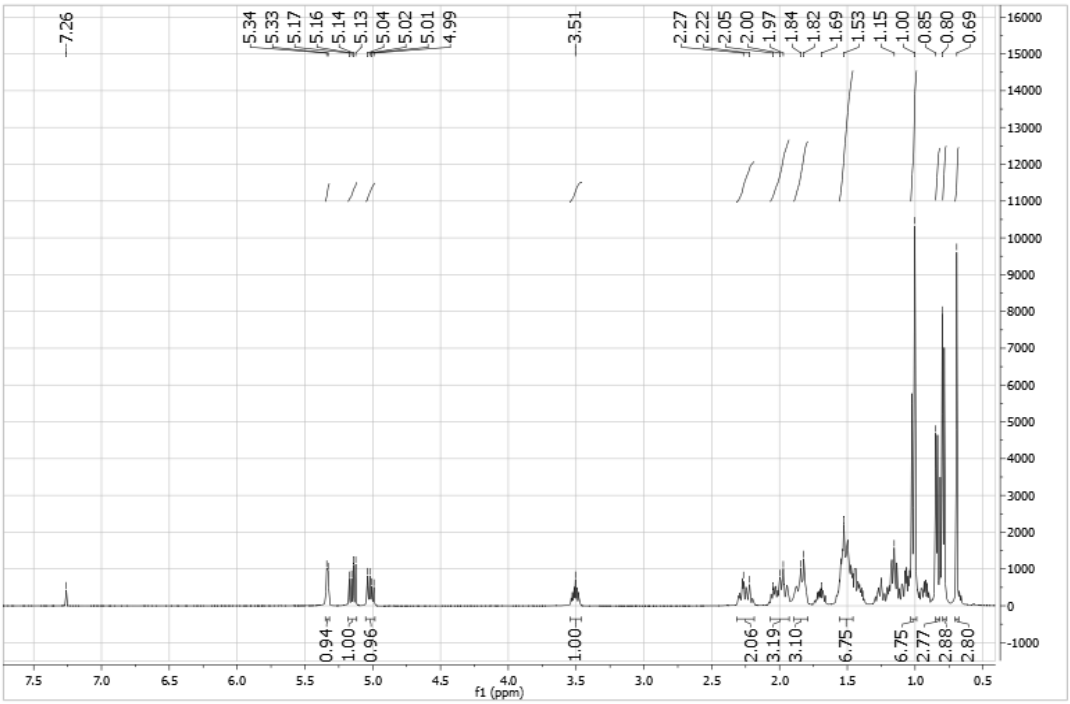

Figure 10. ^{1}H NMR spectrum of compound **2** (stigmasterol) in $CDCI_3$ at 500 MHz.

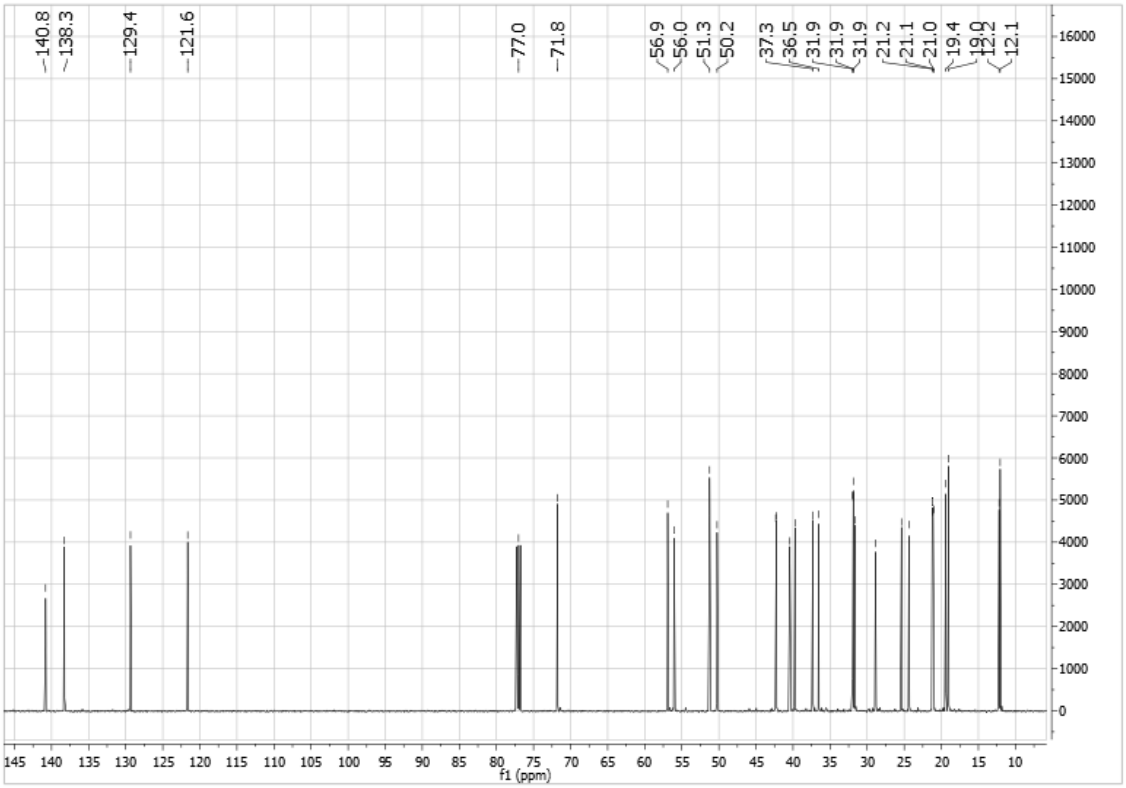

Figure 11. ^{13}C NMR spectrum of compound **2** (stigmasterol) in $CDCI_3$ at 125 MHz.

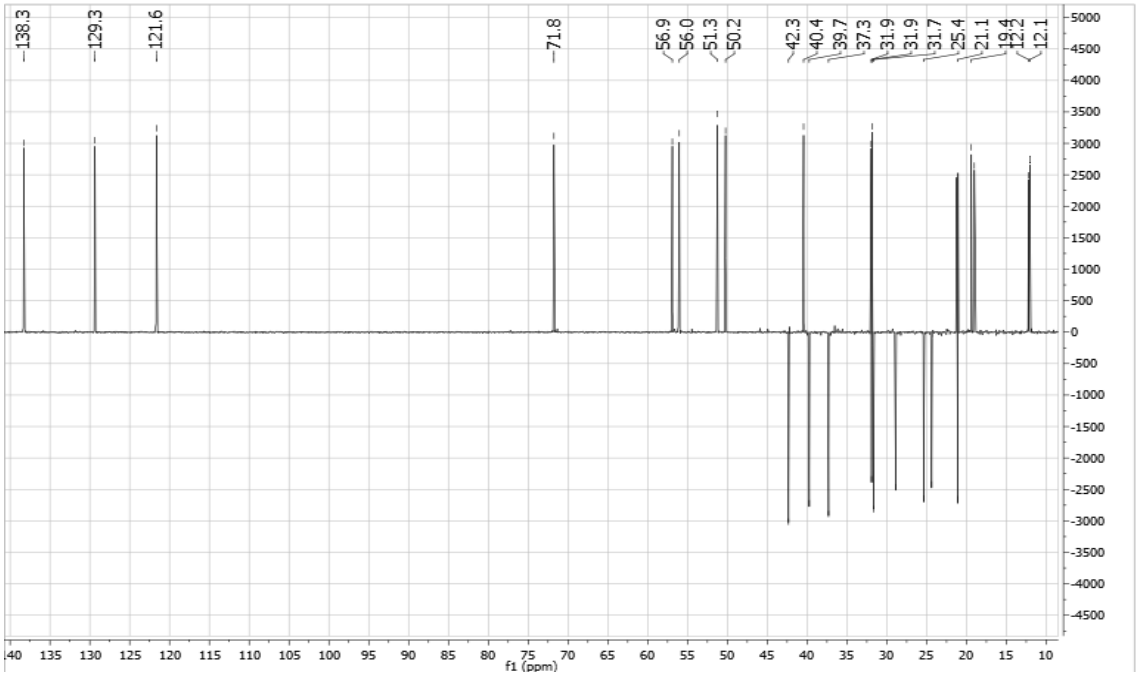

Figure 12. DEPT-135 spectrum of compound **2** (stigmasterol) in $CDCI_3$ at 500 MHz.

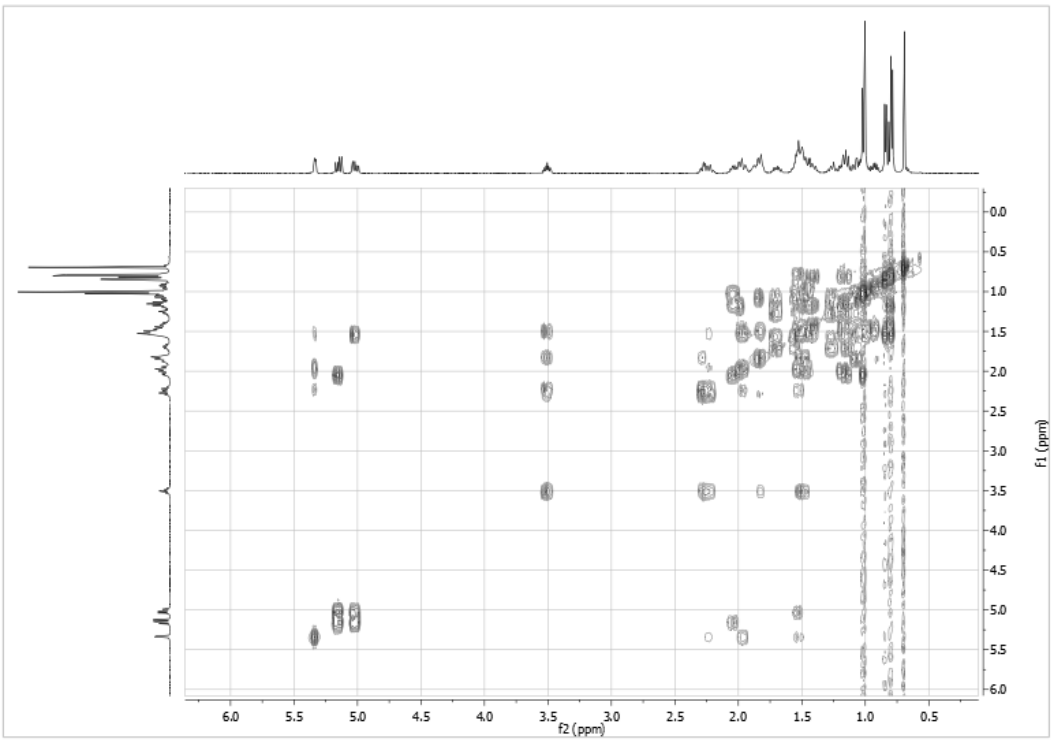

Figure 13. COSY spectrum of compound **2** (stigmasterol) in $CDCI_3$ at 500 MHz.

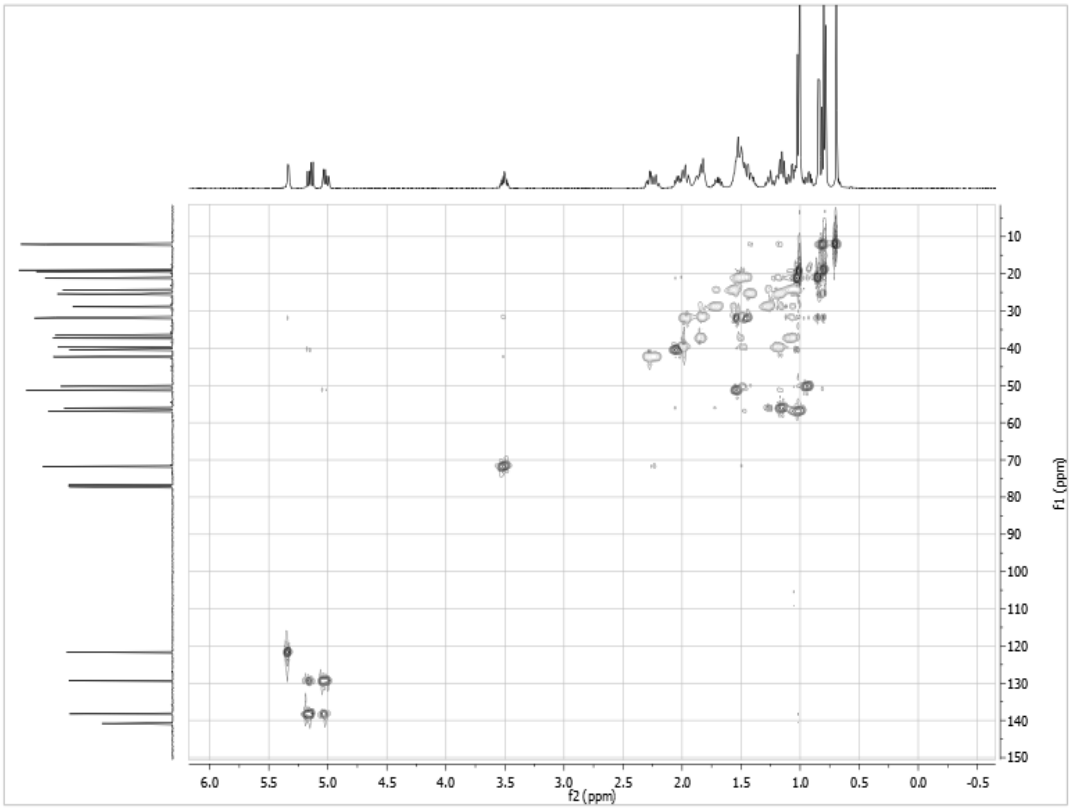

Figure 14. HSQC spectrum of compound **2** (stigmasterol) in $CDCI_3$ at 500 MHz.

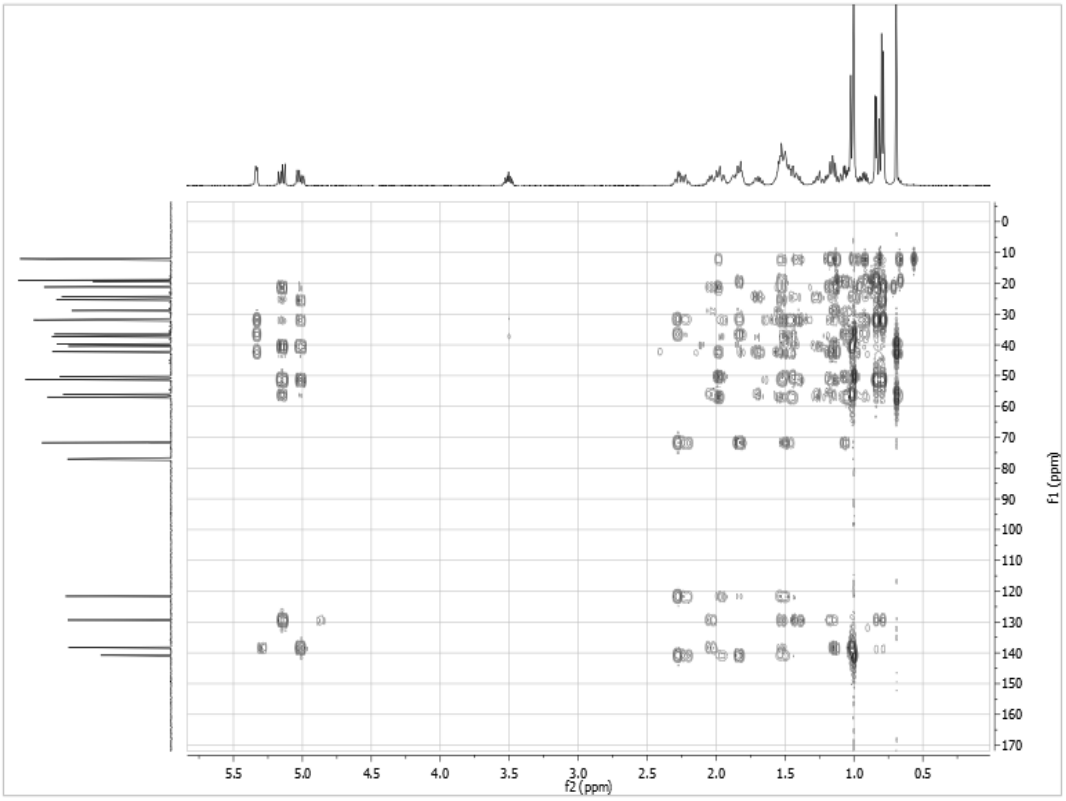

Figure 15. HMBC spectrum of compound **2** (stigmasterol) in $CDCI_3$ at 500 MHz.

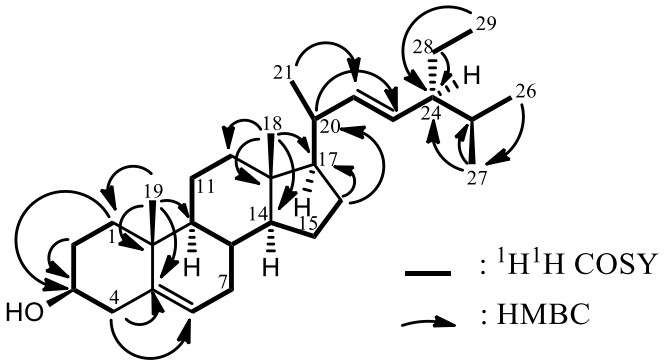

Figure 16. COSY and HMBC correlations of stigmasterol

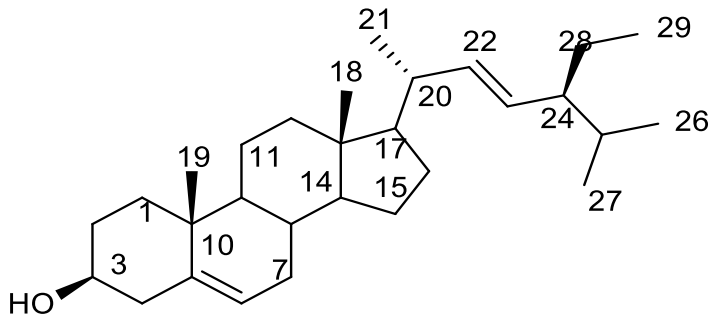

Figure 17. Chemical structure of stigmasterol.

Table 2. Minimum inhibitory concentrations of behenic acid, stigmasterol and standard drugs on the test microbes.

Compound/ Drug	Minimum inhibitory concentrations (MIC) values (µg/mL)					
	Sa	*Ec*	*Kp*	*Pa*	*Ca*	*Tc*
Behenic acid	100.00	>400.00	25.00	200.00	>400.00	25.00
Stigmasterol	25.00	200.00	25.00	200.00	25.00	25.00
Ciprofloxacin	1.25	1.25	1.25	2.50	NA	NA
Clotrimazole	NA	NA	NA	NA	1.25	1.25

The results are by means of triplicate experiments. Ec=*Escherichia coli* (ATCC 25922), Pa=*Pseudomonas aeruginosa* (ATCC 27853), Kp= *Klebsiella pneumonia* (700603), Sa= *Staphylococcus aureus* (ATCC 25923), Ca= *Candida albicans* (10031), Tc= *Tinea corporis.* NA= Not applicable.

Results of behenic acid revealed clear differences in susceptibility with respect to *Candida albicans and Staphylococcus aureus.* The mean minimum inhibitory concentration of behenic acid against *Staphylococcus aureus was* 100 µg/mL with no effect on *Candida albicans*. However, its fungus counterpart *Tinea corporis* was susceptible at 25 µg/mL, which was similar to s *Klebsiella pneumoniae* at the same concentration. However, the compound exhibited low activity against *Pseudomonas aeruginosa* at a relatively high concentration of 200 µg/mL. Also, there was no activity against Gram negative, *Escherichia coli* and a fungal strain *Candida albicans* even at a very high concentration of 400 µg/mL, hence the most resistant in this trend against behenic acid.

From Table 2, *Klebsiella pneumoniae, Staphylococcus aureus, Candida albicans,* and *Tinea corporis* were the most susceptible to stigmasterol with MIC of 25 µg/mL. *Pseudomonas aeruginosa and Escherichia coli* were only sensitive at very high concentrations with a minimum inhibitory concentration of 200 µg/mL, which is eight times, that of the sensitive

organism; revealing the inherent resistance of the Gram negatives to most antimicrobial agents. This scientific research has confirmed the antimicrobial activity of stigmasterol as reported by Gbedema, (2014) that a phytosteroid isolated and determined to be stigmasterol was inactive against *Pseudomonas aeruginosa* but demonstrated moderate antibacterial properties against *Staphylococcus aureus*. Moreover, isolated stigmasterol from *Sida rhombifolia* was active against *Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli* (Woldeyes *et al*., 2012).

Overall, the most resistant organism across all isolates was *Escherichia coli* with MIC range of 200-400 µg/mL. The most susceptible organisms across the isolates were *Klebsiella pneumoneae* and *Tinea corporis* with MIC of 25 µg/mL. Stigmasterol was the active compound against the most resistant *Escherichia coli* with MIC of 200 µg/mL. Also, stigmasterol was very active against *Staphylococcus aureus* and *Candida albicans* at 25 µg/mL.

4. CONCLUSION

This study has shown that the methanolic extract of the silk of *Zea mays* (corn silk) possesses plant secondary metabolites such as alkaloids, flavonoids, coumarins, cardiac glycosides, tannins, saponins, reducing sugars, terpenoids, and sterols. Further phytochemical investigation of the chloroform fraction of the methanolic extract, led to the isolation of behenic acid and stigmasterol. In addition, it is the first report of behenic acid being isolated from corn silk.

Screening the two isolated compounds against the selected microbes, revealed that the most resistant microbe across all isolates was *Escherichia coli* and the most susceptible organisms across the isolates were *Klebsiella pneumoneae* and *Tinea corporis*. The two compounds demonstrated varying degrees of antimicrobial activity and therefore could be useful as leads for future structure optimization towards development for the treatment of some infectious diseases.

Data Availability

The raw data from the experimental study are available with the corresponding author and are available upon request. Previous reports that were used to support this research work have been cited correctly in the text.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Nestor Tordzagla and **Isaac Ayensu** participated in the experimental work, preparation of the manuscript, data analysis, and proofreading; **James Oppong-Kyekyeku** took part in the data analysis and proofreading.

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Research Article

A comparative study of the antioxidant, antibacterial, and cytotoxic activities of different varieties of imported ripe Cavendish banana

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Abstract: Traditionally, Omanis used bananas to treat gastrointestinal disorders, constipation, and diarrhea. The study aims to determine the pharmacological and toxicological activities of two imported banana varieties collected from the local fruit market. Extracts were prepared separately by soaking the bananas in methanol for seventy-two hours. Then, the extracts were fractionated with various solvents with increasing patterns of polarity to give corresponding crude extracts. All extracts were used to determine their antioxidant, antibacterial, and cytotoxic activities using 2,2-diphenyl-1-picrylhydrazyl (DPPH), agar gel diffusion, and brine shrimp lethality methods. In the Indian ripe bananas, the highest antioxidant activity was obtained from the ethyl acetate and the lowest in water crude extract. However, in the bananas from the Philippines, the highest activity was in chloroform extract and the lowest in water extract. Both extracts displayed moderate antibacterial activity at different concentrations. The range of inhibition was 0-19 mm against Gram-positive and negative bacterial strains. Both banana extracts showed significant cytotoxic activity at all working concentrations. Crude extracts killed all nauplii at the highest concentration of 500 µg/mL. In Indian bananas, the highest cytotoxic activity was found in the water crude extract with an LC_{50} value of 27.35µg/mL. The lowest was in ethyl acetate and methanol extracts with an LC_{50} value of 57.54 µg/mL. Almost similar results were obtained from the Philippines. In conclusion, the polar crude extracts prepared from both varieties of ripe bananas have significant pharmacological and toxicological activities. Therefore, polar banana extracts might be agents that can be used as antibiotics.

1. INTRODUCTION

Herbal medicines have been used in traditional healing systems since ancient times to provide the best quality of life for human beings. Alternative medicines are frequently used in primary healthcare in different healthcare systems (Anilreddy, 2009; Barroso *et al*., 2019). People use plant products and fresh plant materials as herbal medicine to treat several chronic diseases.

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Plant-related herbal drugs are a vital resource for treating different serious life-threatening ailments, especially in developing countries.

As reported by the World Health Organization (WHO), more than 60% of the world's population relies on traditional herbal medicines as a safe way of treating severe chronic diseases (Daimari & Swargiary, 2020; Ramzi *et al*., 2010; Reinisalo *et al*., 2015). Due to the importance of alternative herbal medicines, scientists and researchers have to concentrate more and more on improving human health with plant-related resources. Regarding toxicity, the plant materials and their formulated products are safer than pharmaceutical products. In addition, they are cost-effective due to the availability of plants (Anilreddy, 2009; Barroso *et al*., 2019). Most diseases can be cured recently by medicinal plant extracts and their formulated products. Most infections are cured by antimicrobial drugs extracted from plant resources.

Bananas originated from South Asia and are considered one of the oldest crops. (Ayoola-Oresanya *et al*., 2020). The cultivation of bananas has spread all over the tropical world due to their medicinal and nutritive value (Ahmed *et al*., 2016; Pereira & Maraschino, 2015; Zafar *et al*., 2011). In addition to rice, wheat, and maize, bananas are considered primary traded crops in the global market (Oliveira *et al*., 2008). The height of the banana plant is about 3 to 5 meters, depending on the variety of species. The leaves are distributed spirally from the stems.

All parts of this plant species have significant medicinal value and are used in primary health care to treat several chronic diseases. Several previous studies showed that the banana contains different chemical compounds such as unsaturated fatty acids, essential lipids, hydrolyzed sterol esters, different diterpene and triterpene acids such as linoleic acid, and linolenic acid, several monosaccharides and disaccharides, mannose and oleic acids. Most of the chemicals or ingredients mentioned above are present in bananas in substantial amounts (Debabandya *et al*., 2010; Oliveira *et al*., 2008). Several important chemical compounds have been found in bananas, such as vitamin (A-C), oxalic acid, starch, tannin, cardiac glycosides, polyphenolic compounds, triterpenes such as stigmast-7-methylenecycloartanol, stigmast-7-en-3-ol, lanosterol, α-amyrin. All those phytochemicals have significant pharmacological and toxicological activities (Adinarayana & Babu, 2011; Fernanda *et al*., 2016; Natcharee & Rakshit, 2011; Rao *et al*., 2016). Researchers found that the complex constituents in bananas are more active than the single constituent due to the synergistic effect (Liu, 2004).

Traditionally, isolated stem juice is an effective medicine to cure epilepsy, hysteria, dysentery, and diarrhea (Debabandya et al., 2010). The flower extract is used to cure bronchitis, dysentery, and ulcers (Rao et al., 2012). In addition, cooked flowers are also used in different traditional systems in India and China to cure diabetes, epilepsy, leprosy, fevers, hemorrhages, acute dysentery, and diarrhea (Rao *et al*., 2012). Furthermore, root extracts treat dysentery and other ailments (Rao *et al*., 2012). In Oman, green mountain banana is used to treat gastrointestinal problems. Previously, extended research has been conducted on varieties of banana samples. However, no research has been carried out by Omani researchers on imported banana samples. Therefore, the present study was to determine the antioxidant, antibacterial, and toxicological activity of various crude extracts of imported Indian and Filipino ripe bananas.

2. MATERIAL and METHODS

2.1. Chemicals

Dimethyl sulphoxide (DMSO, purity 96.3%)), methanol, and 2,2-diphenyl-1-picrylhydrazyl (DPPH, purity 98.25%) were collected from Sigma-Aldrich Company, Germany. Levofloxacin (Purity 99%) was collected from BDH, UK. All polarity of solvents used in this experiment for the fractionation was collected from BDH, UK. Other chemicals and solvents used in this experiment were of analytical normal grade. Shrimp eggs were from BDH Company, UK. The filter paper was from Whatmann, and refined commercial salts were collected at the local supermarket.

2.2. Microbes

A total of four bacterial strains were used in this experiment: one Gram-positive bacteria, *Staphylococcus aureus* (*S. aureus)*, and three Gram-negative bacterial strains such as *Escherichia coli* (*E. coli*), *Haemophilus influenza (H. influenza)*, and *Enterococcus faecalis* (*E. faecalis*). The strains were cultured in the Microbiology Department, Nizwa Hospital, Nizwa. The bacterial strains were further cultured in the Department of Biological Science, University of Nizwa. All the cultured bacterial strains were brought to the research lab and stored in the freezer at -20 °C until used.

2.3. Instrument

The prepared samples' absorbance was measured by a UV spectrophotometer (Shimadzu, Model-1800, Japan). The rotary evaporator and incubator used for the present experiment were obtained from Yamato Company (Japan) and VWR Company (UK).

2.4. Sample Collection

The imported Indian and Filipino ripe banana samples were obtained from the local fruit and vegetable market in December 2016. Both Indian and Filipino ripe bananas were around 6-7 inches in size. The samples were labeled for processing and carried home for washing, slicing, and drying. The clean samples were identified based on the importer labels on the boxes.

2.5. Sample Processing and Extraction

The sliced Indian and Filipino ripe banana samples (1 Kg each) were dried at room temperature for one week. Both dried banana samples were crushed into rough powder. The rough powder samples (each 500 gm) were soaked with methanol solvent (2 L) for 72 hours. During the extraction, the samples were stirred for complete extraction. Subsequently, they were filtered by a Buchner funnel, and the methanol solvent was evaporated by a rotary evaporator. Then, the methanol extracts (30.00 and 31.45 gm; yield 15% and 15.71%) were dissolved separately in 300 mL water. The samples were fractioned successively with hexane, chloroform, ethyl acetate, butanol, methanol, and water with increasing polarity. (Al Hadhrami & Hossain, 2016). Finally, all the mother solvent was evaporated under reduced pressure.

2.6. Antioxidant Activity

A slightly modified DPPH method was used to evaluate the antioxidant activity of various polarities of the Indian and Filipino ripe banana crude extracts (Tahiya *et al*., 2014). Five concentrations, 12.5, 25, 50, 100, and 200 µg/mL, were prepared from each crude extract (hexane, chloroform, ethyl acetate, butanol, methanol, and water) to measure antioxidant activity. Each crude extract concentration (4 mL) was placed in a pre-cleaned test tube. Then, DPPH solvent (1 mL) was added to the pre-cleaned test tubes, and all tubes were shaken and kept at ambient temperature in a dark place for 45 min. A control sample was prepared in the same way without adding any crude extract. After 45 minutes of incubation of the samples, the absorbance of the incubated crude samples was evaluated at wavelength 517 nm. Gallic acid was used as a standard in the present experiment to calculate antioxidant activity. The inhibition (%) was calculated using the following formula;

$$\% \text{ Inhibition} = \frac{A_{control} - A_{extract}}{A_{control}} \times 100$$

2.7. Antibacterial Activity

A slightly modified usual disc diffusion method was used to evaluate the antibacterial activity of the various polarity crude extracts at four concentrations against one Gram (+) and three Gram (-) bacterial strains (Al Matani *et al*., 2015). Four different concentrations of each polarity crude extract, e.g., 2, 1, 0.5, and 0.25 mg/mL, were used in the present study. Both levofloxacin, a broad-spectrum antibiotic, and DMSO solvent were used in this study as a positive and negative control. Discs of 6 mm size were prepared from filter paper and immersed in each concentration of each extract for 30 minutes. Then, the discs containing samples were placed

on the prepared agar gel plates and kept in an incubator at 37ºC for 24 hours. The zone of inhibition of each concentration of each banana extract was calculated against the four applied Gram (+ and -) bacteria strains.

2.8. Cytotoxic Activity

The prepared crude extracts at varied concentrations were used in this experiment to evaluate cytotoxic activity using the BSL method (Weli *et al*., 2014). At first, the commercial dry shrimp eggs were hatched in artificial seawater in a plastic container. After 24 hours of hatching, the active nauplii was used to evaluate the cytotoxic activity of the prepared crude extracts of the selected plant. Five concentrations of each polarity crude extract, such as 500, 250, 125, 50, and 25 µg/mL, were prepared by dilution techniques with DMSO. From each concentration extract, 50 µl was placed into a 10 mL test tube with 4.95 mL of artificial seawater. It was mixed with a sonicator for 2 minutes. Afterward, 10 active nauplii were added to each test tube with the help of a dropper and magnifying glass. All the test tubes were kept for 24 hours under light. The living nauplii in every test tube were counted, and the percentage of mortality was calculated using the usual formula.

3. RESULTS

3.1. Preparation of Crude Extract

Indian and Filipino ripe bananas were collected from the fruit and vegetable market and extracted with methanol solvent by soaking. The solvent from the extract was evaporated by the usual procedure, and the methanol extract was dissolved with water. The dissolved mixture was transferred to a separatory funnel and extracted with various organic solvents. The percentage yield of each extract is presented in Table 1. The highest amount was obtained in butanol extract, and the lowest was in hexane extract.

Table 1. Yield of crude extracts of Indian and Filipino ripe banana.

Crude extracts	Indian banana (gm)	Filipino banana (gm)
Hexane	2.07	2.54
Chloroform	4.21	3.98
Ethyl acetate	5.81	6.03
Butanol	10.23	7.99
Water	6.03	6.67
Methanol	34.56	29.54

3.2. Antioxidant Activity

The antioxidant activity of various polarity crude extracts, prepared from the Indian and Filipino banana samples, was determined using the well-known DPPH method (Al Matani *et al*., 2015; Daimari & Swargiary, 2020). Using the established formula, the inhibition (%) was determined for all crude extracts at different concentrations of both samples. Among the crude extracts of the Indian ripe banana, the highest antioxidant activity was found in ethyl acetate extract, and the lowest was obtained in water crude extract; however, in the case of the Filipino ripe banana, the highest activity was found in the chloroform extract, and the lowest was found in the water extract (Table 2).

3.3. Antibacterial Activity

The modified disc diffusion method was used to evaluate the antibacterial activity of the various polarity crude extracts. In this experiment, one Gram-positive bacteria strain such as *S. aureus*, and three Gram-negative bacteria strains such as *E. coli*, *H. Influenza* and *E. faecalis* were used to determine the bacterial activity against various concentrations of the extracts through the disc diffusion method. Altogether, six crude extracts of both samples at varied concentrations were used to evaluate their bacterial activity through using the disc diffusion method. Standard levofloxacin and DMSO solvent were used as a control. Most prepared concentrations of each crude extract did not show any activity. The results obtained from this study are presented in Table 3.

Table 2. Antioxidant activity of hexane, ethyl acetate, chloroform, butanol, methanol, and water crude extracts from Indian and Filipino ripe banana.

Extract Conc. (µg/mL)	Water		Methanol		Hexane		Chloroform		Ethyl acetate		Butanol	
	Indian	Filipino	Indian	Filipino	Indian	Filipino	Indian	Filipino	Indian	Filipino	Indian	Filipino
200	68.28±0.12	71.87±0.17	88.76±0.10	92.00±0.09	80.65±0.14	87.37±0.11	84.34±0.10	94.28±0.23	97.22±0.10	75.99±0.08	79.14±0.10	85.40±0.12
100	62.59±0.10	65.87±0.55	78.76±0.17	90.72±0.32	71.29±0.44	83.34±0.18	73.55±0.19	90.65±0.23	91.67±0.19	71.49±0.31	73.55±0.51	79.41±0.11
50	58.54±0.17	63.09±0.09	77.89±0.14	83.89±0.19	66.08±0.87	76.90±0.18	70.92±0.90	81.90±0.78	87.14±0.90	66.23±0.56	64.56±0.44	78.22±0.15
25	55.00±0.13	57.36±0.13	70.28±0.29	75.12±0.18	61.24±0.23	72.71±0.17	68.69±0.23	78.54±0.10	77.15±0.54	60.78±0.50	60.33±0.23	67.34±0.72
12.5	50.98±0.19	50.19±0.22	65.88±0.09	59.03±0.10	53.67±0.16	61.90±0.10	64.09±0.56	71.09±0.54	60.34±0.15	51.23±0.15	54.99±0.19	55.67±0.10

Each value is a mean of three biological replicates.

Table 3. Antibacterial activity of different crude extracts from Indian and Filipino ripe banana samples.

Bacteria	Extract Conc. (mg/mL)	Water		Methanol		Hexane		Chloroform		Ethyl acetate		Butanol	
		Indian	Filipino	Indian	Filipino	Indian	Filipino	Indian	Filipino	Indian	Filipino	Indian	Filipino
E. coli	2	14±0.19	12±0.10	12±0.11	13±0.13	16±0.21	14±0.10	15±0.29	15±0.13	16±0.10	12±0.10	nd	nd
	1	**12**±0.11	10±0.18	13±0.19	12±0.15	14±0.10	12±0.14	14±0.13	13±0.23	14±0.18	9±0.32	nd	nd
	0.5	10±0.09	9.5±0.11	10±0.11	10±0.10	10±0.11	11±0.23	10±0.22	12±0.31	13±0.13	8.5±0.09	nd	nd
	0.25	8±0.16	8.5±0.10	6±0.20	9.5±0.08	9.5±0.17	10±0.24	9±0.15	10±0.22	10±0.17	6±0.15	nd	nd
Control	3	21±0.20	22±0.15	20±0.18	17±0.14	19±0.80	18±0.13	20±0.11	19±0.10	22±0.34	17±0.11	15±0.18	18±0.29
H. influenzae	2	18±0.29	12±0.13	15±0.14	13±0.41	nd	12±0.67	16±0.18	15±0.14	16±0.15	10±0.87	10±0.10	nd
	1	**17**±0.71	10±0.22	13±0.44	11±0.10	nd	10±0.84	15±0.25	12±0.19	15±0.29	10±0.34	9±0.23	nd
	0.5	**12**±0.55	9±0.13	11±0.31	9±0.26	nd	9±0.10	10±0.13	11±0.25	14±0.10	8±0.32	8±0.15	nd
	0.25	9.5±0.10	8±0.20	7±0.67	7±0.31	nd	0±0.13	8.5±0.12	9±0.12	12±0.16	6±0.21	nd	nd
Control	3	20±0.13	25±0.14	21±0.80	16±0.19	16±0.13	12±0.27	21±0.09	18±0.09	20±0.11	19±0.10	18±0.14	17±0.13
S. aureus	2	10±0.13	14±0.14	9±0.12	18±0.10	18±0.67	11±0.17	18±0.15	14±0.29	19±0.23	15±0.15	14±0.25	nd
	1	**10**±0.18	10±0.17	7±0.14	14±0.15	14±0.16	9±0.09	15±0.18	12±0.19	15±0.55	14±0.11	9±0.15	9
	0.5	**9**±0.10	10±0.10	6±0.10	10±0.17	10±0.11	12±0.07	13±0.29	9.5±0.32	14±0.10	10±0.09	7±0.12	nd
	0.25	7±0.14	7±0.44	nd	8.5±0.22	0	9.5±0.19	10±0.10	8±0.14	11±0.12	10±0.25	nd	nd
Control	3	19±0.12	24±0.15	19±0.10	19±0.10	22±0.10	18±0.15	21±0.45	16±0.23	24±0.14	19±0.18	18±0.18	17±0.15
E. faecalis	2	**19**±0.08	15±0.23	10±0.17	15±0.90	nd	10±0.11	13±0.10	13±0.17	14±0.18	15±0.10	nd	nd
	1	**18**±0.13	13±0.29	10±0.22	13±0.51	nd	nd	10±0.72	10±0.18	10±0.20	13±0.16	nd	nd
	0.5	**14**±0.11	10±0.12	8±0.18	10±0.13	nd	nd	9±0.42	10±0.69	10±0.18	10±0.29	nd	nd
	0.25	10±0.55	8±0.11	6±0.13	8±0.10	nd	nd	8±0.10	nd	8±0.27	8±0.17	nd	nd
Control	3	23±0.10	20±0.19	18±0.29	18±0.15	16±0.19	21±0.20	17±0.18	14±0.15	22±0.10	19±0.10	15±0.15	19±0.10

nd= not detected; Each value is a mean of three biological replicates.

3.4. Cytotoxic Activity

This study used a modified BSL method to evaluate the cytotoxic activity against different concentrations of the prepared hexane, chloroform, ethyl acetate, butanol, methanol, and water crude extracts. The mortality (%) of the shrimp is shown in Table 4. Most prepared concentrations of each extract showed substantial cytotoxic activity; the LC_{50} values of different crude extracts at varied concentrations are given in Table 5.

Table 4. Percentage of mortality and lethal concentration (IC_{50}) of different polarity banana extracts.

Crude extract	Conc.	Mortality (%)		LC_{50} (µg/mL)	
	µg/mL	Indian	Filipino	Indian	Filipino
Hexane	500	100	100	33.38±0.19	36.68±0.12
	100	70	60		
	50	40	50		
	10	20	10		
	Control	0	0		
Chloroform	500	100	100	48.12±0.17	26.19±0.31
	100	60	80		
	50	30	50		
	10	10	20		
	Control	0	0		
Ethyl acetate	500	100	100	57.54±0.10	70.17±0.44
	100	50	40		
	50	30	30		
	10	10	10		
	Control	0	0		
Butanol	500	100	100	29.73±0.09	41.81±0.18
	100	70	60		
	50	50	40		
	10	20	10		
	Control	0	0		
Methanol	500	100	100	57.54±0.25	34.11±0.29
	100	50	70		
	50	30	50		
	10	10	30		
	Control	0	0		
Water	500	100	100	27.35±0.11	49.39±0.65
	100	80	50		
	50	40	40		
	10	30	10		
	Control	0	0		

Each value is a mean of three biological replicates.

Table 5. LC_{50} of different extracts of ripe banana samples against brine shrimp larvae (n = 10).

Extract	Ripe banana LC_{50} (µg/mL)	
	Indian	Filipino
Hexane	33.38±0.19	36.68±0.12
Chloroform	48.12±0.17	26.19±0.31
Ethyl acetate	57.54±0.10	70.17±0.44
Butanol	29.73±0.09	41.81±0.18
Methanol	57.54±0.25	34.11±0.29
Water	27.35±0.11	49.39±0.65

4. DISCUSSION and CONCLUSION

Natural products are the core sources of new drugs in the today's world. These products include plants, animals, and different microorganisms. Plants are considered a major source in terms of reliability and safety. Plants and plant products are the primary sources for the health care system in some areas of the world. Therefore, in the past few decades, there has been a growing research interest in plants to find therapeutic agents that might lead to new drugs to treat diseases.

4.1. Antioxidant Activity

The most popular DPPH method was used to evaluate the antioxidant activity at five varied concentrations of six various polarities of banana extracts (Al Matani *et al*., 2015). After the incubation of the samples, the absorbance of each extract was measured with a UV spectrophotometer, and their activity was calculated using the established formula. Based on the experimental results, the highest antioxidant activity of the Indian ripe banana was found in ethyl acetate crude extract, and the lowest was in water crude extract in the following order: ethyl acetate>chloroform>methanol >butanol>hexane>water extract. On the other hand, in the Filipino ripe banana, the chloroform extract showed the highest activity, while the lowest was in water extract, in the following order: chloroform>methanol>hexane>butanol>ethyl acetate>water extracts. The literature searches reveal that the banana extracts contain different bioactive compounds like fatty acids, vitamins, oxalic acid, starch, complex of tannin, cardiac glycosides, polyphenolic compounds, triterpenes and α-amyrin, which are responsible for different biological activities (Adinarayana & Babu, 2011; Debabandya *et al*., 2010; Natcharee, 2011; Oliveira *et al*., 2008). All of these active ingredients could reduce DPPH color by their proton donation capability (Aziza & Hossain, 2015). The experiment results showed that the activity of ripe banana crude extracts was higher than the reported values of crude extracts elsewhere. The variation in antioxidant results might be due to the varied polarities of fractionation, active compounds, and processing methods. Furthermore, during extraction by solvent, some low molecular weight compounds were damaged or vaporized from the samples. That is why the antioxidant activity results in our study are different. Similar results and relationships were obtained between the antioxidant activity of crude extracts and active ingredients, as has previously been reported by several authors (Ahmed *et al*., 2016; Nessma, 2015; Rafaela *et al*., 2010).

4.2. Antibacterial Activity

All prepared crude extracts of ripe imported bananas displayed substantial activity against the applied bacterial strains at varied concentrations with 0-19 mm values. The highest activity was obtained in the Indian water crude extract against *E. faecalis*, and the lowest activity was obtained in the Indian methanol extract against *S. aureus*. All six crude extracts at varied concentrations showed activity against *E. coli* except the butanol extract. On the other hand, all extracts also give significant activity against *S. aureus* and *H. influenza* bacterial strains. However, the butanol and hexane extracts showed no activity against *E. faecalis*. Our results differ completely from the previously reported values (Ahmed *et al*., 2016; Fagbemi *et al*., 2009). This difference may be due to the extraction procedure and sample processing.

4.3. Cytotoxic Activity

At the highest concentration of 500 µg/mL, all six crude extracts of Indian and Filipino ripe bananas killed all shrimps (mortality 100%). The mortality percentage (%) for the extracts at varied concentrations is presented in Table 4. The highest cytotoxic activities were shown in the water and butanol extracts of the Indian ripe bananas and the lowest in ethyl acetate in both Indian and Filipino extracts. Our observation showed that the percentage of mortalities increases with concentration. As presented in Table 5, all crude extracts exhibited moderate toxicity against the BSL method. In the Indian ripe banana, butanol extract was the most biologically active, exhibiting an LC_{50} value of 20.12 µg/mL. Among the Filipino ripe banana

extracts, the chloroform extract was the most active, exhibiting an LC_{50} value of 26.19 µg/mL, and the lowest was in ethyl acetate, with an LC_{50} value of 70.17 µg/mL (Table 5). The experimental results contradict what has been reported for the cytotoxic activity of banana extract elsewhere (Juliana *et al*., 2014). This variation of LC_{50} value in this study might be due to the varied methodologies of sample collection, process, and extraction. In our experiment, we used the most common BST assay, while in other experiments reported in the literature by other authors, an *in-vitro*-based assay was used. In addition, our extraction procedures and preparation of crude extracts are entirely different from other studies.

In this study, all prepared crude extracts from the Indian and Filipino ripe bananas showed moderate antioxidant, antibacterial, and cytotoxic activities against DPPH, agar gel, and BST methods compared to the reported values. However, most of the crude extracts from both types of bananas showed moderate activity against the selected bacterial strains. All of the applied methods in this present experiment have been found to be quick and versatile for evaluating the biological activities of plant extracts. Further studies designed to isolate constituents from the active banana extracts and confirm their antioxidant, antibacterial, and cytotoxic compounds and *in vivo* studies are also needed to prepare new drugs.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Faisal Said Hamed Al-Abri: Data curation; Data analysis; **Salem Said Jaroof Al Touby**: Edit manuscript, Literature survey; Reviewing and Editing. **Mohammad Amzad Hossain:** Supervision, Planning, draft writing, interpretation.

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Research Article

Deciphering the therapeutic actions of *Brenania brieyi* (Rubiaceae) fractions on oxidoinflammatory anomalies

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Abstract: A decline in the antioxidant network during the inflammatory response plays a critical role in the pathogenesis of numerous diseases. We designed this study to decipher the therapeutic efficacy of *Brenania brieyi* in reducing oxidative stress caused by the inflammatory response to cotton pellets. Graded doses of methanol and chloroform fractions of *B. brieyi* (MFBB and CFBB) and indomethacin were administered to Wistar rats for seven days after implanting sterilised cotton pellets (20 mg). Thereafter, biochemical indices of oxidative stress were determined using blood samples taken through cardiac puncture. Furthermore, molecular interactions, drug-likeness, and toxicity features of *B. brieyi phytochemicals* were also assessed. Compared with the untreated group, the groups treated with MFBB and CFBB had a significant ($p < 0.05$) decrease in granuloma tissue weight and MDA levels while increasing glutathione levels, SOD, and CAT activities. In addition, a substantial increase in inflammatory-induced changes in antioxidant nutrients, together with a decline in liver enzymes, was obtained in the treated groups. The docking tests revealed that the top-scoring phytoconstituents of *B. brieyi,* n-hexadecanoic acid, and 9-octadecanoic acid interacted well with catalase, having docking scores of -6.19 and -7.58 kcal/mol, respectively. Moreover, the hits had good oral drug-likeness features and a safe toxicity profile. The findings of the study provide evidence that *B. brieyi* has antioxidant and anti-inflammatory properties, suggesting that it could be used as an alternative therapy to regulate oxidative stress-related diseases.

1. INTRODUCTION

The inflammatory cascade is essentially initiated by microbial agents, mechanical, chemicals, thermal stimulation, trauma, or autoimmune illnesses to defend the body from injury (Ashenafi *et al.*, 2023). Sadly, disproportionate or excessive inflammatory responses elicit an overwhelming increase in reactive oxygen species generation which skewed the redox equilibrium to an oxidative state (Belahcene *et al.*, 2024) Oxidative stress provokes oxidative

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modification of biomolecules (lipid, protein, and DNA) through lipid peroxidation, protein carbonylation, nitration, sulfoxidation, and carbonyl adduct formation (Alothaid, 2022; Chukwuma *et al*., 2023). Altogether, this interferes with redox signalling for cellular processes, resulting in defects in cell differentiation, proliferation, genomic stability loss, and biomolecule functionalities (Pisoschi *et al*., 2021).

Oxidative stress and inflammation are intertwined and intrinsically involved in the pathophysiology of several disease conditions, including cancer, diabetes, alcoholic liver diseases, chronic kidney disease, and cardiovascular and neurological diseases (Awan *et al*., 2023; Ezeorba *et al*., 2024) Emerging evidence from epidemiological and experimental studies has shown that the signal transduction pathway triggers inflammatory gene expressions, such as kinases, cytokines, and transcription factors, which mediate the interdependent relationship between the two (Pisoschi *et al*., 2021). Conversely, these processes act as a vicious cycle (Belahcene *et al*., 2024). For instance, in a disease state where inflammation is a primary event, oxidative stress usually develops as a consequence, which will further exacerbate inflammation and vice versa (Pisoschi *et al*., 2021). This potentiates the need to target both to subvert the onset and progression of chronic diseases since they work in concert. Although many orthodox drugs have been designed to address these pathologies, their adverse effects, including immune suppression, ulcers, gastrointestinal bleeding, and an increase in blood sugar and pressure, limit their usage (Chukwuma *et al*., 2021). As such, natural agents have recently been the subject of increased investigation as remedies for human diseases due to their great therapeutic value, affordability, and biocompatibility (Radi *et al*., 2023; Rodrigues *et al*., 2024). The medicinal plants' therapeutic potentials are hinged on the presence of various secondary metabolites (Nkwocha *et al*., 2022; Radi *et al*., 2023).

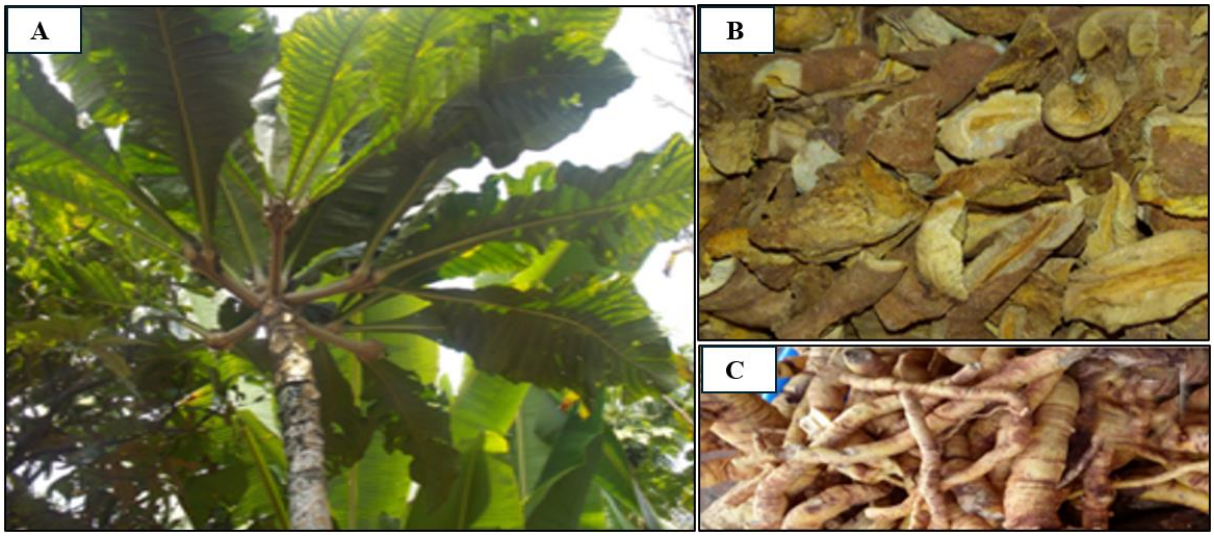

Figure 1. Pictorial view of *B. brieyi* plant (a), root (b) and root bark (c).

In the milieu of plants highly sought for inflammatory-mediated pathologies in ethnomedicinal practice is *Brenania briefyi* (De Wild.) E.M.A.Petit taxonomically classified under the Rubiaceae family of flowering plants. *B. brieyi* is predominantly distributed in Cameroon, Congo, Nigeria, Gabon, and the Central African Republic (Ezeala *et al*., 2023). The large leaves look similar to those of some species of the genus *Anthocleista* Afzel. ex R.Br. The leaves are rounded at apex while the stipules, which in most Rubiaceae help to identify the family, are small and fall early in this species. *B. brieyi* plant grows up to 30 m tall (Ezeala et al., 2023) (Figure 1). It has excellent value as an herbal therapy for swelling, pain, fever, and endocrine disorders. Previous studies identified pharmaceutical-relevant secondary metabolites such as phenols, flavonoids, saponins, terpenoids, alkaloids, and tannins in the root bark of *B. brieyi* (Chukwuma *et al*., 2022). Gas-chromatography characterization of the root bark also revealed the presence of 9-Ocadecanoic acid, hexadecanoic acid, octadecanoic acid, and

squalene, among others (Odo *et al*., 2017). These metabolites could be the basis for the plant's registered antipyretic, anti-inflammatory, analgesic, and *in vitro* antioxidant effects (Chukwuma *et al*., 2022). Given all the various applications of *B. brieyi* in the treatment of inflammatory-related diseases, its rich bioactive compounds, and the already established *in vitro* antioxidant activity of *B. brieyi* fractions, this study was designed to investigate the therapeutic efficacy of the fractions and their secondary metabolites against inflammation-mediated oxidative stress in Wistar rats.

2. MATERIAL and METHODS

2.1. Collection and Identification of Plant Materials

The roots bark of *B. brieyi* root used for this study was collected from Njikoka, Anambra State, Nigeria and identified by Mr. Felix Nwafor, a plant taxonomist in the Department of Pharmacognosy and Environmental Medicine. Voucher specimens with identification numbers PCG/UNN/0327 were deposited in the department's herbarium.

2.2. Preparation of the Fractions

The air-dried and grounded *B. brieyi* root bark (1793 g) was prepared at ambient temperature using methanol and chloroform (1:2 v/v) as extracting solvents for 48 h. The macerate was filtered using Whatman No. 1 filter paper, measured, and mixed with distilled water (20% volume). Then, the resulting solution was separated into two organic layers after vigorous shaking and separated using a separating funnel. The upper and lower layers were designated methanol fraction of *B. brieyi* root bark (MFBB) and chloroform fraction of *B. brieyi* root bark (CFBB), respectively, based on their molecular weight. The fractions were concentrated and kept in a refrigerator at 4 ºC.

2.3. Research Animals

The forty-five (45) Wistar rats of both sexes (average weight of 120.11 g; 8–12 weeks old) used for this investigation were housed in a cage at 25°C with a 12-hour dark/light cycle and given free access to rodent feed and water throughout the study. The rodents were randomized into nine groups (n =5) as follows:

Group 1: (No injection)
Group 2: Cotton pellet (20 mg) (not treated)
Group 3: Cotton pellet (20 mg) + indomethacin (10 mg/kg)
Group 4: Cotton pellet (20 mg) + MFBB (50 mg/kg)
Group 5: Cotton pellet (20 mg) + MFBB (100 mg/kg)
Group 6: Cotton pellet (20 mg) + MFBB (200 mg/kg)
Group 7: Cotton pellet (20 mg) + CFBB (50 mg/kg)
Group 8: Cotton pellet (20 mg) + CFBB (100 mg/kg)
Group 9: Cotton pellet (20 mg) + CFBB (200 mg/kg)

The protocol of Mosquera *et al*., (2011) was used to assess the effects of the fractions on cotton pellet-induced inflammation. The animals were treated for seven days and anaesthetized following the procedure of the American Veterinary Medical Association (AVMA) on the eighth day. Subsequently, their blood samples were collected in plain bottles and centrifuged, and the serum was used to determine biochemical markers of oxidative stress. Finally, the pellets surrounded by granuloma tissue were disserted and weighed after oven drying at 60 ºC.

2.4. Determination of Antioxidants and Biomarkers of Oxidative Stress

The status of antioxidants and oxidative stress biomarkers were measured using the following methods: Malondialdehyde levels (Wallin *et al*., 1993), activities of antioxidants enzymes such as catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) were assayed using, (Aebi, 1983), (Fridovich, 1989), and (Paglia & Valentine, 1967) methods, respectively. The non-enzymatic antioxidants were measured with these methods: Reduced

glutathione (GSH), (Beutler *et al*., 1963); total protein, (Tietz, 1995); total bilirubin and direct bilirubin, (Jendrassik & Grof, 1938); albumin, (Doumas *et al*., 1971); vitamin E, (Pearson, 1976); vitamin C, (Goodhart & Shils, 1973); zinc, (Johnsen & Eliasson, 1987); selenium, (Krishnaiah *et al*., 2003); iron (Teco diagnostic, USA). Experimental protocols of (Reitman & Frankel, 1957) were used to assay serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphate activities.

2.5. Molecular Docking Studies

2.5.1. *Protein preparation*

The protein preparation wizard panel of Schrodinger Suite was used to prepare the crystallographic structure of catalase (PDB ID: 1DGB), which was obtained from the Protein Data Bank (PDB) in the following way: disulfide bonds were formed, bond orders were assigned, hydrogen atoms were added, side chains and loops that were missing were filled, and water molecules larger than 3.0 Å of heteroatoms were eliminated. OPLS2005 and PROPKA were used for the structure's minimization and optimization, respectively.

2.5.2. *Ligand preparation*

The compounds identified from *B. brieyi* (Odo *et al*., 2017) and ascorbic acid (a standard antioxidant) imported in the project table of Schrödinger Suite, 2020-3, from the PubChem database, were prepared for molecular docking via the Lipprep wizard panel.

2.5.3. *Protein-ligand docking*

Based on the site map analysis findings, the produced ligands were docked into the protein's optimal binding pocket using the glide docking tool of Schrödinger Maestro 12.5. A flexible ligand sampling method was used to get samples of all the set functional groups, ring conformations, and nitrogen inversions. The partial charge cut-off for ligand atoms was set at 0.15, and the vdW radius scaling factor was scaled at 0.80. Finally, the 2D and 3D interactions of ascorbic acid and the two top-scoring ligands (best docking scores) were examined.

2.6. ADMET Prediction

The ADME properties of compounds from *B. brieyi and* standard antioxidant (ascorbic acid) were obtained with canonical smiles of each compound using the Swiss ADME server, while the ProTox-II online server was used for toxicity screening.

2.7. Statistical Analysis

One-way analysis of variance helped to calculate the mean and standard deviation (SD) of the datasets obtained in the study. The differences between the means were measured at $P < 0.05$, at least significant level, using the Duncan post hoc multiple comparisons of SPSS Inc., Chicago, IL, USA.

3. RESULTS and DISCUSSION

3.1. Effects of MFBB and CFBB on Granuloma Tissue Weight

The cotton pellet inflammatory model is widely used to measure the efficacy of therapy against proliferative and exudative phases of chronic inflammation (Ashenafi *et al*., 2023). We found out that the weight of granuloma tissue formation was significantly lower ($p < 0.05$) in the groups that were given the fractions and the standard drug indomethacin compared to the group that was not treated (Figure 2). Interestingly, the group that was given 200 mg/kg of MFBB had the smallest granuloma tissue weight, which shows that it is very good at stopping chronic inflammation (Figure 2). Foreign agents, such as cotton pellets, incite foreign body granulomas, which incite macrophage, phagocytosis, and T cell-mediated immune responses (Chukwuma *et al*., 2021). It induces the infiltration of macrophages and inflammatory mediators into the inflamed region, which forms mast cells to wade off the foreign agent. Nevertheless, the majority of cells and mediators released may quicken the formation of ROS and RNS,

contributing to oxidative stress and more tissue damage (Kaufmanova *et al*., 2021). Therefore, the capacity of fractions to inhibit granuloma tissue formation suggests its effectiveness in suppressing the proliferative phase of the inflammation cascade, granuloma fibroblast formation, and oxidative stress after cotton pellet implantation, thereby making it a remedy for inflammation. This finding concurs with the reports of (Ashenafi *et al*., 2023), who also observed reduction in granuloma weight in rats treated with *Vernonia auriculifera* (Asteraceae) fractions.

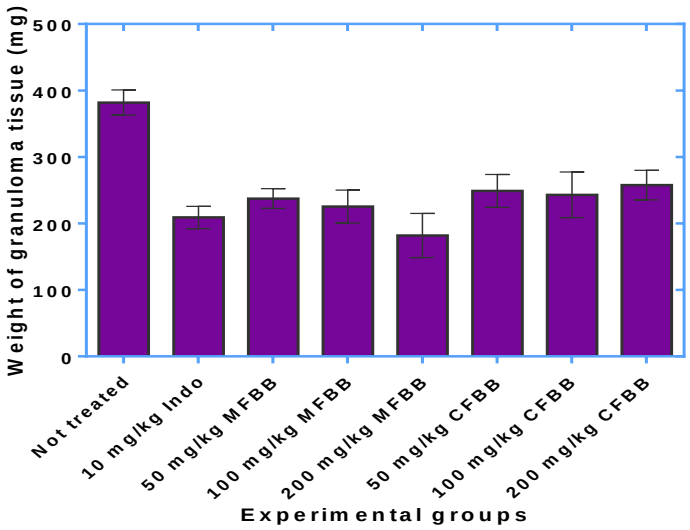

Figure 2. The weight of granuloma tissues (mg) formed after cotton pellet implantation.

3.2. Effect of MFBB and CFBB on Lipid Peroxidation and Antioxidant Enzymes Activities

Malondialdehyde is an oxidative stress biomarker used to quantify the extent of lipid peroxidation (Alothaid, 2022). Herein, a significant ($p < 0.05$) increase in the levels of MDA was recorded in rats not treated (group 2) compared to group 1 (no injection). However, a dose-dependent decrease in MDA levels was obtained in groups treated with 50, 100, and 200 mg/kg b.w. of MFBB and CFBB, as well as the standard drug (Figure 3). The fractions' bioactive compounds likely scavenged free radicals, stopping lipid peroxidation from starting, or they converted peroxy radicals to hydroperoxide before they could start a chain reaction, which triggers the propagation of radical chain reactions (Ezeorba *et al*., 2024). This finding is supported by the significant ($p < 0.05$) increase in the activities of SOD and CAT following treatment with the fractions relative to the untreated group. The drop in antioxidant enzymes in the untreated group could be because of a change in redox equilibrium, which causes these antioxidants to be over-utilized due to the escalated generation of oxidant species by inflammatory enzymes like leukotrienes and cyclooxygenase (Belahcene *et al*., 2024). Considering the functions of these antioxidants in the inactivation of reactive oxygen species, their restoration in the treated groups will subvert oxidative damage mediated by chronic inflammation. Thus, this suggests that the fractions might have served as scavengers of the free radical products of granular inflammation or improved the production of antioxidant enzymes, which shielded the cells from reactive substances, making them a target for stemming the health challenges associated with oxidative stress. This may be because of the phytochemicals—phenols, flavonoids, and tannins that were previously found in the fractions. These compounds have been shown to exhibit antioxidant activity through a variety of mechanisms, including metal ion chelation, scavenging of radicals, and stimulation of endogenous antioxidant expression (Chukwuma *et al*., 2023; Onyesife *et al*., 2023). It is worthy to note that other nonphenolic compounds are also implicated in the antioxidant actions of plant extracts (Gashaye & Birhan, 2023).

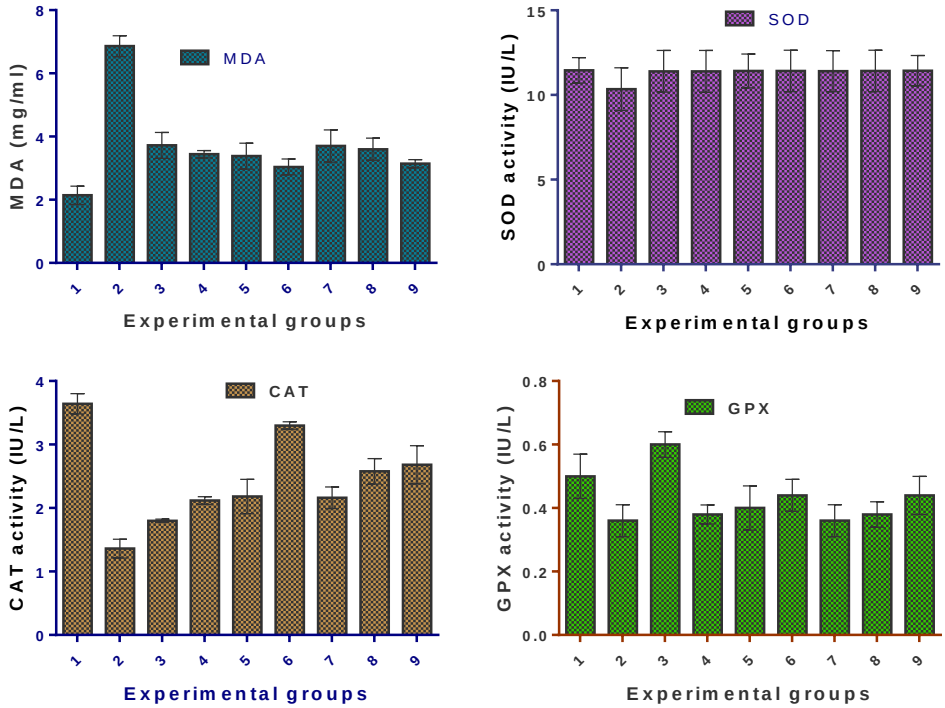

Figure 3. Effects of the MFBB and CFBB on serum MDA and antioxidant enzyme activities.
Group 1: no injection, Group 2: not treated, Group 3: Standard group, 10 mg/kg b.w indomethacin. Groups 4-6 received 50, 100, and 200 mg/kg b.w of MFBB, whereas Groups 7 -9 received 50, 100, and 200 mg/kg b.w of CFBB.

3.3. Effects of the MFBB and CFBB on Serum Metabolic Antioxidants

The results in Table 1 revealed that the cotton pellets elicited a decline in GSH, total protein, and albumin while increasing the levels of bilirubin in group 2 (not treated) compared with group 1 (no injection). The reduced levels of these endogenous antioxidants in the untreated group could be associated with the inflammatory-mediated generation of free radicals, which could impair immune response and susceptibility to infection (Hussen & Endalew, 2023). Notably, treatment with MFBB and CFBB attenuated these anomalies, with the effect being more pronounced in the group administered 200 mg/kg b.w. of MFBB (Table 1). Since studies have shown that glutathione is essential for defending cells against damage caused by free radicals, increasing glutathione levels by the fractions confers an additional therapeutic benefit in many inflammatory disorders (Alothaid, 2022). Similarly, the rise in total protein and albumin suggests that the fractions might have increased protein synthesis or reduced ROS-mediated protein modification. Elevation of bilirubin levels is a strong indication of hepatic injury due to impaired biliary function of the liver (Alothaid, 2022). Excess ROS can change the electrical charge of proteins, break peptide chains, cross-link proteins, and oxidise particular amino acids, creating protein adducts. It is worth mentioning that antioxidants inhibit bilirubin synthesis via inactivating heme oxygenase-1 (HO-1), an essential enzyme in bilirubin synthesis (Ryter, 2022).

Table 1. Effects of the MFBB and CFBB on Serum Metabolic Antioxidant Concentrations.

Groups	Glutathione (mg/ dL)	Total protein (g/ dL)	Direct bilirubin (mg/ dL)	Total bilirubin (mg/ dL)	Albumin (g/ dL)
1	2.62 ± 0.08^{f}	5.80± 0.45^{cd}	0.58 ± 0.04^{a}	1.38 ± 0.04^{a}	3.74 ± 0.11^{ab}
2	0.68 ± 0.08^{a}	3.86±0.24^{a}	1.78 ± 0.15^{e}	3.76 ± 0.17^{e}	3.48 ± 0.26^{a}
3	1.14 ± 0.18^{b}	5.18± 0.75^{bc}	0.94 ± 0.15^{b}	1.62 ± 0.084^{b}	4.06 ± 0.19^{bc}
4	2.24 ± 0.05^{e}	5.48 ± 0.43^{bcd}	1.46 ± 0.90^{d}	2.46 ± 0.15^{d}	4.26 ± 0.15^{c}
5	2.54 ± 0.13^{f}	5.56±0.68^{bcd}	0.84 ± 0.06^{b}	2.40 ± 0.07^{d}	4.06 ± 0.18^{bc}
6	2.58 ± 0.13^{f}	5.64±0.38^{bcd}	0.62 ± 0.13^{a}	1.92 ± 0.08^{c}	5.48 ± 0.43^{d}
7	1.58 ± 0.05^{c}	4.82±0.59^{b}	1.42 ± 0.04^{d}	2.04 ± 0.11^{c}	3.90 ± 0.43^{bc}
8	2.00 ± 0.12^{d}	5.28± 0.70^{bcd}	1.16 ± 0.11^{c}	1.96 ± 0.18^{c}	4.12 ± 0.19^{c}
9	2.22 ± 0.16^{e}	6.16±1.20^{d}	0.90 ± 0.10^{b}	1.72 ± 0.08^{b}	4.20 ± 0.10^{c}

Results are reported as mean ± standard deviation (n = 5*)*. The mean values in the same column containing different superscript alphabets indicate a significant difference ($p < 0.05$).
Group 1: no injection, Group 2: not treated, Group 3: Standard group, 10 mg/kg b.w indomethacin.Groups 4-6 received 50, 100, and 200 mg/kg b.w of MFBB, whereas Groups 7 -9 received 50, 100, and 200 mg/kg b.w of CFBB.

3.4. Effects of the MFBB and CFBB on Serum Nutrients Antioxidants

For optimum cellular activities, especially during situations of oxidative stress, nutrient antioxidants support endogenous antioxidants. Although group 2 (not treated) had a decrease in vitamins E and C, zinc, and selenium while increasing iron levels compared with group 1 (no injection), treatment with the fractions and indomethacin restored these anomalies (Table 2), with MFBB being more effective. Emerging evidence from previous studies has reported that vitamin C not only acts directly as a scavenger of ROS and RNS but also reduces both the tocophenol radical and the tocophenyl quinone. The generated tocopherol will, in turn, protect membrane lipids from lipid peroxidation induced by inflammatory reactions and peroxynitrite (Pisoschi *et al*., 2021). In the same vein, zinc helps to maintain the redox state by regulating transduction pathways, activator genes, as well as the antioxidant enzymes. In addition, selenium is an integral part of selenoproteins and some antioxidant enzymes, which enhance vitamin C regeneration, Cu^{+} chelation, and protect the cells against free radicals and DNA damage (Pisoschi *et al*., 2021). Reducing iron levels after treatment with the fractions is vital for hemostasis since excess iron can cause metabolic dysfunction and severely threaten cell survival in oxidative environments. Conversely, restoration of these antioxidant nutrients will help to restore redox equilibrium and cellular homeostasis under an inflammatory response, which is the basis for preventing metabolic diseases (Ashenafi *et al*., 2023; Awan *et al*., 2023).

Table 2. Effects of the MFBB and CFBB on Serum Nutrient Antioxidants.

Groups	Vitamin C (mg/dL)	Vitamin E (mg/dL)	Iron (µg/dL)	Zinc (µg/dL)	Selenium (µg/dL)
1	1.42 ± 0.10^{d}	0.62 ± 0.02^{cd}	107.46 ± 7.18^{a}	170.36 ± 7.53^{d}	2.86 ± 0.11^{d}
2	1.02 ± 0.04^{a}	0.42 ± 0.03^{a}	210.44 ± 8.96^{e}	134.72 ± 7.15^{a}	1.72 ± 0.33^{a}
3	1.06 ± 0.05^{a}	0.56 ± 0.04^{abcd}	120.14 ± 2.60^{b}	159.62 ± 8.37^{cd}	2.08 ± 0.04^{bc}
4	1.08 ± 0.08^{ab}	0.46 ± 0.07^{ab}	206.72 ± 4.27^{e}	152.54 ± 5.60^{bc}	2.02 ± 0.04^{bc}
5	1.12 ± 0.04^{ab}	0.52 ± 0.03^{abc}	157.46 ± 7.15^{d}	194.00 ± 11.25^{e}	2.08 ± 0.04^{bc}
6	1.22 ± 0.08^{c}	0.70 ± 0.04^{d}	160.44 ± 5.90^{d}	198.88 ± 5.19^{e}	2.22 ± 0.04^{c}
7	1.04 ± 0.05^{a}	0.56 ± 0.05^{abcd}	162.68 ± 7.72^{d}	144.40 ± 9.17^{ab}	2.10 ± 0.10^{bc}
8	1.04 ± 0.11^{a}	0.60 ± 0.05^{bcd}	138.14± 6.43^{c}	190.94 ± 6.80^{e}	1.96 ± 0.11^{b}
9	1.18 ± 0.04^{bc}	0.56 ± 0.02^{abcd}	126.32 ± 10.05^{b}	192.90 ± 13.76^{e}	2.16 ± 0.18^{bc}

Results are reported as mean ± standard deviation (n = 5*)*. The mean values in the same column containing different superscript alphabets indicate a significant difference ($p < 0.05$).
Group 1: no injection; Group 2: not treated, Group 3: Standard group, 10 mg/kg b.w indomethacin.Groups 4-6 received 50, 100, and 200 mg/kg b.w of MFBB, whereas groups 7 -9 received 50, 100, and 200 mg/kg b.w of CFBB.

3.5. Effects of the MFBB and CFBB on Serum Liver Enzymes Activities

The liver function panel measures the activities of AST, ALT, and ALP, which are significant and sensitive markers of hepatic assault (Alothaid, 2022). Hence, we assessed the activities of liver enzymes (AST, ALT, and ALP) to ascertain the hepatocellular status of the rats after chronic inflammation. Our results showed a significant ($p < 0.05$) elevation in AST, ALT, and ALP activities in the untreated group relative to baseline (Table 3). Accordingly, the elevation of these liver enzymes in the untreated group suggests liver injury and an alteration in membrane permeability, which released these enzymes into the blood. This is possible since there was also an increase in MDA, a commonly used indicator of lipid peroxidation, in the untreated rats (Alothaid, 2022). Interestingly, indomethacin (group 3), MFBB, and CFBB-treated groups (5, 6, 8, and 9) registered a decline in AST, ALT, and ALP activities (Table 3). The decrease in the liver enzymes assayed in the treated groups could be hinged on the phytochemicals present in the fractions. Studies have shown that phenols and flavonoids affluent in the fractions exert antioxidant and hepatic protective effects since they prevent membrane hemolysis, lipid peroxidation, and, ultimately, leakage of liver enzymes into the blood.

Table 3. Effects of the MFBB and CFBB on Serum Liver Enzymes Activities.

Groups	Treatments	Doses (mg/kg b w)	AST (u /l)	ALP (u/l)	ALT (u/l)
1	Baseline	-	39.40 ± 3.21^{e}	64.28 ± 2.32^{a}	22.00 ± 4.18^{a}
2	Control		67.00 ± 2.23^{f}	124.64 ± 19.96^{e}	76.00 ± 6.89^{e}
3	Indomethacin	10	57.00 ± 2.54^{cd}	96.18 ± 12.15^{cd}	51.00 ± 6.12^{c}
4	MFBB	50	64.00 ± 7.03^{ef}	93.40 ± 9.84^{bcd}	71.80 ± 6.87^{de}
5		100	52.20 ± 4.49^{bc}	81.20 ± 6.54^{bc}	69.60 ± 1.14^{de}
6		200	50.40 ± 3.65^{b}	72.66 ± 16.50^{b}	65.40 ± 5.31^{d}
7	CFBB	50	61.00 ± 3.39^{def}	123.76 ± 17.21^{e}	69.20 ± 6.41^{de}
8		100	59.60 ± 5.98^{de}	102.28 ± 17.73^{d}	45.60 ± 3.71^{c}
9		200	52.60 ± 5.50^{bc}	74.48 ± 20.67^{b}	31.60 ± 3.85^{b}

Results are reported as the mean ± standard deviation (n = 5). The mean values in the same column containing different superscript alphabets indicate a significant difference ($p < 0.05$)

3.6. Molecular Interaction of *B. brieyi* Phytochemicals with Catalase

Molecular docking studies show that the binding affinity of - 6.19 and 7.58 kcal/mol was obtained for hexadecenoic and 9-octadecanoic acids, respectively, which is higher than the -6.07 kcal/mol obtained for ascorbic acids (Figure 4). The docking studies established two hydrogen bonds in both compounds with positively charged HP 362 and ARG 365 amino acid residues using carbonyl groups attached to their structures (Figure 4). Similarly, ascorbic acids formed two hydrogen bonds between positively charged ARG 72 and GLY 147 using carbonyl and hydroxyl groups attached to the straight chain (Figure 4). According to (Apeh *et al*., 2023), enzyme catalysis and the structural stability of biomolecules both depend on hydrogen bonding with ligands. Interestingly, the report of this study is in agreement with previous studies by (Sierra-campos *et al*., 2020) who also reported the interaction of *Moringa oleifera* (Moringaceae) with catalase. The distance between the random structure and the α-helix structure increases through this contact, which in turn boosts catalase's activity (Sierra-campos *et al*., 2020).

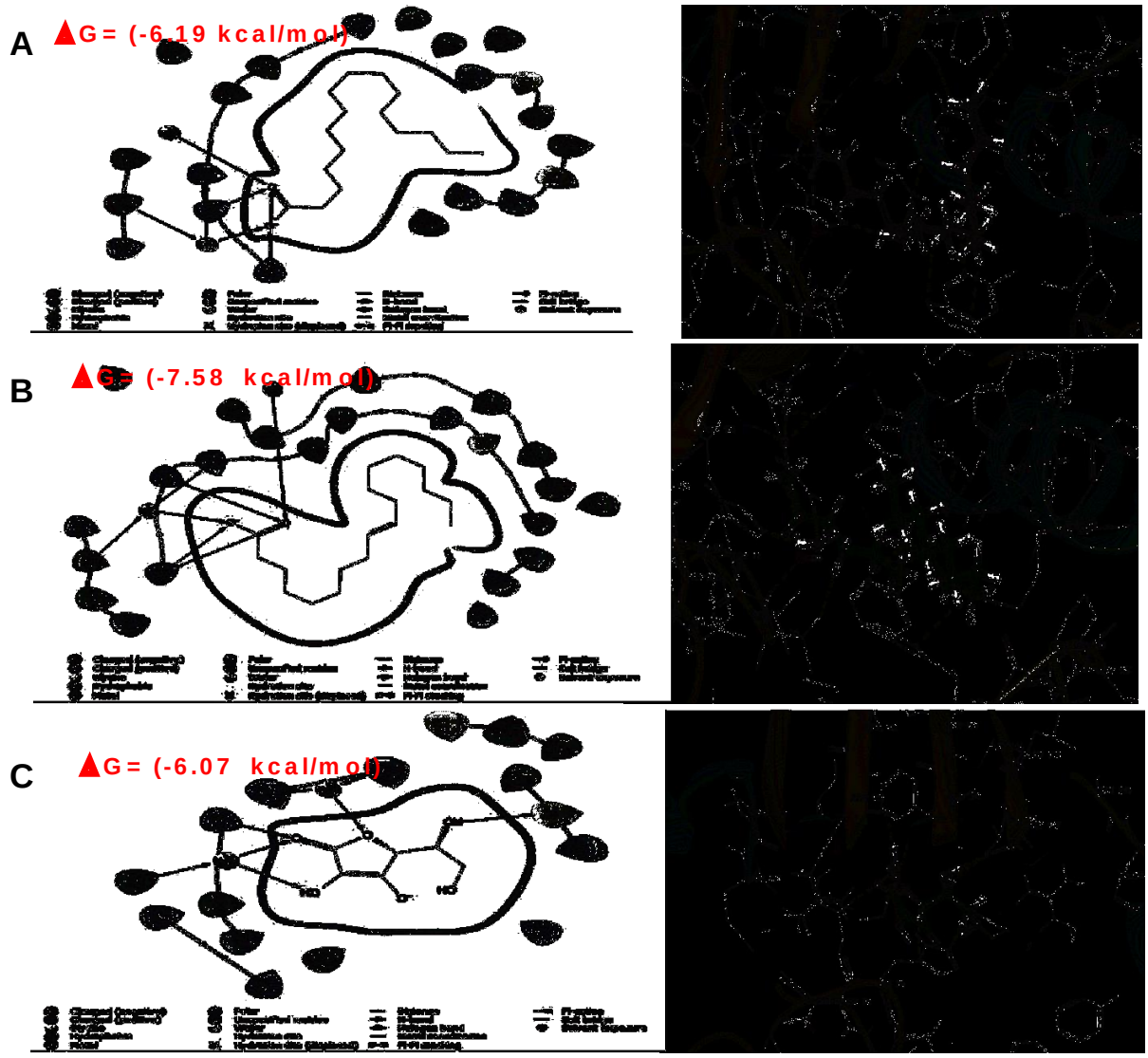

Figure 4. Molecular interaction of hexadecenoic (A), 9-octadecanoic acids (B) and ascorbic acid (C) with catalase. 2D left; 3D right.

3.7. ADMET Prediction

The ADME prediction for oral drugs is crucial in evaluating the drug-likeness and pharmacokinetics of oral medications (Yusuf *et al*., 2023). Hexadecenoic and 9-octadecanoic acids, just like the standard antioxidants (ascorbic acid) and anti-inflammatory (prednisolone, and indomethacin) drugs, passed the Lipinski rule of drug-likeness based on their < 500 molecular weight, < 5 iLog P, < 5 hydrogen bond donor and < 5 hydrogen bond acceptor (Apeh *et al*., 2021). Besides, the toxicity profile shows that the LD_{50} of hexadecenoic and 9-octadecanoic acids were 900 and 1925, respectively, making them safe within these dosage ranges. They were also safe based on their carcinogenicity, immunotoxicity, mutagenicity, and cytotoxicity profiles, except for 9-octadecanoic acid, which is hepatotoxic. However, the high LD_{50} values indicate that consumption of lower doses will be safe, thereby averting hepatoxicity. Hence, they are safer than indomethacin with low LD_{50} values (12 mg/kg), belong to the high toxicity class (class 2), and are also hepatotoxic and immunotoxic (Table 4).

Table 4. ADMET Prediction of *B. brieyi* compounds and standard drugs.

	A	B	C	D	E
Drug likeness					
Molecular weight ≤ 500	256.42	282.46	176.12	360.44	357.99
Hydrogen bond donor ≤ 5	1	1	4	3	1
Hydrogen bond acceptor ≤ 5	2	2	6	5	4
Octanal water partition coefficient					
Solubility class	Moderately soluble	Moderately soluble	Highly soluble	soluble	Moderately soluble
Mean log P	3.85	4.16	0.39	2.19	2.76
ESOL log S	-5.02	-5.7	0.23	-2.96	-4.86
Lipinski violation	1	1	0	0	0
Toxicity profile					
LD_{50} (mg/kg)	900	1925	3367	1680	12
Toxicity class	4	4		4	2
Hepatotoxicity	-	+	-	-	+
Carcinogenicity	-	-	-	-	-
Immunotoxicity	-	-	-	+	+
Mutagenicity	-	-	-	-	-
Cytotoxicity	-	-	-	-	-

Active = (+), Inactive = (-), A = n-hexadecanoic acid, B = 9-octadecanoic acid, C = Ascorbic acid, D = Prednisoline and E = indomethacin.

4. CONCLUSION

The findings from this research study disclosed that *B. brieyi* fractions have astonishing antioxidant and anti-inflammatory activities. The fractions inhibited granuloma tissue formation and lipid peroxidation and also restored the inflamed rats' endogenous and exogenous antioxidant status. Collectively, both the experimental-based and molecular studies validated the antioxidant actions of the fractions. This implies that it might be used as a therapeutic agent for oxidative stress pathologies.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s). The study was approved by the Faculty of Biological Sciences Ethics and Biosafety Committee, University of Nigeria. **Ethics Committee Number:** UNN/FBS/EC/1049.

Authorship Contribution Statement

IFC: Conceptualization. **IFC, LUSE** and **VNO**: Study design. **IFC, VOA, NFN, AEA** and **MOO**: Investigation. **VOA** and **AEA**: Analysis and docking, **IFC, VOA, NFN, AEA**, and **MOO**: Writing - original draft. **LUSE** and **VNO**: Writing - review and editing. All authors read and approved the final manuscript before submission.

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Research Article

Evaluation of potential anti-aging effects of *Achillea phrygia* Boiss. & Balansa (Asteraceae)

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Abstract: This study aims to determine the anti-aging effects of *Achillea phrygia*, an endemic plant, by evaluating its sun protection factor (SPF) level, antioxidant activity, total phenolic content, extracellular matrix-degrading enzymes (ECM) inhibition, genotoxic/anti-genotoxic, and cytotoxic activities. The SPF level was assessed using an *in vitro* quantitative method, while antioxidant capacity was determined through DPPH, β-carotene, and hydroxyl-radical (H_2O_2) scavenging assays. The total phenolic content was quantitatively conducted using the Folin Ciocalteu reagent. The inhibition of ECM-degrading enzymes was determined using matrix metalloproteinase-1 (MMP-1), hyaluronidase, and elastase enzymes. Genotoxic/anti-genotoxic properties were assessed using the AMES *Salmonella*/microsome assay, and cytotoxicity effects were assessed through the MTT assay. The results indicated that *A. phrygia* showed moderate SPF activity (SPF = 4.013) and exhibited IC_{50} values of 0.183 ± 0.03, 0.079 ± 0.51, and 1.18 ± 0.35 mg/mL for DPPH, β-carotene, and hydroxyl-radicals, respectively. The total phenolic content was measured to be 23.56 ± 1.42 mg GAE/g dry extract. Furthermore, the extract demonstrated inhibition of MMP-1 (47.98%) and elastase (39.2%) activities. Importantly, it did not induce DNA damage and showed antigenotoxic activity ranging from 10% to 65.6%. The cytotoxicity assay revealed an IC_{50} value of 42.41±4.05 µg/mL. These findings suggest that *A. phrygia* could be utilized as a cosmetic ingredient in skincare products due to its ability to protect against UV radiation, exhibit antioxidant properties, prevent extracellular matrix degradation, and inhibit DNA damage.

1. INTRODUCTION

Skin aging is a time-dependent, quite complicated, and natural process, and constitutes one of the important problems for both women and men. It is influenced by various internal and external factors, leading to structural and physiological changes in each layer of the skin. Internal aging progresses gradually, involving the production of reactive oxygen species (ROS) through oxidative metabolism (Lephart, 2016). ROS-induced oxidative stress is a key

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contributor to skin proteins, lipids, and DNA damage, contributing directly to skin aging (Pientaweeratch *et al*., 2016). Another significant aspect of internal skin aging is the decline in cellular replicative abilities and the increased degradation of extracellular matrix (ECM) components such as collagens, elastin, proteoglycans, and glycosaminoglycans. Disruption of the ECM results from increased expression of enzymes like matrix metalloproteinases (MMPs), which degrade the dermal ECM (Millis *et al*., 1992). This ECM damage leads to decreased elasticity, strength, and moisture retention in the skin. These processes can also be triggered by ROS.

Exposure to UV rays, also called photoaging, is the primary cause of external skin aging. UV radiation serves as a potent inducer of ROS formation in the skin (Masaki, 2010). Chronic exposure to low-grade UV radiation accounts for over 80% of skin aging. Repeated exposure to UV rays accelerates the degradation of ECM components and disrupts the synthesis of new components (Madan & Nanda, 2018).

Achillea L. is one of the significant genera of the Asteraceae family, which includes more than 115 species that grow in many parts of the world (Başer, 2016). The use of this genus in traditional medicine has a long-standing tradition in various countries and cultures. It has been utilized as a natural remedy for conditions such as bleeding, pain, burns, wounds, gastrointestinal disorders, skin infections, eczema, inflammation, hemorrhoids, and dysentery, as well as for its carminative, tonic, diuretic, and diaphoretic properties (Mohammadhosseini *et al*., 2017; Salehi *et al.*, 2020). *Achillea millefolium* L. is a widely distributed plant with extensive medicinal use worldwide. *A. millefolium* extract, also called Yarrow extract, is also one of the most well-known ingredients of cosmetic products (Aronson, 2016). This species alone is currently being used in 135 cosmetic products as active ingredients for personal care products and cosmetic formulations (Becker *et al.*, 2016). However, there is not enough research reported on extracts of other *Achillea* species for their potential use in skincare products. Recent studies suggest that other *Achillea* species may also yield safe compounds with beneficial biological properties, making them potential multifunctional ingredients for cosmetics.

Achillea phrygia Boiss & Balansa is a species that is endemic to Anatolia (Huber-Morath, 1975) and it is used in Western Anatolia as an appetizer, to aid digestion, to treat abdominal pain and nausea and wound healing (Deniz *et al.*, 2010). According to the studies performed on polyphenolic components, *A. phrygia* extracts obtained with different solvents were found to contain chlorogenic acid, rutin, luteolin, sinapic acid, ferulic acid, apigenin, quercetin, catechin, epicatechin, and kaempferol (Zengin *et al.*, 2017a). Furthermore, recent studies have demonstrated the enzyme inhibitory effects of *A. phrygia* extracts against tyrosinase, cholinesterases, amylase, lipase, and glucosidase (Zengin *et al*., 2017a) as well as their antioxidant activity (Zengin *et al*., 2017a; Doğan *et al*., 2022). Additionally, these plant extracts have shown anti-nociceptive properties (Küpeli *et al*., 2007) and potential anticancer activity (Doğan *et al*., 2022). The potential use of *A. phrygia* as an ingredient in skincare products has not been investigated. This study aims to determine the suitability of *A. phrygia* for cosmetic and dermatological applications by evaluating its sun protection factor, antioxidant activity, total phenol content, inhibition of ECM-degrading enzymes, and genotoxic/antigenotoxic and cytotoxic activity.

2. MATERIAL and METHODS

2.1. Plant Sample

The aerial parts of *Achillea phrygia* were harvested during flowering in August 2020 at Karaisalı, Çevlik village (altitude: 1650m) located at coordinates 37°22'16.2660"N and 35°4'3.6228"W in Adana, Türkiye. *A voucher specimen was* identified by Dr. Volkan Eroglu and deposited in Muğla Sıtkı Koçman University herbarium (ARB-A011). Air-dried aerial parts (35 g) were extracted using an ultrasonic-assisted extraction method using ethanol (350 mL) at

30 °C for 3 h and filtered through filter paper. Following filtration, the solvent was evaporated, and stored at 4 °C in a dark place until analysis.

2.2. Evaluation of the SPF

The *in vitro* photoprotective efficacy test was conducted using a UV-visible spectrophotometer (Saraf & Kaur, 2010). Hydroalcoholic dilutions (50% v/v) of *A. phrygia* (500 µg/mL) were prepared, and sample absorbance values were detected at 5 nm intervals within the mid-wave ultraviolet light range (290-320 nm). SPF values were estimated using the formula below:

$$\mathrm{SPF}_{\mathrm{spectrophotometric}} = \mathrm{CF} \times \sum_{290}^{320} \mathrm{EE}(\lambda) \times \mathrm{I}(\lambda)\ \mathrm{Abs}(\lambda)$$

where: CF is a correction factor (10), EE(λ) is an erythemogenic effect of wavelength radiation, I is the intensity of the sun, and Abs (λ) is the absorbance of the samples.

2.3. Antioxidant assays

The scavenging ability against DPPH radicals was assessed following a previously established method (Ebrahimabadi *et al*., 2010). DPPH solution (0.1 mM, 100 µL) in methanol was combined with the extract (100 µL) at various concentrations and incubated in darkness for 30 minutes. After incubation, abs were measured at 517 nm. α-tocopherol and ascorbic acid were used as references. The results were expressed as IC_{50} values.

Total antioxidant capacity was applied using the β-carotene/linoleic acid bleaching method as described by Rauter *et al.* (2012). β-carotene/linoleic acid solution was added to 250 µL of different concentrations of the extract or positive control solutions. The mixture tubes were then incubated at 50°C in the dark, and abs changes were recorded every 15 minutes at 470 nm. The results were expressed as IC_{50} values.

The hydroxyl-radical scavenging activity was investigated using the method described by Zhang *et al.* (2015). Different concentrations of the extract (250 µL) and salicylic acid (9 mmol/L) were added to a reaction mixture containing H_2O_2 (20 mmol/L), 500 µL of dH_2O and $FeSO_4$ (9 mmol/L). After 30 min incubation, the abs were evaluated at 510 nm. The *results are expressed* as IC_{50}.

2.4. Total phenolic content

In brief, a 50 *µ*L extract was incubated with 25 *µ*l Folin–Ciocalteu *reagent for 3 min.* Subsequently, Na_2CO_3 was added to the mixture, followed by an additional 2-hour incubation period. Abs readings were taken at 760 nm (Singleton *et al.*, 1999). The outcomes were expressed as mg GAE/g dry weight using the standard curve.

2.5. Enzyme Inhibitory Activity

2.5.1. MMP-1 inhibition

The reaction mixture consisted of *Clostridium histolyticum* collagenase (MMP-1), tricine buffer (pH 7.5), and the extract solution. After 20 min, N-(3-[2- Furyl]-acryloyl)-Leu-Gly-Pro-Ala was mixed with the test solution and abs were recorded at 2-min intervals on a plate reader at 335 nm (Barrantes & Guinea, 2003).

2.5.2. Elastase inhibition

For elastase inhibition, 25 µL of elastase and 25 µL of Tris–HCl buffer were mixed with 50 µL of the sample and pre-incubated for 20 minutes in the dark at 25°C. Subsequently, the mixture was inoculated with 125 µL of N-Succinyl-Ala-Ala-Ala-p-nitroanilide and again incubated under the same conditions. Abs were recorded at 410 nm on a plate reader (Lee *et al.*, 1999).

2.5.3. Hyaluronidase inhibition

Regarding hyaluronidase inhibition, the extract's effect on bovine hyaluronidase was determined by pre-incubating the hyaluronidase at 37°C for 30 minutes. Then, 100 µL of CaCl2 and sodium hyaluronate were added to the test mixture and incubated for 40 minutes. Following this, the mixture was supplemented with sodium borate and NaOH, heated in hot water, cooled, and mixed with p-dimethyl amino benzaldehyde. The resulting mixture was then incubated away from light at 37°C for 20 min, and abs was detected at 585 nm (Lee *et al.*, 1999). The enzyme inhibition abilities were determined using the following equations:

$$\text{Inhibition (\%)} = [(\beta - \alpha) - (\delta - \gamma)] / (\beta - \alpha) \times 100$$

Where β is the abs without the sample, α is the abs without the sample and enzyme, δ is the abs with the sample, and γ is the abs with the sample without the enzyme.

2.6. Genotoxicity and Antigenotoxicity Assay

The genotoxic/antigenotoxic effects of the extract were assessed using the *Salmonella typhimurium*/microsome test with *S. typhimurium*TA98 and *S. typhimurium*TA100 strains. Before testing, the original mutations of the test strains were verified for test reliability, and the spontaneous mutation rates as well as the cytotoxic doses of the sample extract were determined (Maron & Ames 1983).

For the genotoxicity test, sodium-phosphate buffer (pH 7.4), fresh overnight bacterial culture, and test compounds (ranging from 0.1 µg to 1 mg per plate) were combined in test tubes containing top agar supplemented with histidine/biotin. The mixture was then spread onto plates containing minimal glucose agar (MGA) and incubated at 37°C for 48 hours. The number of His+ colonies was subsequently counted. Positive controls included 4-nitro-o-phenylenediamine and sodium azide.

In the anti-genotoxicity test, fresh overnight bacterial culture, sodium-phosphate buffer, the test substance, and mutagens were mixed in test tubes containing top agar supplemented with histidine/biotin. The resulting mixture was spread onto plates containing MGA and incubated at 37°C for 2–3 days. The number of His+ colonies in the positive control was considered as 100%, and any decrease in the number of revertant colonies obtained from the extracts in the presence of mutagen was evaluated as anti-mutagenicity

2.7. Cell Culture

NIH-3T3 mouse fibroblast cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and antibiotics (100 µg/mL streptomycin and 100 IU/mL penicillin) under conditions of 5% CO2 at 37°C in a humidified atmosphere.

2.7.1. Cytotoxicity assay

To initiate the experiment, fibroblast cells were initially seeded into individual wells of 96-well plates at a density of $1x10^4$ cells per well. Subsequently, the cells were exposed to the test extract and allowed to preincubate for 24 hours. Following this incubation period, the culture medium was carefully aspirated, and the wells were gently washed with phosphate-buffered saline (PBS). Next, 200 µL of MTT solution was added to each well, followed by the addition of 200 µL of DMSO to dissolve the resulting MTT crystals. The plates were then placed in an incubator for 20-30 minutes to facilitate the reaction. After incubation, the absorbance of each well was measured at 540 nm using a spectrophotometer (Mosmann, 1983).

2.8. Statistical Analysis

Readings for all SPF, enzyme inhibition tests, total phenol, and antioxidant assays were taken in triplicate. Genotoxicity and anti-genotoxicity experiments were conducted in 3 repetitions, 2 in parallel. The results were averaged with their standard deviations. A one-way ANOVA followed by Tukey's test was used to investigate statistical significance. The statistical significance was indicated by $p < 0.001$.

3. RESULTS

To assess the SPF activity of the *A. phrygia* extract, the UV absorbance method was employed. Sample absorbance values were measured between 290 and 320 nm at 5 nm intervals. The extract demonstrated moderate protection activity, with an SPF value of 4.013 (see Table 1).

Table 1. Spectrophotometrically calculated sun protection factor value of *A. phrygia*

Wavelength (nm)	EE(λ) x I(λ) (normalized)	*A. phrygia*	
		Absorbance	SPF
290	0.0150	0.437	0.065
295	0.0817	0.429	0.350
300	0.2874	0.413	1.201
305	0.3278	0.395	1.295
310	0.1864	0.389	0.725
315	0.0837	0.372	0.311
320	0.0180	0.369	0.066
Total	=1.000		4.013

EE: Erythemal efficiency spectrum;
I: Solar simulator intensity spectrum.

The antioxidant activity of the sample was evaluated through three different methods and compared with α-tocopherol and ascorbic acid (see Table 2). The methods employed were the DPPH assay, β-carotene-linoleic acid bleaching test, and H_2O_2 scavenging test, yielding IC50 values of 0.183 ± 0.03, 0.079 ± 0.51, and 1.18 ± 0.35 mg/mL, respectively. Ascorbic acid showed IC_{50} values of 0.035 ± 0.17, 0.021 ± 0.7, and 0.35 ± 0.041 µg/mL for the same tests. Additionally, the total phenolic content of the extract was measured as 23.56 ± 1.42 mg GAE/g dry extract (see Table 2).

Table 2. Antioxidant activity and total phenol content of the *A. phrygia*.

Samples	Test Systems			
	DPPH scavenging activity[a] IC_{50} (mg/mL)	β-carotene–linoleic acid test activity [a] IC_{50}(mg/mL)	Hydroxyl-radical scavenging activity[a] IC_{50} (mg/mL)	Total phenol[a] (mg GAE/g extract
A. phrygia	0.183 ± 0.03	0.079 ± 0.51	1.18 ± 0.35	23.56 ± 1.42
α-tocopherol[b]	0.015 ± 0.21	–	–	–
Ascorbic acid[b]	0.035 ± 0.17	0.021 ± 0.7	0.35 ± 0.041	–

[a]IC_{50} values are presented as the means ± standard deviation from three parallel measurements (p< 0.001).
[b] Reference compounds

The anti-aging potential of *A. phrygia* was assessed based on its inhibition activities against MMP-1, elastase, and hyaluronidase. The extract demonstrated inhibition of MMP-1 (47.98%) and elastase (39.2%), while exhibiting a weaker inhibitory effect on hyaluronidase (16%) (see Table 3). EGCG exhibited MMP-1 and elastase inhibitory activities of 20.36% and 25.3%, respectively. At a concentration of 100 µg/mL, tannic acid exhibited a 62.2% inhibition of hyaluronidase activity.

Table 3. Extracellular matrix-degrading enzyme inhibition of the *A. phrygia*.

		Inhibition (%)		
Samples	Concentrations	MMP-1	Elastase	Hyaluronidase
A. Phrygia	1000 µg/mL	47.98 ± 0.17[a]	39.2 ± 0.35 [a]	16 ± 0.23 [a]
EGCG	100 µg/mL	20.36 ± 0.58 [a]	25.3 ± 0.42 [a]	–
Tannic acid	100 µg/mL	–	–	62.2 ± 0.72 [a]

[a] Standard error meaning,
Significance compared to control at p<0.001

To assess the genotoxic potential of *A. phrygia* (ranging from 0.1 µg/plate to 1000 µg/plate), the Ames *Salmonella*/microsome test was conducted. None of the tested doses of the extract induced an increase in the number of revertant colonies compared to the control (data not shown). Subsequently, the same doses were evaluated to determine the antigenotoxic effect of *A. phrygia*. Notably, it exhibited a strong anti-mutagenic effect (44.5%) against 4-NPD mutagenicity, while demonstrating moderate anti-mutagenic effects at concentrations of 100 µg/plate and 10 µg/plate. Furthermore, against NaN_3 mutagenicity, a strong inhibition effect (ranging from 65.6% to 46.6%) was observed for TA100 using all concentrations (see Table 4).

Table 4. Anti-genotoxicity of the *A. phrygia*.

Samples	Concentration	TA98		TA100	
		Number of colonies	Mutagenicity inhibition (%)	Number of colonies	Mutagenicity inhibition (%)
Negative control		20 ± 4.1[a]		150 ± 6.6[a]	
4-NPD	3.0 µg/plate	654 ± 21.03			
NaN_3	8.0 µg/plate			888 41.7	
A. phrygia	1000 µg/plate	372 ± 11.5[b]	44.5	455 ± 10.0	65.6
	100 µg/plate	425 ± 18.4	36.2	506 ± 11.1	60.0
	10 µg/plate	410 ± 15.3	38.5	591 ± 23.8	50.3
	1 µg/plate	556 ± 21.8	15.5	575 ± 19.1	52.0
	0.1 µg/plate	590 ± 20.3	10	624 ± 21.0	46.6

[a] Values expressed are means ± standard deviation.
[b] $p < 0.001$

The cytotoxic analysis of the extract was conducted on 3T3 fibroblast cells. Fibroblasts were exposed to various extract concentrations (ranging from 15.625 µg/mL to 500 µg/mL) for 24 hours. The results revealed that the extract did not exert a significant negative effect on cell viability. The IC_{50} value for 3T3 cells was determined to be 42.41 µg/mL.

4. DISCUSSION and CONCLUSION

According to recommendations from the US Food and Drug Administration (FDA), sunscreen formulations should ideally have an SPF value exceeding 2 to be considered effective (FDA, 2013). Consequently, many natural compounds are investigated to ascertain if they meet these criteria and can be categorized as 'green sunscreens' (Cefali *et al*., 2016). In this study, the SPF level was evaluated by an *in vitro* quantitative method, and the SPF value of the 500 µg/mL concentration of *A. phrygia* was determined as 4.013 (see Table 1). This value falls within the 4–6 category on the SPF scale (Jaradat *et al*., 2018), indicating moderate protection. In another study, *A. millefolium* (14.04%) and *A. biebersteinii* Afan (11.67%) extracts exhibited notable UV protective properties at a concentration of 5%, while they demonstrated the lowest SPF values at a concentration of 1.25% (*A. millefolium* (1.90%) and *A. biebersteinii* (1.85%)). (Gaweł-Bęben *et al*., 2020).

If the skin is consistently exposed to the sun, its ability to repair damage diminishes. UV radiation can penetrate deep layers of the skin, breaking down collagen and elastin fibers, and reducing the synthesis of pro-collagen. Additionally, UV exposure can generate reactive oxygen and nitrogen species, disrupting cell metabolism and functions, and triggering genetic changes (Krutmann, 2001). Natural ingredients have been explored as potential sources of sunscreen due to their antioxidant activities and ability to prevent UV ray absorption (Cefali *et al*., 2016; Fibrich & Lall, 2018; Jaradat *et al*., 2018). To our knowledge, the SPF protective activity of *A. phrygia* was examined for the first time in this study. The SPF effect of *A. phrygia* may be attributed to active components such as apigenin, quercetin, rutin, or luteolin. These

components have chemical groups and conjugated bonds capable of absorbing UV radiation (Choquenet *et al*., 2008; Wölfle *et al*., 2012; Saewan & Jimtaisong, 2013).

The scavenging activity of free radicals DPPH, H_2O_2, and the total antioxidant effect of *A. phrygia* were determined and are presented in Table 2. The test results were compared with α-tocopherol and ascorbic acid. In the DPPH test, the extract exhibited antioxidant potency with an IC_{50} value of 0.183 ± 0.03 mg/mL. Considering that raw plant extracts contain various ingredients, the synergistic effect between these components may contribute to the antioxidant effect. Subsequently, the activity of *A. phrygia* was analyzed using the β-carotene method and compared to ascorbic acid. The total antioxidant activities of *A. phrygia* and ascorbic acid were determined to have IC_{50} values of 0.079 ± 0.51 mg/mL and 0.021 ± 0.7 mg/mL, respectively. The hydroxyl radical is known to be one of the most powerful oxidants, capable of attacking organic molecules and causing mutagenesis, cytotoxicity, carcinogenesis, and other diseases (Sun *et al*. 2020). The IC_{50} inhibitory concentration of *A. phrygia* was measured as 1.18 ± 0.35 mg/mL, while for ascorbic acid, it was 0.35 ± 0.041 mg/mL.

Several studies have been carried out to explore the antioxidant properties of various *Achillea* species. However, only two studies have been identified concerning the antioxidant activity of extracts from *A. phrygia* (Zengin *et al*., 2017a; Doğan *et al*., 2022). Zengin *et al*. (2017a) focused on *A. phrygia*, assessing the antioxidant capacities of its methanol, water, and ethyl acetate extracts, with the methanol extract displaying the highest DPPH scavenging activity and the water extract showing the highest ABTS radical scavenging ability. Another study by Doğan *et al*. (2022) evaluated eight sub-extracts derived from *A. phrygia*, revealing varying antioxidant activities with DPPH IC_{50} values ranging from 0.399 to 1.399 mg/mL, CUPRAC values ranging from 0.422 mM to 2.149 mM, and FRAP values ranging between 28.050 to 40.984 mM.

Antioxidants play a crucial role in alleviating skin aging by neutralizing ROS that have already formed. They also serve to prevent photodamage and offer protection against skin cancer. Among the ingredients utilized in cosmetics, antioxidants hold significant importance. While sunscreens provide essential UV protection, they alone cannot offer complete defense against UV damage. Therefore, antioxidants are often incorporated into the formulation of sun protection products to prevent and mitigate skin wrinkles induced by UV radiation. The primary claim regarding antioxidants revolves around their anti-aging effect, which involves reducing wrinkles by combatting free radicals generated by sunlight exposure (Ramos-e-Silva *et al*., 2013).

The phenolic contents were examined using the Folin-Ciocalteu assay, yielding a value of 23.56 ± 1.42 mg GAE/g extract, as detailed in Table 2. Previous studies have highlighted *Achillea* species as rich sources of phytochemical compounds (Agar *et al*., 2015; Zengin *et al*., 2017b; Afshari *et al*. 2018; Barda *et al*., 2021). For example, Zengin *et al*. (2017a) examined various *A. phrygia* extracts and determined that the highest total phenolic content was in the methanol (41.13 mg GAE/g extract)> water (35.53 mg GAE/g extract)>ethyl acetate (23.62 mg GAE/g extract) extracts, respectively.

In vitro enzyme inhibitory activity experiments have revealed that *A. phrygia* exhibits significant inhibitory effects on elastase and MMP-1 enzymes, with percentages of 39.2% and 47.98% observed at a concentration of 1 mg/mL, respectively (Table 3). Collagen, hyaluronic acid, and elastin, which are key components of the ECM, play crucial roles in maintaining skin moisture, elasticity, and structural integrity. Therefore, depletion or damage to these components can lead to skin wrinkling and other signs of aging (Madan & Nanda, 2018). The increased activation of ECM-degrading enzymes contributes to the degradation and irregularity of these components, which is a primary cause of skin aging. There exists a delicate balance between the degradation and synthesis of these components in young and healthy skin, a balance that diminishes with age (Fibrich & Lall, 2018). Additionally, UVB exposure disrupts

biological processes that support the expression of extracellular matrix components (Madan & Nanda, 2018).

Inhibition of elastase and collagenase activities can prevent the loss of skin structural integrity or sagging of the skin. In a study involving *A. clypeolata* Sibth. & Sm., a methanolic extract from this species exhibited inhibitory effects against hyaluronidase, collagenase, and elastase enzymes (Barak *et al*., 2023). Similarly, aqueous and ethanolic extracts of *A. sintenisii* Hub.-Mor demonstrated concentration-dependent inhibition of hyaluronidase activity (Anlas *et al*., 2023). Additionally, ethanolic extracts of *A. sintenisii* exhibited dose-dependent inhibitory effects against collagenase, hyaluronidase, and elastase enzymes (Eruygur *et al*., 2023). The enzyme inhibition observed in *A. phrygia* may be attributed to its phenolic and flavonoid compounds. For example, in a study, apigenin inhibited MMP-1 induction in human dermal fibroblasts (Lee *et al*., 2007). Similarly, in another study, it displayed inhibition of collagenase activity. Quercetin, another active ingredient, exhibited elastase and collagenase inhibition activities (Lim & Kim, 2007). Furthermore, luteolin has been shown to inhibit MMP-1 expression and hyaluronidase activity (Wölfle *et al*., 2012).

According to genotoxicity test results, *A. phrygia* can be considered genotoxically safe. Anti-aging facial moisturizers containing a synthetic chemical product can be highly genotoxic (Alnuqaydan & Sanderson, 2016). Genotoxicity assessment of compounds with cosmetic potential is a fundamental need at an early stage of any cosmetic formulation (Meena & Mohandass, 2019). In the study, none of the tested doses of the extract had genotoxic effects. Additionally, *A. teretifolia* Willd. aqueous extract showed no mutagenic effects on *S. typhimurium* strains TA98 and TA100 in the Ames test (Akyil *et al*., 2012).

The extract showed a strong anti-mutagenic effect (44.5%) against the 4-NPD mutagenicity at 1000 µg/plate concentration, demonstrating strong inhibition effects (ranging from 65.6% to 46.6%) against NaN_3 mutagenicity across all concentrations (Table 4). The extract showed better activity in preventing NaN3-induced nucleotide substitution mutations compared to 4-NPD-induced frameshift mutations. When DNA damage, whether endogenous or exogenous, is not adequately repaired, mutations can occur. In somatic cells of multicellular organisms, these mutations may contribute to aging. Compounds with anti-genotoxic properties have the potential to prevent DNA damage by reducing mutagenic effects. As far as current knowledge goes, there is a lack of studies specifically investigating the antimutagenic effects of *A. phrygia*. However, research suggests that various *Achillea* species, such as *A. millefolium*, might harbor antimutagenic properties (Düsman *et al*., 2013; Hussein *et al*., 2019).

According to cytotoxic activity results of *A. phrygia*, demonstrated no significant negative impact on cell viability in 3T3 fibroblasts, with an IC_{50} value of 42.41±4.05 µg/mL. In a study, it was found that the methanolic extract from *A. wilhelmsii* C. Koch. did not induce negative effects on fibroblast viability even at concentrations ranging from 10 to 200 µg/mL. Additionally, this extract notably stimulated fibroblast proliferation starting from a concentration of 25 µg/mL (Anlaş *et al*., 2022). Similarly, within the concentration range of 50-200 µg/mL, both the ethanolic and aqueous extracts of *A. sintenisii* did not exhibit a significant decrease in fibroblast viability compared to untreated cells (Anlaş *et al*., 2023). Furthermore, Agar *et al*. (2015) observed potent proliferative effects of *A. kotschyi* Boiss. on mouse fibroblasts over a concentration range of 2.5–20 µg/mL.

A. phrygia shows promise in delaying skin aging by potentially reducing the signs of aging through various mechanisms. Its SPF property, inhibition of MMP-1 and elastase enzymes, free radical scavenging activity, and antigenotoxic effect suggest its ability to counteract skin aging. Moreover, it demonstrated genotoxic and cytotoxic safety at tested concentrations. These findings suggest that *A. phrygia* could be utilized as an anti-aging agent in cosmetic products for skincare purposes.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Rukiye Boran Gülen: Investigation, methodology, resources, visualization, writing—review and editing, **Nurdan Saraç:** methodology, validation, review and editing, **Aysel Uğur:** supervision, validation, review and editing

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Research Article

Unlocking traditional remedy: Gulkand-enhanced mucoadhesive gel for canker sore relief

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Buccal mucoadhesive gel.

Abstract: This study focuses on the development and evaluation of mucoadhesive gel formulations containing different Gulkand and its extract with a specific emphasis on their potential for managing oral health conditions, including the discomforting issue of mouth ulcers. The formulations underwent a thorough analysis, encompassing the assessment of phenolic compounds responsible for antioxidant activity, in addition to comprehensive physical, chemical, and pharmacological evaluations to determine their suitability for commercial utilization. Mouth ulcers are a prevalent oral health concern that can cause significant discomfort and inconvenience. In this study, Gulkand extract exhibited remarkable characteristics with its high phenolic content and robust antioxidant activity. It demonstrated rapid drug release, positioning it as a promising new for addressing the immediate relief needs of individuals suffering from mouth ulcers. Conversely, gel formulation showcased a sustained release profile suggesting the potential for longer therapeutic benefits, presents an intriguing option for oral health applications, capitalizing on the antiulcer properties associated with liquorice. An innovative aspect of this study is the compatibility assessment, which employed antioxidant and phenolic content analysis to verify the harmonious interaction between herbal constituents and excipients. This approach introduces novel perspective on compatibility testing, particularly critical for formulations designed to alleviate oral discomfort effectively. Furthermore, stability studies are warranted to be evaluated prior to their potential for commercialization. The present study revealed the promising potential of the prepared mucoadhesive gels in managing not only mouth ulcers but also a spectrum of oral health conditions, holding broader implications for advancements in the field of medicine.

1. INTRODUCTION

People around the globe suffer from oral ulcers once or many times during their lifetime. Mouth ulcers are the lesions that develop within the tender tissue bordering the lips, palate, inner cheek, or gums (Burley *et al.*, 2021). Canker lesions are another indication of their use. Mouth ulcers can affect people of all ages, typically more of an annoyance than a significant scientific challenge (Rai *et al.*, 2022). Even though it is no longer harmful, but are found to be linked to various diseases and conditions,including diabetes, immune disorders, inflammatory bowel

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disease, celiac disease, HIV/AIDS, and Behçet's disease (Abed *et al*., 2019; Baarah *et al.*, 2017; Seoudi *et al*., 2015). Mouth ulcers are easy to detect, usually developing on the lips, roof of the mouth, or inner cheeks of younger individuals (Ussher *et al*., 2003). Their centers are typically white, yellow, or gray, and one or more may be present in the pharynx (Dudding *et al*., 2019). Symptoms include swelling around the ulcer, prolonged tenderness when brushing the teeth, and pain that worsens after consuming highly spiced, salted, or acidic foods (Ballen *et al*., 2016; Qu *et al*., 2022). The exact cause of the buccal ulcer remains uncertain, with multiple factors contributing to their formation, such as allergic reactions to specific microbes, vitamin deficiency, using abrasive toothpaste, consuming an abundance of acidic foods, hormonal fluctuations, stress, and lack of sleep (Zhou *et al*., 2022; Orcina & Santos, 2021; Ussher *et al*., 2003).

1.1. Types of Mouth Ulcers

Based on size and quantity, mouth ulcers are classified into:

• Minor ulcers: Diameter of 2.8 mm, typically resolving in 10–13 days (Dudding *et al*., 2019).

• Major ulcers: Larger, deeper, may leave a scar, and may take few weeks to heal (Ballen *et al.*, 2016).

• Herpetiform ulcers: A collection of several tiny and, pinhead-sized lesions (Dudding *et al.*, 2019; Amin & Marouf, 2022).

1.2. Treatment and Side Effects of Mouth Ulcers

Common treatments includes application of antiseptic, local anesthetic gels, steroid ointments, and medicinal mouth ulcer rinses (Fan *et al.*, 2022). Drugs that reduces the oxidative stress by reducing the level of free radicals also plays an important in the treatment of mouth ulcers. Oral health, especially mouth ulcers, requires antioxidant activity. Studies link periodontal disease to reduced salivary antioxidant capacity, leading to increased oral cavity oxidative damage (Bhattacharya *et al*.,2014). Antioxidants known for their anti-ulcer properties, and their chemical composition have also been studied for gastric ulcers (Danisman *et al*.,2023). Antioxidants like beta carotene, selenium oxide, and zinc sulfate have been studied for treating oral submucous fibrosis, demonstrating their potential benefits (Rao *et al.*,2020). However, these may result in side effects such as inadequacy, immunosuppressant effects, osteoporosis, hyperglycemia, and gastrointestinal disturbances (Mittal *et al.*, 2023; Ballen *et al.*, 2016). Commercial formulations with synthetic and semi-synthetic active components may cause local discomfort and stains due to high alcohol content and chemical compounds (Mittal *et al*., 2023). The use of plant-based medicines is gaining significance globally due to increased patient compliance and demand (Proestos *et al.*, 2013). Herbal remedies of mouth ulcer includes chewing, consumption or application of *Basella alba, Glycyrrhiza glabra*, *Psidium guajava*, *Aloe barbadensis*, *Capsicum* sp., *Carica papaya*, *Curcuma longa*, various species of *Rosa*, *Bathinda variegate*, and *Hibiscus rosa sinensis*, are considered as safer alternatives (Çiçek *et al*., 2022; Proestos *et al*., 2013; Kumar *et al.*, 2009). In various parts of India, it was observed that Gulkand is generally applied on the mouth ulcer to treat. Gulkand, a sweet preserve made from rose petals, involves the use of rose petals from several rose species, with *Rosa damascena* being a key component (Çiçek *et al*., 2022; Fascella *et al*., 2022; Yap *et al*., 2011). Gulkand, also known as rose petal jam, is derived from the Persian words gul (rose) and qand (sweet/sugar) and is served at room temperature in the Indian subcontinent (Bhatt *et al*., 2000). Damask rose, with its powerful scent, is frequently used in the manufacture of Gulkand recipes and blossoms only twice a year, making it exclusive and sugars are generally used as a sweetening agents in the preparation of Gulkand (Çiçek *et al.*, 2022). The ideal roses employed for preparing gulkand are *Rosa centifolia* and *Rosa damascena* (Proestos *et al.*, 2013; Hajizadeh *et al*., 2023).

Hence, the primary objective of the present study was to prepare the Gulkand, and its formulations containing sugars and *Glycyrrhiza glabra* as an alternate to sugar and evaluating

their antioxidant efficacy. Secondarily an effort was also made to develop a simple method to evaluate compatibility of Gulkand and the excipients using UV and FTIR spectroscopy. Additionally, diffusion study was also conducted to confirm the amount of antioxidants available for the therapeutical efficacy.

2. MATERIAL and METHODS

2.1. Materials

In the preparation of Gulkand, a traditional confectionary delicacy, a blend of natural ingredients was employed, sourced meticulously from local markets. The petals of *Rosa damascena*, roots of *Glycyrrhiza glabra* (liquorice), and rock sugar played pivotal roles in crafting the distinctive flavor profile of the preparation. Alongside these botanical elements, a range of solvents and chemicals, including alcohol, methanol, DPPH, sodium carbonate, Folin-Ciocalteu reagent, hydrochloric acid, and vanillin were also employed in the present study. Pharmaceutical excipients such as carboxymethyl cellulose, polyethylene glycol 4000, methyl paraben, propyl paraben, and triethanolamine were carefully selected and sourced from reputed suppliers like E-Merck and Fischer. The use of various instrumental techniques, such as a freeze dryer, sonicator, UV spectrometer, Fourier-transform infrared spectroscopy (FTIR), and a rotary evaporator, ensured precision in the preparation process. This comprehensive combination of plant materials, solvents, chemicals, and instruments establishes a robust foundation for a detailed investigation into the properties and potential applications of the developed buccal mucoadhesive gel.

2.2. Methods

2.2.1. *Preparation of different types of Gulkand using different sweetening agents*

2.2.1.1. Method 1 Rose petal + Rock sugar (R+R). *Rosa damascena* fresh rose petals that have been picked are properly cleansed in clean flowing water and RO water before being dried in the shade. After drying, rose petals are crushed and combined with rock sugar in a 2:1 ratio in a glass jar with a wide mouth that is clean, dry, and airtight for a period of five days while exposed to the sun. During this period, the sugar totally melted and was thoroughly blended with the rose petals that makes the rose petals' nutrients intact.

2.2.1.2. Method 2 Rose petal + Liquorice (R+L). The collection and preparation of rose petals was carried out as the procedure given in the section 2.2.1.1. Exactly to the 50 g of dried liquorice (*Glycyrrhiza glabra*) powder, 500 mL of water was added, and then it was boiled at 100°C to produce the liquid extract. A magnetic stirrer was used to stir continuously for 30 minutes. In a mixing dish, 200 g of crushed rose petals were combined with 200 mL of the aforementioned liquorice extract and along with some preservatives. The prepared mixture was placed in a clean, dry, and airtight glass jar with a wide mouth till the formation of Gulkand.

2.2.1.3. Method 3 Rose petal +Liquorice prepared on frying pan (R+L, pan). In this method, liquorice Gulkand was prepared by using a frying pan. Hundred grams of the rose petals were gathered and added to the pan. In a clean skillet, 100 mL of the liquorice extract prepared in step 2 was added and cooked until it turned to brown colour. This procedure was aimed to maintain the nutrients found in rose petals. The resulting mixture was stored in a clean, dry, and airtight glass jar with a wide opening.

A pilot study was performed to determine the stability of the prepared samples prior to the use of methods 2 and 3.

2.2.2. *Evaluation of gulkand*

The prepared Gulkands' colour, flavour, stability, and consistency were assessed at regular intervals.

2.2.3. *Drying, powdering and extraction of gulkand composed of liquorice*

2.2.3.1. Drying. The aforementioned preparations (R+R, R+L, and R+L pan) were dried in a lyophilizer after few days. Below given steps are followed to dry the prepared samples:
Step 1: Samples are maintained in a beaker with aluminium foil covering them.
Step 2: Samples are stored in the primary drying chamber for 11 hours and the secondary drying chamber for 12 hours after being pre-frozen in the freezer.

2.2.3.2. Powdering. While powdering the freeze dried samples, a temperature of 35° C was maintained throughout the process in order to preserve the bioactive phytoconstituents present in the prepared samples. To keep the samples fresh for a long period, the powdered samples are placed in a centrifuge tube, sealed with parafilm, and preserved there.

2.2.3.3. Extraction. Only sample 2 (R+L) underwent extraction since it had higher antioxidant activity than the other samples.

Preparation of extract of R+L sample: *Rosa damascena* petals powdered gulkand and 60g of liquorice were placed in a conical flask and extracted for 1 hour using the sonication process with 80% of 1200 mL alcohol (300 mL X 4 times). The extracted solvent was transferred into a rotary evaporator operated at 50° C. The dried extract was collected after the concentrated viscous extract was once again maintained in a water bath at a temperature of 50^{o} C.

2.2.4. *Determination of antioxidant activity*

The DPPH test measures the antioxidant capacity of the herbal extract, as well as its composition, standards as well as also employed for the first time to study the compatibility of samples. DPPH was dissolved in methanol to create the DPPH reagent, which was then further diluted to get the final concentration of 0.4 mm DPPH. Gallic acid was utilised as a positive control while various concentrations of extract, powder, petals, and formulations were made by dissolving in methanol. One mililitre of the DPPH reagent was added to the various sample concentrations used in the test, vortexed, and left in the dark for 30 minutes. A UV visible spectrophotometer was used to evaluate the reduction in absorbance caused by the scavenging of the DPPH free radical at a wavelength of 517 nm (Proestos *et al*., 2013).

The antioxidant activity in terms of percentage inhibition was determined using equation

$$\% \text{ inhibition} = (Ac\text{-}As/Ac) \times 100$$

Where, As an Ac is the absorbance of the sample solution and control, respectively.

2.2.5. *Total phenolic content*

Using the Folin-Ciocalteu reagent, a complicated combination of heteropolyphosphotungstate molybdate, the total quantity of phenolic-related chemicals contained in the sample was calculated. In the presence of sodium carbonate, phenols undergo FC reagent treatment and produce a blue colour complex. The quantity of reactive phenolic chemicals in the sample directly relates to how intense the blue colour is. This approach can measure the overall polyphenolic content of the extracts, which ranges from 5 to 100% (w/w), in terms of standard gallic acid.

Procedure:

➢ The standard calibration curve was prepared using gallic acid.

➢ Folin-Ciocalteau reagent was added after sample stock solutions were created at a concentration of 10 g/mL.

➢ 1.6 mL of Na_2CO_3 (7.5%) was added as an alkaline medium after a 6 minute break.

➢ The mixture was completely vortexed before being left to incubate in the dark for a full hour.

➢Using a UV-Visible Spectrophotometer set at 765 nm, the color developed by the samples was examined, and the absorbance was recorded (Kumar *et al*., 2009).

2.2.6. *Crude drug- excipient interaction studies*

Gulkand powders of R+R, R+L, and extract of R+L are utilised to create gel formulations. Prior to formula preparation, the following procedures were used to assess compatibility:

2.2.6.1. Drug –Excipients interaction (compatibility) study by FTIR. Fourier Transform Infrared spectrometer (FTIR) analysis is performed on samples containing R+R, R+L gelling agents such as carbopol 940, sodium CMC, Chitosan, Polyethylene glycol (PEG), and preservatives such as methyl paraben and propyl paraben. The aforementioned gulkand samples and excipients were weighed, combined, and delivered to CRI (centre of research innovation), ACU, and B. G. Nagara for FTIR for the compatibility studies (Shaw *et al.*, 2017; Zou *et al.*, 2020).

2.2.6.2. Drug –Excipients interaction (compatibility) study by antioxidant activity. The DPPH technique was used to measure antioxidant activity after dissolving the weighted quantity of samples and excipients in different amounts of water.

2.2.7. *Preparation of buccal mucoadhesive gel*

Methyl and propyl paraben were accurately weighed and dissolved in water heated to 80°C. A weighed amount of sodium CMC was dissolved in water at 50°C for 30 min while being continuously stirred with a mechanical stirrer spinning at 2000 rpm. In accordance with its solubility, PEG 4000 has transformed into liquid form. With steady stirring, the weighed amount of samples and extract are dissolved in PEG 4000 solvent, and then they are added to the gel base. To create a uniform gel, constant stirring is helpful. Triethanolamine was used to adjust the pH to 7, and the mixture was carefully agitated until a clear gel was produced (Aslani *et al*., 2013). Information related to the composition of mucoadhesive gel is given in the Table 1.

Table 1. Composition of mucoadhesive gel.

Ingredients	Formulations			Uses
	R+R (p) in g	R+L (p) in g	R+L (e) in g	
Sodium CMC	1.5	1.5	1.5	Gelling agent
Methyl paraben	0.09	0.09	0.09	Preservative
Propyl paraben	0.01	0.01	0.01	Preservative
PEG 4000	6.5	6.5	6.5	Osmotic agent
R + R powder	10			Active ingredient
R + L powder		10		Active ingredient
R + L extract			1	Active ingredient

R + R (p): Rose petal and rock sugar powder, R + L (p): Rose petal and Licorice powder and R + L (e): Rose petal and Licorice extract.

2.2.8. *Physical and chemical evaluation of buccal mucoadhesive gel*

2.2.8.1. Physical appearance of gel formulations. Clearness, color, homogeneity, consistency, and the presence of particles in gel compositions were all visually evaluated. A microscope was used to check homogeneity. A small amount of gel was pushed between the thumb and index fingers in order to examine the formulations' consistency. The gel's consistency was then noted.

2.2.8.2. Determination of pH in the gel formulations. The produced gels' pH was assessed using a pH meter, which was calibrated with standard buffer solutions at pH 4 and 7 before each usage. In 10 mL of pure water, each gel formulation was precisely weighed and mixed.

The pH of the samples was recorded after the electrode had been put into the sample for ten minutes at room temperature. The pH was measured after 48 hours, one week, two weeks, and one month.

2.2.8.3. Centrifugal test. The formulation after 48 hours of preparation were transferred into centrifuge tubes and centrifuged at 2000 rpm for 60 min using a centrifuge device (Centrifuge 5430). The stability of the formulations was assessed at the times of 5, 15, and 60 min to investigate the stability of the formulations against the centrifugal force.

2.2.8.4. Drug content determination in gel formulations. After 48 hours of preparation of gel, one gram of the gel was taken and dissolved in water in a volumetric flask with a 10 mL capacity. The Folin-Ciocalteu technique and the DPPH method were carried out to assess the total phenolic content and antioxidant activity of the prepared formulation.

2.2.8.5. Determination of viscosity. The gel formulations were studied for viscosity 30 mins after the preparation at room temperature. Brookfield DV-II viscometer was used to evaluate the produced gels' viscosity at 100 rpm and 25°C using spindle number 7.

2.2.9. *Ex vivo drug release study*

In order to carry out the drug release study goat buccal mucosa membrane was purchased from a slaughterhouse in Bellur, Mandya was employed as a barrier membrane. The gels were tested using a Franz diffusion cell for drug release. Buccal mucosal membrane was mounted between the donor and receptor compartments. The diffusion cell was positioned in simulated saliva that was kept at a constant 37 °C. A magnetic bead was used to stir at 300 rpm while 50 mL of phosphate buffer (pH 7.4) was added to the receptor compartment to maintain hydrodynamics. In order to maintain the volume of the liquid medium, 5mL of the sample was removed, and the tube was then filled with 5mL of fresh medium. The material was examined in a UV spectrophotometer at a wavelength of 226 nm.

Composition of saliva: To get a saliva of pH 6.75, add phosphoric acid to 2.38 g of Na_2HPO_4, 0.19 g of KH_2PO_4, and 8.00 g of NaCl per litre of distilled water.

Composition of Phosphate buffer solution: To 393.4 mL of 0.1M sodium hydroxide, add 250 mL of 0.2M potassium dehydrogenate phosphate (Aslani *et al.*, 2013).

2.2.10. *Statistical analysis*

Statistical analyses, including One-way ANOVA, Tukey's HSD post-hoc test, Student's t-test, correlation analysis, Chi-square test, and descriptive statistics, were conducted to evaluate experimental data. These methods compared means, identified significant differences, explored relationships between variables, and assessed compatibility. By employing these statistical techniques, the study enhanced the robustness and validity of results, providing valuable insights into the effectiveness of different gulkand samples and gel formulations.

3. FINDINGS

3.1. Preparation of Different Types of Gulkand Using Different Sweetening Agents Such as Rock Sugar and Liquorice

The collected rose petals underwent a thorough cleaning before air drying for three to five days, and the the prepared gulkand was kept in an airtight container.

3.2. Evaluation of Gulkand

3.2.1. *Morphological evaluation*

Table 2 provides the results of a morphological examination of several types of Gulkand based on their flavour, consistency, texture, and particle size. The Table 2 shows that while all of the gulkand samples had the same aromatic odour, consistency, texture, and particles, they had different tastes because the gulkand prepared with liquorice solvent had a sweet and mildly sour flavour. The semisolid consistency and pungent odour of each batch of gulkand were the same.

Table 2. Morphological evaluation of different gulkand samples.

Sample	Odour	Taste	Consistency	Texture	Particles
R+R	Aromatic	Sweet & soothen	Semi solid	Thick semi solid	Broken petals found
R+L	Aromatic	Sweet & sour	Semi solid	Thick semi solid	Broken petals found
R+L pan	Aromatic	Slightly sweet	Semi solid	Thick semi solid	Broken petals found

3.3. Antioxidant Activity of Various Samples

Using the DPPH technique, the antioxidant activity of the prepared Gulkand stored at room temperature, stored in refrigerator, freeze-dried gulkand and the extract of the Gulkand was assessed and the results of the study was depicted in the Figure 1. It is evident from the Figure 1 that among prepared Gulkand, Gulkand stored at room temperature possess good antioxidant activity at a concentration of 2.5 mg/mL, and the activity was increased after storing in refrigerator and after freeze-drying. Among the different Gulkand extracts, Gulkand of R + L freeze dried powder extract at the concentration of 2.5 ug showed significant antioxidant activity followed by R + L Gulkand, R + R Gulkand and R + L pan Gulkand at the concentration of 2.5 mg/mL. However, the activity was found to significantly lesser than gallic acid but significantly higher than ascorbic acid at the same concentration.

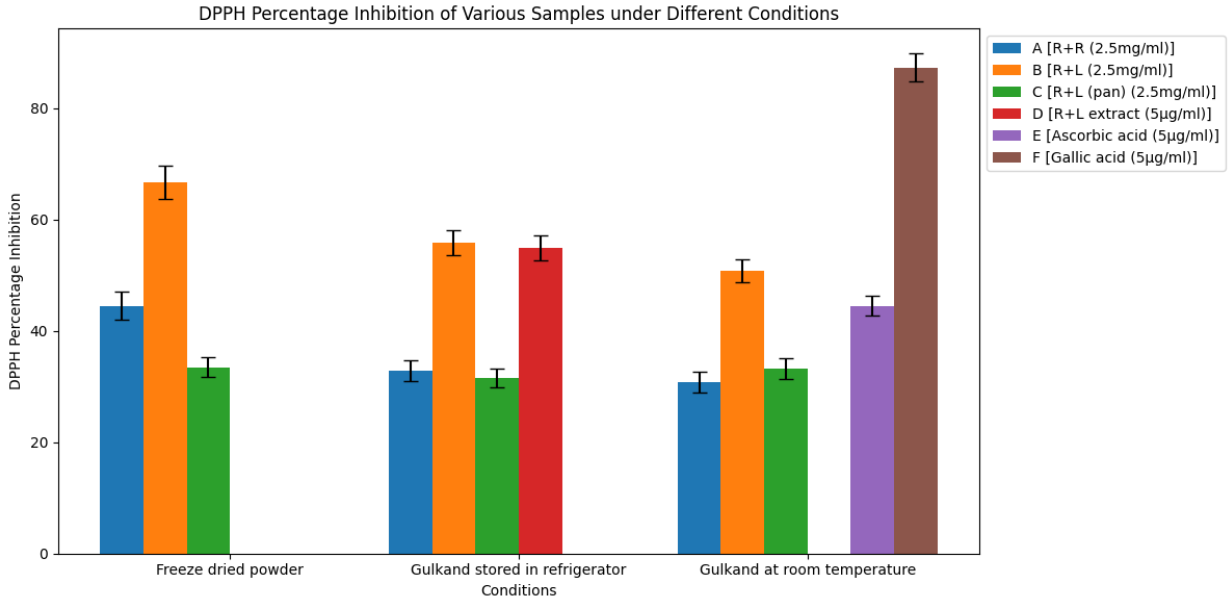

Figure 1. DPPH Percentage Inhibition of various samples under different conditions.

3.4. Total Phenolic Content

After determining the antioxidant activity of the Gulkand samples, the total phenolic compounds present in them were analyzed. Results of the same is represented in Table 3. Extract of Gulkand (R+L) contained significant amount of phenolic compounds, followed by R+L and R+R.

Table 3. Total Phenolic content of different Gulkand (n = 3) samples.

Samples	mg GAE/g extract
R+L	23.04±1.60
R+R	20.91±0.32
R+L (E)	43.25±0.54

3.5. Crude Drug - Excipient Interaction Studies

3.5.1. *Antioxidant activity of different samples with excipients*

The compatibility study results for numerous Gulkand samples and various excipients employed in the the preparation of formulations are shown in Figure 2. The antioxidant activity was reported for the first time as a factor in determining compatibility. The combinations of gulkand samples and excipients were evaluated for their antioxidant activity, and compatibility was determined based on either decrease or increase in the antioxidant activity or remained unchanged.

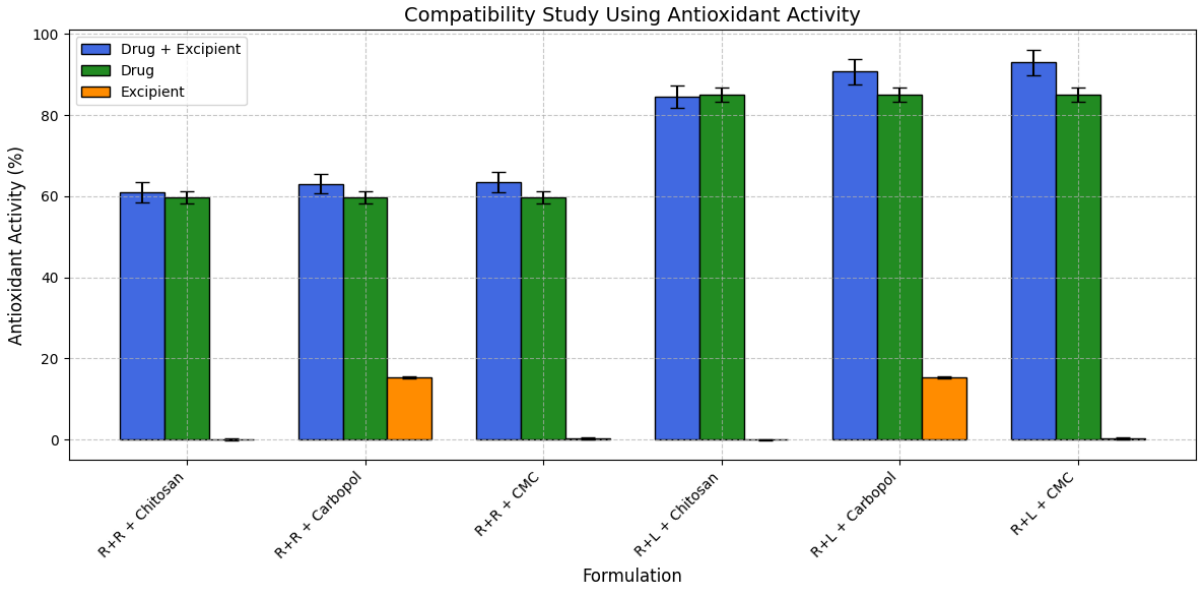

Figure 2. Compatibility study using antioxidant activity.

3.5.2. *Compatibility study of gulkand samples and excipients by FTIR*

In order to substantiate the results of the antioxidant compatibility study, FTIR analysis was performed for the same samples. The detector on the FTIR spectrometer was a deuterated triglyceride sulphate (DTGS), and IR spectra were recorded in the range 400–4000 cm^{-1} with a resolution of 4 cm^{-1}. The room was maintained at a controlled temperature of 25 degrees Celsius and relative humidity of 30%. Dry gulkand powder of sample R+L (S1) and R+L extract (S2) are weighed and compared with excipients S3 and S4. This is their graphical representation. Figure 3 depict the IR spectrum of the R+R powder sample alone and in combination with CMC. The characteristic peaks derived from the FTIR of R+R powder alone and in combination with the CMC excipient have been analyzed and reported in Table 4. This table demonstrates that the characteristic peaks of the R+R powder were unaffected by the presence of CMC excipients, indicating that the R+R powder and CMC excipients are compatible. Figure 4 depict the IR spectrum of the R+L(E) sample alone and in combination with CMC. Table 5 details the presence of characteristic FTIR peaks in R+L(E) alone and in combination with the CMC excipient. It is evident from this Table 5 that none of the characteristic peaks of the R+L(E) changed in the presence of CMC excipients, indicating that the R+L powder is compatible with CMC excipients.

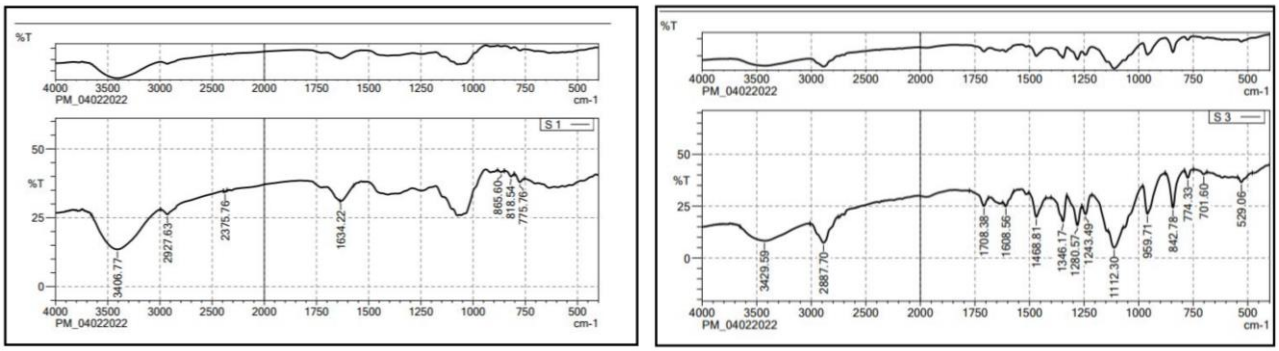

Figure 3. FTIR spectra of R+R (P) and R+R (P) with CMC.

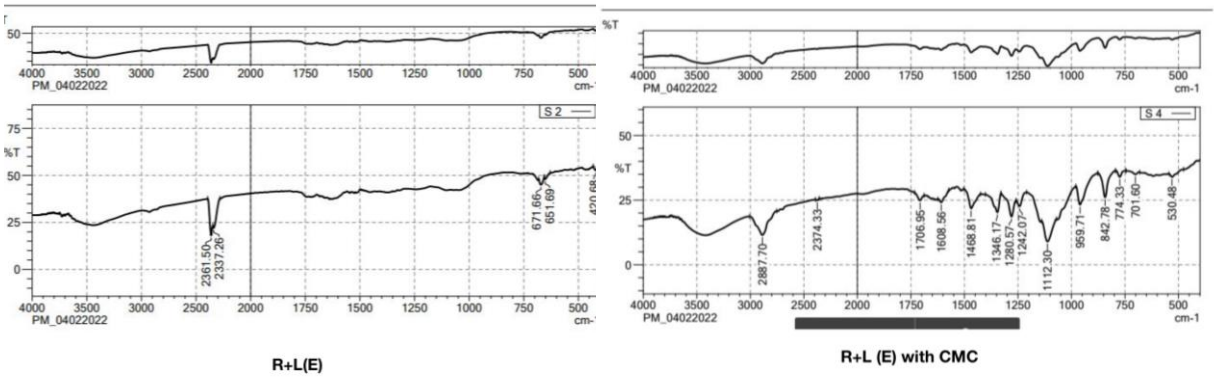

Figure 4. R+L (E) and R+L (E) with CMC.

Table 4. Comparison of R+R (P) and R+R (P) with CMC.

R+R (P)		R+R (P) and CMC	
Peaks	Functional group	Peaks	Functional group
3406.77	NH	3429.59	NH
2927.63	O-H and COOH	2887.70	O-H and COOH
2375.76	C≡N	2380.32	C≡N
1634.22	C=C, N-H, C=O	16008.56	C=C, N-H, C=O
865.6	C-H	842.78	CH,
818.54	CH, NH	842.78	CH, NH,
775.56	CH, NH, C-Cl	774.33	CH, NH, CCl

Table 5. Comparison of R+L (E) and R+L (E) with CMC.

R+L (E)		R+L (E) and CMC	
Peak	Functional group	Peak	Functional group
2361.5	C≡N	2374.33	C≡N
2337.26	C≡N	2372.26	C≡N
671.66	CH, CCL,	701.6	CH, CCl
651.69	CH, CCl, C-BR, Cl	580.6	CH, CCl, C-BR, Cl

3.7. Physical Evaluation of Gel Formulation

Buccal mucoadhesive gels were prepared utilising R+R Gulkand, R+L Gulkand, and R+L Gulkand extracts with CMC excipients and subjected to physical evaluation methods including physical appearance, centrifugation test, pH, and viscosity.

3.7.1. *Physical appearance*

The physical characteristics of the formulated gel, including its color, clarity, homogeneity, consistency, and particle presence, are detailed in Table 6. The formulations R+R (p) and R+L (p) shared identical characteristics due to the inclusion of the soluble extract in the formulations prepared in the present study. However, the gel of R + L extract was found be very clear, homogenous and with no particles present.

Table 6. Physical appearance of the different formulated gels.

Samples	Clarity	Colour	Homogeneity	Consistency	Presence of particles
R+R (p)	Not clear	Brown	Not homogeneous	Gritty	Thick particles
R+L (p)	Not clear	Dark brown	Not homogeneous	Gritty	Fine particles
R+L (e)	Not clear	Yellowish brown	Homogeneous	Clear	No particles

3.7.2. *Determination of pH and thermal tests of the prepared formulations*

The pH of all mucoadhesive gels was evaluated to ensure their suitability for the delicate buccal cavity. As indicated in the Table 7, pH of the gels remained within the neutral range specified by regulatory agencies at different tested temperature and at different duration of intervals.

Table 7. pH of the formulations at different temperature and duration.

Samples	Day 1	Day 2	Week 1	Week 2	1 month
R+L (E) 4° C	ND	6.50	6.48	6.48	6.29
R+L (E) 25° C	6.58	6.58	6.52	6.56	6.47
R+L (E) 45° C	ND	6.66	6.58	6.59	6.57
R+R (P) 4° C	ND	7.16	7.12	7.13	7.15
R+R (P) 25° C	7.11	7.10	7.10	7.09	7.10
R+R (P) 45° C	ND		7.21	7.23	7.26
R+L (P) 4° C	ND	6.81	6.80	6.81	6.82
R+L (P) 25° C	6.81	6.78	6.85	6.81	6.82
R+L (P) 45° C	ND	6.71	6.62	6.68	6.61

ND: Not Determined

3.7.3. *Centrifugal test*

Formulations were subjected to a centrifugal test to determine their stability against centrifugal force. The results indicate that none of the formulations exhibited sedimentation at different time intervals confirming their stability.

3.7.4. *Determination of viscosity*

The viscosity of the prepared gels was measured using a Brookfield DV-III viscometer. The gel prepared with rock sugar (R+R) exhibited the highest viscosity, followed by the gel prepared with liquorice and liquorice extract and the result is shown in the Table 8.

Table 8. Viscosity of the formulations.

Sample	Viscosity
R+L (P) gel	7760 cP
R+L (E) gel	2680 cP
R+R (P) gel	29720 cP

P: Powder, E: Extract and cP: Centipoise

3.8. Antioxidant Activity and Total Phenolic Content

Antioxidant activity and total phenolic content of the prepared gels were evaluated and shown in Table 9 and 10. The gel prepared with liquorice extract [(R+L (E)] exhibited higher antioxidant activity and phenolic content compared to the gels prepared with rock sugar [(R+R (P)] and liquorice [(R+L (P)].

Table 9. Antioxidant activity of different formulated gels (n=3).

Samples	% of inhibition DPPH
R+L (E) gel	86.23 ±0.93^{b}
R+L (P) gel	77.12 ±2.73^{a}
R+R (P) gel	78.09 ±0.73^{a}

Values are represented as mean ± standard deviation. Values in the same column with different alphabets in superscripts represent significant differences.

Table 10. Phenolic content determination (n=3).

Sample	GAE
R+L (E) gel	504.5 ±8.24^{c}
R+L (P) gel	375.1 ± 4.82^{a}
R+R (P) gel	391.4 ±0.47^{b}

Values are represented as mean ± standard deviation. Values in the same column with different alphabets indicates the presence of significant differences.

3.9. *In- vitro* Drug Release Study

In-vitro drug release studies was conducted using Franz diffusion cells. The release profiles of the gels are illustrated in Figure 5. The gel prepared with Liquorice extract [(R+L (E)] exhibited a sustained drug release profile compared to the other gels.

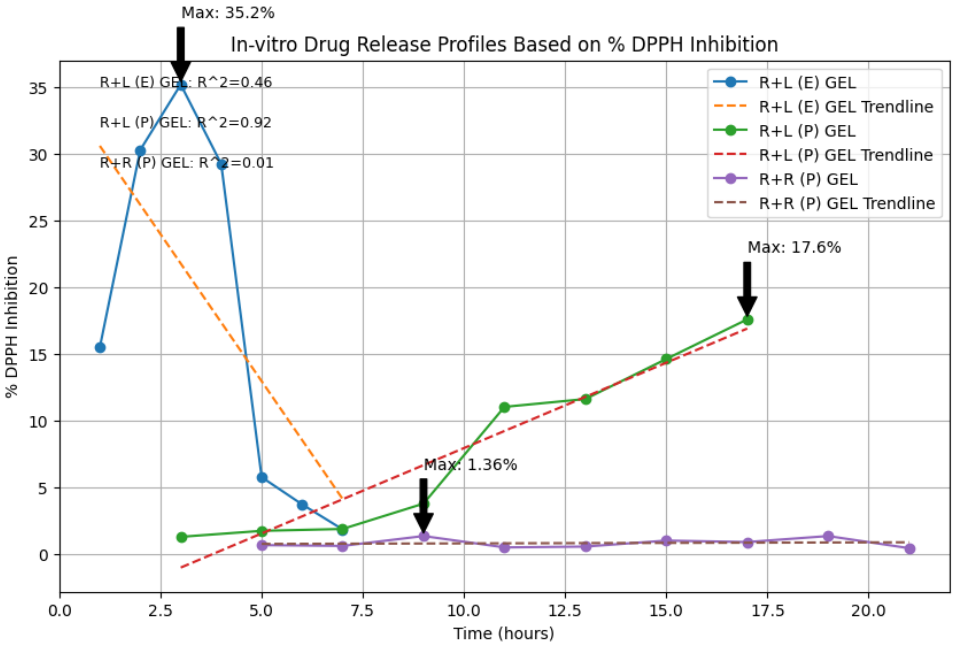

Figure 5. In-vitro drug release profile based on % DPPH Inhibition.

4. DISCUSSION

In the "Results" section, we presented an extensive evaluation of the different types of Gulkand and their characteristics, including morphological evaluation, antioxidant activity, total phenolic content, compatibility with excipients, physical evaluation of gel formulations, antioxidant activity, and phenolic content of gels, *in-vitro* drug release studies, and buccal permeation studies. The following is a discussion of the key findings and their implications: The morphological evaluation of different gulkand samples revealed intriguing differences in taste while maintaining consistent odours, texture, and the presence of broken rose petals. The variations in taste between samples can be attributed to the different sweetening agents used in their preparation, with liquorice-based gulkand exhibiting a sweet and mildly sour flavour. Despite these taste differences, the semisolid consistency and pungent odour remained consistent across all batches of Gulkand. This suggests that the base ingredients of rose petals and sugar, in different forms, provide the core characteristics of Gulkand.

4.1. Antioxidant Activity and Total Phenolic Content of Gulkand Samples

The assessment of antioxidant activity and total phenolic content provided valuable insights. Gulkand prepared with liquorice extract (R+L) demonstrated the highest antioxidant activity and phenolic content among all the samples tested. This can be attributed to the presence of liquorice, a known strong antioxidant due to the presence of various bioactive constituents (Pastorino *et al.*, 2018). Furthermore, the freeze-dried Gulkand powder exhibited superior antioxidant activity compared to the raw and frozen forms, indicating that the lyophilization process enhances the antioxidant potential compared with other drying process (Annegowda *et al.*, 2013; González *et al.*, 2023). Even the lyophilzed samples easily powdered and have smaller particle size and increased surface area that enhances the release of potent phenolic content responsible for antioxidant activity. Additionally, raw and frozen Gulkand samples are composed of higher moisture content that may be contributing for the weight of the samples compared with the freeze dried samples along with deterioration of bioactive compounds present in the Gulkand samples during the processing.

4.2. Compatibility Studies

Compatibility is crucial to ensure the stability and effectiveness of the final product. The innovative approach of using antioxidant activity to assess compatibility between Gulkand samples and excipients proved useful. This is the first study to report the application of DPPH

driven antioxidant activity to determine the compatibility of the samples and excipients used in the preparation of formulations. The results demonstrated that most combinations of gulkand samples and excipients were compatible as there was no change in the antioxidant activity of the samples after mixing with the excipients, making them suitable for the preparation of mucoadhesive gel formulations. In addition, the present method employed for the evaluation of compatibility study was found to be simple, cost effective and rapid.

4.3. Physical Evaluation of Gel Formulations

The physical evaluation of the gel formulations highlighted differences in clarity, color, homogeneity, consistency, and the presence of particles. Notably, the addition of soluble extract in the R+L (e) gel resulted in improved characteristics compared to other formulations ensuring the application of extracts rather than the crude Gulkand to be used as an ingredient in the preparation of gel. These physical properties play a role in the patient acceptability and usability of the gels for buccal administration.

4.4. pH and Stability

The pH values of the gel formulations is a crucial factor for their suitability. Results of the present study indicated that pH of the formulation remained within the neutral pH range. Additionally, the stability of the formulations against centrifugal force was also confirmed, indicating that they can withstand handling and transportation.

4.5. Viscosity

Viscosity measurements showed that the gel prepared with rock sugar (R+R) had the lowest viscosity, followed by the gel prepared with licorice extract [(R+L (E)]. Viscosity is a critical factor in mucoadhesive gels, as it influences their ability to adhere to the buccal mucosa and diffusion of the same.

4.6. Antioxidant Activity and Phenolic Content of Gel Formulations

The gels retained their antioxidant activity, and the gel prepared with licorice extract [(R+L (E)] exhibited the highest antioxidant activity among the gel formulations. This suggests that the gel formulation process did not compromise the gulkand's antioxidant properties. The determination of phenolic content further confirmed the presence of bioactive compounds in the gel formulations that may be responsible for the found antioxidant activities too.

4.7. *Ex-vivo* Drug Release Study

The *ex-vivo* drug release study provided crucial insights into how these gel formulations perform when applied to buccal mucosal membranes. The results indicated that the gel formulated with liquorice extract [(R+L (E)] achieved rapid drug release within one minute and maintained this release profile for up to five minutes. This rapid release can be attributed to the small particle size and high diffusion potential of the formulation. It suggests that R+L (E) may be well-suited for providing quick relief from oral ulcers. In accordance with our results, Jiang *et al.* (2021) reported that increased viscosity decreased the diffusion. On the other hand, the gel prepared with rock sugar [(R+R (P)] exhibited slower drug release, which may be attributed to its higher viscosity and larger particle size. This slow release could potentially lead to a longer-lasting therapeutic effect, making it a suitable option for individuals seeking sustained relief.

4.8. Antioxidant Activity and Phenolic Content of The Formulated Gels

After the gel formulations were prepared, their antioxidant activity and phenolic content were assessed. These evaluations are critical because they provide information about the formulations 'ability to retain the beneficial properties of Gulkand and its formulations. The results demonstrated that all three gel formulations retained excellent antioxidant activity. This is a promising finding, as it suggests that the gel preparation process did not compromise the formulations 'ability to scavenge free radicals and provide potential health benefits.

Furthermore, the determination of phenolic content showed that the gel prepared with gulkand [R+L (E) gel] contained the highest concentration of phenolic compounds. This aligns with the superior antioxidant activity observed in this formulation. The high phenolic content indicates that the Gulkand was effectively integrated into the gel and maintained its bioactive constituents.

4.9. Overall Implications

The comprehensive assessment of Gulkand samples carried out in the present study revealed their incorporation into mucoadhesive gel formulations as several important implications. First, it highlights the potential of gulkand, especially when prepared with liquorice extract, as a valuable component for herbal oral ulcer therapy. The different release profiles of the gel formulations (rapid vs. sustained) provide flexibility in tailoring treatment options to individual patient needs.

Second, the compatibility studies conducted using antioxidant activity as a criterion offer a novel approach to ensuring the stability of gulkand-based formulations. This approach can be applied to future research involving herbal formulations to assess their suitability for specific applications as it is found to be very simple, fast and cost effective as well as reliable.

Third, the retention of antioxidant activity and phenolic content in the gel formulations suggests that gulkand-based gels can provide not only relief from oral ulcers but also potential antioxidant and health-promoting effects.

In conclusion, the findings from this study demonstrate the potential of gulkand-based mucoadhesive gels as effective and versatile options for oral ulcer treatment. The ability to modulate drug release profiles and maintain antioxidant activity and phenolic content makes these formulations promising candidates for further research and development in the field of herbal medicine.

5. CONCLUSION

In the present investigation, three oral mucoadhesive gel formulations containing gulkand powder and extract were created. At each stage of gel preparation, samples were analysed for the presence of phenolic compounds responsible for antioxidant activity as well as the formulations' antioxidant activity. In addition, physical, chemical, and pharmacological evaluations were performed on the prepared formulations to determine their commercial viability. All of the prepared mucoadhesive gel preparations met the physical requirements. The extract of gulkand produced a substance with a high phenolic content and antioxidant activity. Within a minute, even it is released quicker from the mucosal membrane. Therefore, it will be the optimal formulation if rapid drug release is required. Even after 21 minutes, the R+R gel phytoconstituents do not discharge. This may be due to the larger particulate size of the gulkand powder. Even the R+L gel demonstrated the release of antioxidants at 11 minutes, with the process concluding at 17 minutes. Thus, both formulations assure their suitability as mucoadhesive gels for the release of antioxidant compounds necessary for the treatment of various types of mouth wounds/ulcers. Because liquorice is a potent anti-ulcer agent, the outcome of the present study was a correlation between antioxidant and anti-ulcer activity. The herbal constituents and excipients were found to be compatible through a compatibility study. To the best of our knowledge, this is the first study to report compatibility testing using antioxidant and phenolic content analysis. Further research is required to examine the in vivo anti-ulcer activity of these formulations in animal models and to determine their stability for commercialisation.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Manoj Madanahalli Ramesh: Investigation, Resources, Visualization, Software, Formal Analysis, and Writing - original draft. **Annegowda Hardur Venkatappa:** Methodology, Supervision, and Validation.

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Research Article

Wound healing and coagulant activity of crude extract metabolites from fungal endophytes

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Abstract: Bleeding from a wound as a result of physical injury is a life-threatening condition. In pursuing new drug structures, the effect of crude fungal extracts fungal isolated from *Jatropha multifida* on wound healing and coagulation of mouse whole blood was investigated. *Jatropha multifida* leaves were sterilized, cut into small segments, and then incubated in Potato Dextrose Agar for seven days. Four isolates were purified and their morphologies were characterized. Identification of isolates was confirmed by a molecular protocol. Two crude extracts from *Phlebiopsis gigantea* (OK021602) and *Phyllosticta* sp (OK021603), which exhibited higher phytochemicals composition, were selected and evaluated using wound excision and coagulation of mouse whole blood, by administering 30 µg/mL, 50 µg/mL and 70 µg/mL crude extracts respectively. The percentage of wound healing in mice was higher (p<0.05) for the crude extracts of *Phlebiopsis gigantea* (OK021602) as compared to that of *Phyllosticta* sp (OK021603). The highest percentages of wound contraction were 99% at 70 µg/mL, and 53% at 70 µg/mL for *Phlebiopsis gigantea* (OK021602) and *Phyllosticta* sp (OK021603), respectively as compared to the control group which had 42% wound contraction at day 15 post-treatment. The results of the present study clearly indicate that *Jatropha multifida* leaves harbor endophytic fungi that produce pharmacologically important bioactive secondary metabolites with wound and hemostatic effects; therefore, further exploration is inevitable, particularly for the purification and identification of specific chemical structures of bioactive compounds.

1. INTRODUCTION

Bleeding from a wound as a result of physical injury and hemorrhage is a life-threatening condition. For example, in the United States of America only, it was estimated that about 60,000 reported deaths every year are attributed by bleeding due to hemorrhage. Notably, it has been extrapolated that about 1.9 million deaths attributed by bleeding are reported every year worldwide (Cannon, 2018). The problem is even more alarming in sub-Saharan (SSA). For example, among 480,000 maternal deaths were attributed to bleeding worldwide between 2003 and 2004, 41.6% were reported from SSA (Tochie *et al*., 2019). Likewise, Tanzania is not exceptional, the problem of bleeding is very significant, astonishingly, about 21% of deaths

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among women of reproductive age were associated with bleeding related to postpartum hemorrhage (Bishanga *et al*., 2018).

Although bleeding often stops on its own in minor injuries, in many situations, the use of mechanical barriers, thermal and hemostatic drugs is essential for the rapid prevention of life-threatening bleeding (Spotnitz, 2007). Pharmacological drugs such as tranexamic acid (TXA) have been used for the prevention of excessive blood loss from major trauma, postpartum bleeding, surgery, and heavy menstruation (Chauncey &vWieters, 2020). However, similar to other synthetic pharmacological drugs, most hemostatic agents have side effects. For instance, an anti-fibrinolytic agent (aprotinin) has nonspecific serine protease inhibitory effects, which may cause serious allergic reactions (Ebrahimi *et al*., 2020). Furthermore, there is a paucity of discovery of novel drug structures with coagulation and wound-healing effects. Existing challenges have triggered the search for new drug molecules, for example by exploring bioactive compounds generated by endophytic fungi isolated from medicinal plants with wound healing and coagulation activity.

Traditional medicines have been used for centuries worldwide for the treatment and prevention of diseases such as malaria, cholera, tuberculosis, and asthma *(Rakotoarivelo et al*., 2015). Traditional medicine in Eastern and Southern Asia has been used for at least 3000 years (Park *et al*., 2012). For example, the roots of *Alkanna tinctoria* have been used for antiviral treatment in India (Shaheen *et al*., 2020). Some medicinal plants have been reported to have wound healing and hemostatic effect (Kumar *et al*., 2007). A notable medicinal plant is *Jatropha multifida*, a member of the family Euphorbiaceae, which has been reported to have bioactive compounds with pharmaceutical effects (Anani *et al*., 2016; Kumar and Sharma, 2008). Various studies have been conducted to evaluate the effectiveness of *Jatropha multifida* leaf latex and crude leaf extracts on wound healing and coagulation of whole rat blood (Anani *et al*., 2016; Dougnon *et al*., 2012; Victorien *et al*., 2012). Information from these studies suggests that endophytes residing in *Jatropha multifida* may produce bioactive secondary metabolites with similar pharmacological activities to the wound and hemostatic effects.

Endophytic fungi belong to a class of plant symbionts that reside within plant tissues (Guo *et al*., 2008). Fungal endophytes are fungi that under normal circumstances live within living plant tissues without causing any noticeable symptoms (Tadych and White, 2009). Endophytes produce bioactive compounds that enable plants to adapt to abiotic and biotic stressors (Rana *et al*., 2019). They are known to produce a multitude of bioactive compounds with medicinal values (Dhayanithy *et al*., 2019). However, information on the wound healing and the coagulant effects of crude extract of endophytic fungi isolated from *Jatropha multifida* was lacking; therefore, this study aimed to evaluate the wound healing and coagulant activity of crude extracts of endophytic fungi isolated from the leaves of *Jatropha multifida*.

2. MATERIAL and METHODS

2.1. Plant Materials and Authentication

Plant materials were collected between January and April 2021 in the Ilala and Kisarawe districts of the Dar es Salaam and Coastal (Pwani) regions Tanzania, respectively. Initially, representative leaf samples were taken to the Department of Botany at the University of Dar es Salaam (UDSM) for identification and authentication (voucher number: FMM 4184). Following authentication, the samples were collected and packed in sterile well-labeled plastic bags placed in a container containing silica gel, and immediately transported to the Microbiology laboratory of the Department of Molecular Biology and Biotechnology (UDSM) for further processing.

2.2. Description of Experimental Animals

Six-weeks-old Swiss albino mice were obtained from the Tanzania Veterinary Laboratory Agency (TVLA) and taken to the Zoology Department of the University of Dar es Salaam,

where they were further housed for two weeks before experimentation. The mice were fed a layer mash (FUGA mills, Dar es Salaam, Tanzania) diet at *ad libitum*.

2.3. Isolation and Characterization of Endophytic Fungi

Leaf samples were sterilized as previously described (Kjer *et al.*, 2010) with some modifications. Briefly, leaf samples were washed in running water for 10 min to remove soil particles and stick debris followed by washing in 95% ethanol for 1 min, 2% sodium hypochlorite for 10 seconds, and in 95% ethanol for 1 min. Finally, samples were washed with sterile distilled water for 2 min. To test the efficiency of sterilization a non-sterilized leaf segment (control) and a segment of sterilized leaf were inoculated into growth media. The absence of epiphytic fungi and bacteria on the sterilized leaf segments indicated a high efficiency of surface sterilization. Sterilized leaves were cut into small segments of approximately 2 to 3 mm^2 and then inoculated onto Petri dishes with potato dextrose agar (PDA) and incubated at room temperature for seven days. Pure colonies were obtained after sub-culturing on PDA. The colonies were selected based on their color, texture, elevation, and morphology.

2.3.1. Molecular characterization of endophytes isolated

Fungal genomic DNA was extracted using the ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research, USA), according to the manufacturer's instructions. Initially, genomic DNA was quantified by using Nanodrop Spectrophotometer 2000 (Thermo Fisher Scientific), which was then followed by quality evaluation by running the genomic DNA on 0.8% agarose gel electrophoresis and observed under UV light.

The nuclear ribosomal DNA (rDNA) internal transcribed spacer (ITS) of the fungal was amplified using the forward primer, ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and the reverse primer, ITS-4 (5′- TCCTCCGCTTATTGATATGC-3′) (Hamzah *et al*., 2018). The PCR thermal protocol was as follows: initial denaturation was at 95 ^oC for 2 min, denaturation at 95 ^oC for 30 seconds, annealing at 55 ^oC for 30 seconds, extension at 72 ^oC for 30 seconds, and the final extension at 72 ^oC for 10 min (Manter and Vivanco, 2007). The expected amplicon size for the ITS region was about 600 bp.

2.3.2. Sequencing of amplicons and Bioinformatics analysis

Sanger sequencing of PCR amplicons was done at Inqaba Biotec™ (South Africa). The raw sequences were then transferred electronically for Bioinformatics analysis. First, consensus DNA sequences were reconstructed by using Geneious Prime (Biomatters, New Zealand). Then, the phylogenetic tree was constructed by using the neighbor-joining tree algorithm to establish species relatedness (Araújo *et al*., 2018).

2.4. Fungal Mass Cultivation and Extraction of Crude Extracts

Mass cultivation of the fungal endophytes was performed as previously described (Mbilu *et al*., 2018). Briefly, agar blocks of actively growing pure isolates were inoculated in a 500 mL conical flask containing 300 mL of sterile potato dextrose broth (PDB) followed by incubation at room temperature in a shaker at 90 rpm for 14 days. After the incubation, the fungal mycelia were filtered using Whatman No. 1 filter paper. The filtrate was mixed with an equal volume of ethyl acetate and shaken for 10 minutes. Ethyl acetate was evaporated to dryness using a vacuum rotary evaporator at 35 ^oC to obtain a crude extract. The crude extracts obtained were stored at −4 ^oC for further analysis (Mbilu et al., 2018). The selection of crude extracts of fungal secondary metabolites for wound healing and coagulant activity testing was based on the presence and abundance of the tested functional groups.

2.5. Phytochemical Analysis of Fungal Crude Extract

Fungal crude extracts were assessed for the presence of natural compounds (bioactive secondary metabolites) qualitatively by using method previously described by Fitokimia *et al.*,

(2016). The presence of alkaloids, flavonoids, phenolics, saponins and tannis was assessed in the present study. To test the presence of alkaloids, flavonoids and phenolics, for each, 1 mL of fungal crude extracts were placed in test tube, which was then followed by addition of few drops of Wagner's reagent (iodine, potassium iodide, and distilled water solution), 10% ferric chloride ($FeCl_3$) and 5% ferric chloride, respectively. The crude extract colour change to brown, dark green and dark green was an indication of the presence of alkaloids, flavonoids and phenolics in the fungal crude extracts, respectively. Likewise, to test tannins, 2 mL of 5% $FeCl_3$ solution added in test tube containing 2 mL of fungal crude extract, and the extract colour change to greenish-black precipitate indicated the presence of tannins. On the other hand, the presence of saponins was determined using frothing test. The fungal crude extract was vigorously shaken with distilled water and allowed to stand for 10 minutes. The formation of fairly emulsion indicated the presence of saponins.

2.5. Wound Healing Activity of the Fungal Crude Extract

Two fungal crude extracts (FUCE) named FUCE 1 and FUCE 2, which exhibited the highest phytochemical content, were selected for the evaluation of wound healing and whole mice blood coagulation effectiveness. Forty mice were divided into eight groups: control, FUCE 1 (30 µg/mL) FUCE 1 (50 µg/mL) FUCE 1 (70 µg/mL), FUCE 2 (30 µg/mL), FUCE 2 (50 µg/mL), FUCE 2 (70 µg/mL) and the vehicle group. Each group consisted of five mice (n=5). Mice in the control group were not treated, whereas mice in the FUCE groups were treated with 0.1mL of 30 µg/mL, 50 µg/mL and 70 µg/mL of the respective extract. Mice in the vehicle group were treated with 10% DMSO, solvent used to reconstitute extracts.

The mice were anesthetized with chloroform (Ezike et al., 2010). The dorsal thoracic central region of the animals was shaved, and the entire thickness of the skin was excised using a circular biopsy punch of with a diameter of 1 cm to obtain a wound of approximately 80 mm^2 (Masson-Meyers et al., 2020). The mice were then treated daily from day zero to the day after wound healing. The wound area was measured at three-day intervals. The following formula was used to calculate the wound contraction percentage (%):

$$\text{wound contraction (\%)} = \frac{W_{AO} - W_{AT}}{W_{AO}} \times 100\ \%$$

where W_{AO} = wound area at day zero, and W_{AT} = wound area on a specific day (Ezike et al., 2010).

2.6. Coagulation Time of Whole Blood

Hemostasis is a physiological process that aids in preventing blood loss from damaged blood vessels (Blanco & Blanco, 2017). Thirty-five mice were randomly divided into seven groups. The control group which was not treated, FUCE 1 (30 µg/mL) group, FUCE 1 (50 µg/mL) group, FUCE 1 (70 µg/mL) group and FUCE 2 (30 µg/mL) group, FUCE 2 (50 µg/mL) group, FUCE 2 (70 µg/mL) group. Each animal was anesthetized with chloroform and the thoracic cavity was open to expose the aorta (Ezike *et al.,* 2010). The aorta was cut and 1 mL of blood was quickly withdrawn using a plastic disposable syringe, and then transferred into paraffin-coated plastic tubes containing small amount of 30 µg/mL, 50 µg/mL and 70 µg/mL fungal crude extracts. The plastic tube was swirled every 15 s to check the fluidity of the contents. The interval between the introduction of the blood and the time of clot formation was taken as the coagulation time in seconds (Ezike *et al.,* 2010).

2.7. Statistical Analysis

Descriptive and inferential statistical analyses were performed using the origin pro software (Seifert, 2014). One-way analysis of variance (ANOVA) was conducted to compare means among wound contraction groups. Results were expressed as mean ± standard error of means

(SEM). Pairwise comparison of means was performed by Tukey's HSD, and a significant difference was taken at $p < 0.05$.

3. RESULTS

3.1. Phenotypic and Molecular Characteristics of Endophytic Fungi

In this study, four endophytic fungi were isolated and characterized. Phenotypic characteristics based on color, texture, shape, edges, and elevation of mycelia on agar surface were variable (Figure 1).

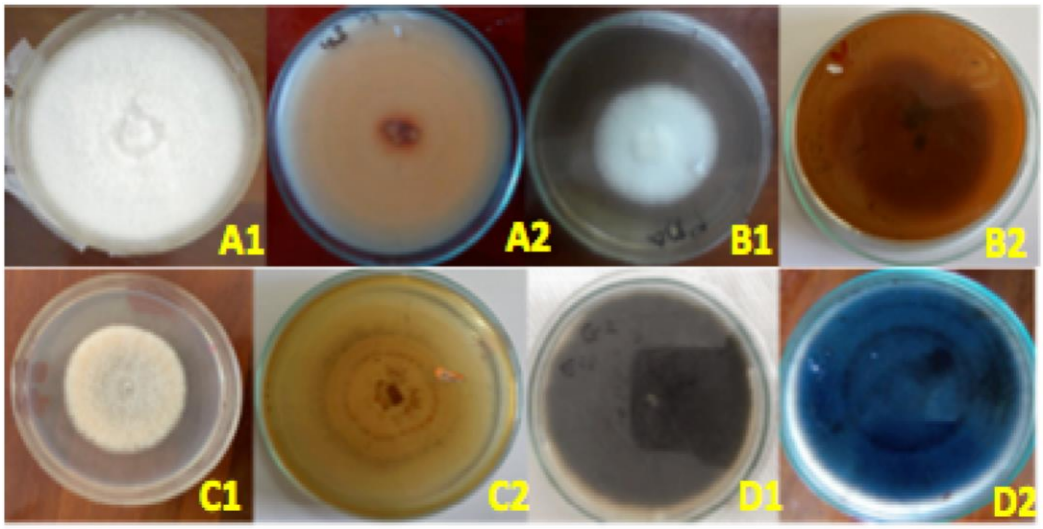

Figure 1. Morphological appearance of endophytic fungi isolated from *Jatropha multifida. Phlebiopsis gigantea (*OK021602) front (A1) and back view (A2); *Phyllosticta* sp (OK021603) front (B1) and back (B2) view*; Colletotrichum* sp (OK021604) front (C1) and (C2) view; *Phyllosticta elongata (*OK021605) front (D1) and back (D2) view.

The phenotypic characterization was further confirmed by genotypic characterization by sequencing the ITS region of the isolates by using ITS1 and ITS4 primers. The Accession numbers and suggested scientific names are presented in Table 1. In addition, DNA sequences were used to generate a phylogenetic tree to establish genetic relatedness between endophytic fungi that were isolated in the present study with the ones reported in GenBank (Figure 2).

Table 1. Name and accession numbers of endophytic fungi that were isolated from *Jatropha multifida* and successfully characterized in the present study.

Isolate ID	Accession number	Scientific name
FC 1	OK021602	*Phlebiopsis gigantea*
FC 2	OK021603	*Phyllosticta sp*
FC 4	OK021604	*Colletotrichum sp*
FC 6	OK021605	*Phyllosticta elongata*

Note: FC stands for fungal culture, so FC1 indicates fungal culture 1 for pure isolate 1.

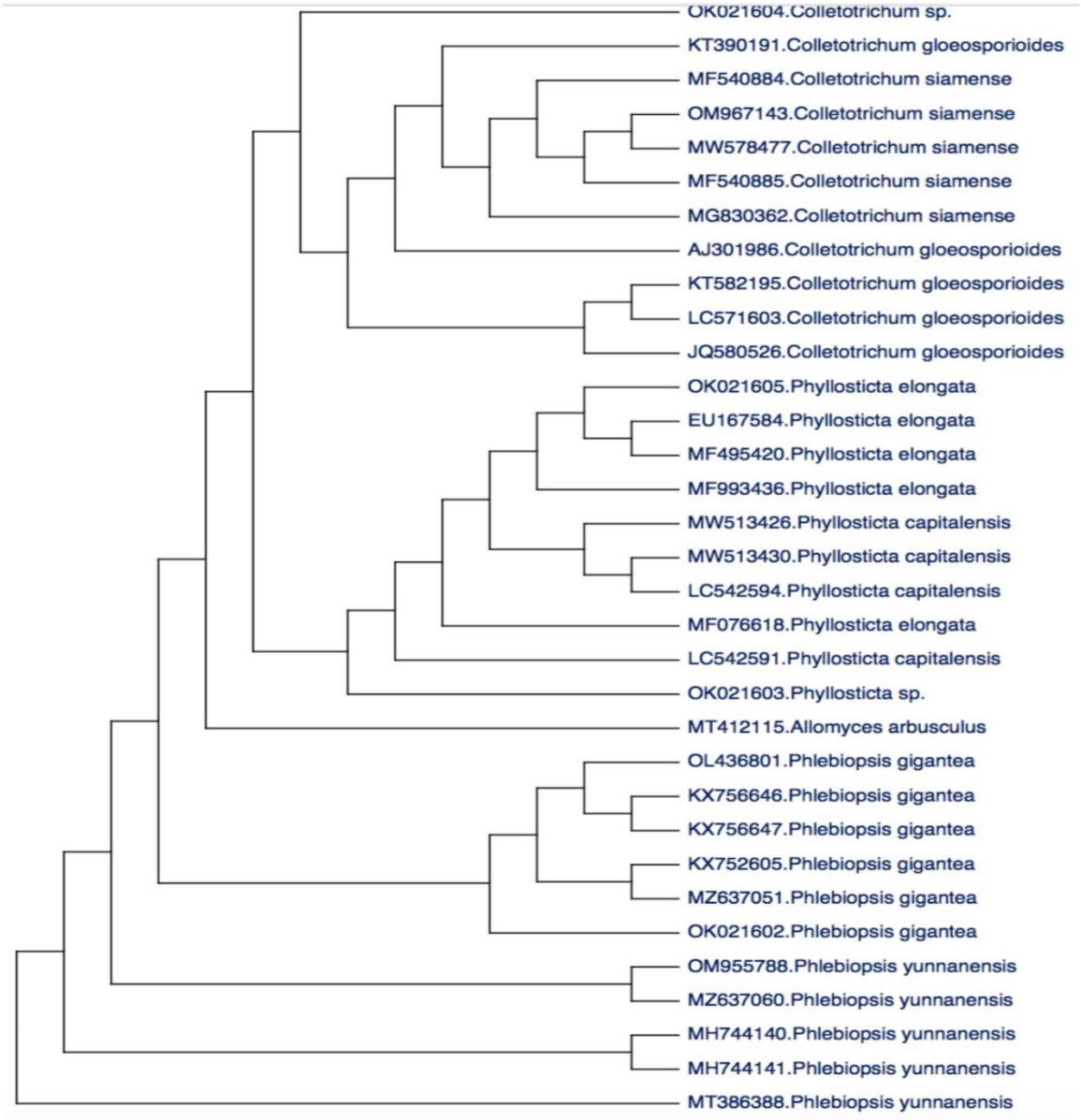

Figure 2. A neighbor-joining phylogenetic tree depicting the relationship of *Phlebiopsis gigantea* (OK021602), *Phyllosticta sp* (OK021603), *Colletotrichum sp* (OK021604), *Phyllosticta elongata* (OK021605) with other related fungi obtained from GenBank. The *Allomyces arbusculus (*MT412115) was used as an out-group.

3.2. Qualitative Phytochemical Analysis

Initial screening for the performance of crude extracts on wound healing and hemostatic activity was done by evaluation of the presence of essential classes of bioactive compounds. Therefore, phytochemical analysis was conducted, and the results revealed the presence of tested classes of bioactive compounds in all four fungal crude extracts (FUCE) as summarized in Table 2.

Table 2. Qualitative phytochemical analysis of fungal crude extracts

Compound test	alkaloid	flavonoid	saponin	tannin	phenol
FUCE 1	++	++	++	++	++
FUCE 2	+	+	+	+	+
FUCE 3	–	–	–	+	+
FUCE 4	+	–	–	–	–

FUCE stands for fungal crude extract, + means present, – means absent, and the number of + indicates the abundance of a particular phytochemical

3.3. Wound Healing Activity of Crude Extracts

Animals treated with crude fungal extract (FUCE 1) had the highest percentage of wound contraction in the fifteenth day post-treatment. Animals treated with FUCE 1 showed a significant reduction in wound diameter (p<0.05) compared to animals in the control group as shown in Table 3.

Table 3. effect of fungal crude extracts on wound contraction in mice wounds.

Treatment group	Concentration µg/mL	Wound contraction (%)				
		The day of observation post treatment				
		Day 3	Day 6	Day 9	Day 12	Day 15
Control	-	2^a ±2.00	4^a ±1.87	12^a ±2.55	24^a ±1.87	42^a ±1.22
FUCE 1	30	5^a ±2.2	9^a ±4.00	25^a ±3.53	35^a ±2.73	53^a ±2.54
	50	19^a ±1.00	39^a ±1.00	58^a ±1.22	79^a ±1.00	98^a ±2.00
	70	27^b ±2.54	47^b ±2.00	63^b ±2.73	83^b ±3.39	99^b ±3.39
FUCE 2	30	1^a ±1.00	4^a ±1.87	13^a ±2.54	26^a ±2.91	41^a ±2.91
	50	3^a±2.00	10^a±2.74	21^a±2.45	36^a±2.92	53^a±3.39
	70	2^a ±1.22	11^a ±2.91	25^a ±3.16	42^a ±3.00	58^a ±2.54
Vehicle	-	1^a±1.00	4^a±2.00	10^a±1.87	23^a±1.22	40^a±2.45

[a] no significance difference; [b] there is a significance difference (p<0.05); FUCE stands for fungal crude extract.

3.4. Coagulation Effect of Crude Fungal Extract

The reference range of mice's whole blood clotting time was about 100 seconds. FUCE 1 demonstrated higher blood clotting activity as compared to FUCE 2. Surprisingly, FUCE 2 demonstrated anticoagulant activity (Table 4). Interestingly, the pattern of coagulation and anticoagulation activity of FUCE 1 and FUCE 2, respectively was correspondingly to the increase in extract concentration (Table 4), demonstrating dose-response relationship.

Table 4. Effect of fungal crude extracts in mice whole blood coagulation.

Treatment	Dose (µg/mL)	Coagulation time (s)
Control	-	98.6
FUCE 1	30	42.6
	50	32.33
	70	14.6
	30	986
FUCE 2	50	$2.67x10^3$
	70	$3.04x10^3$

FUCE stands for fungal crude extract, for FUCE1 is fungal crude extract of isolate 1 under the present study

4. DISCUSSION and CONCLUSION

A multitude of reports have demonstrated wound healing and the hemostatic effect of *Jatropha multifida* (Anani *et al.*, 2016; Ezike *et al.*, 2010). Although not supported by empirical evidence, *Jatropha multifida* is used in most of Tanzania communities for the prevention of bleeding and treatment of wounds. Notably, there is a plethora of evidence, which suggests the relationship between endophytes and their host plant in terms of bioactive secondary metabolites. Based on this reality, it was hypothesized that endophytic fungi isolated from *Jatropha multifida* generate bioactive secondary metabolites with wound healing and coagulant activity. Results of the present study corroborated wound healing activity of crude extracts of fungal endophytes from *Jatropha multifida*; however, the coagulation activity of the fungal endophytes is still debatable based on the present findings (Tables 3 and Table 4).

In this study *Jatropha multifida* leaves were collected from six different plants that were used for the isolation of endophytic fungi. Based on the molecular identification, the genus

Phyllosticta was observed to be the dominant genus on *Jatropha multifida* leaves, compared to other genera isolated. The *Phebiopsis* genus was the only Basidiomycota isolate identified in this study whereas other genera belong to the class Dothideomycetes and Sordariomycetes in phylum Ascomycota. All genera isolated are pathogens to other plant hosts but there are no reports of the pathogenesis of these species in *Jatropha multifida*. In addition, all endophytes isolated in this study have previously been isolated in other host plants (Rampadarath et al., 2018; Wikee et al., 2013). The abundance, diversity, and species composition of endophytes vary according to host species, tissue types, site characteristics, local microclimates, and anthropogenic factors (Torres *et al.*, 2011). It is strongly believed that the isolation of a relatively small number of endophytes in this study could have been due to the aforementioned reasons. Interestingly, this is the first study report on the isolation and identification of endophytic fungi isolated from *Jatropha multifida*.

Phytochemical analysis results revealed that *Phlebiopsis gigantea* extract had a high level of bioactive compounds compared to other extracts. The similarity between bioactive compounds present in this study and the ones that have been previously reported from the crude extract of *Jatropha multifida* leaves justifies the hypothesis that fungal endophytes and their host plants might have been producing similar bioactive compounds. Results from this study are in accordance with the previous report (Devi *et al*., 2012) wherein endophytes have shown the presence of different bioactive compounds like flavonoids, saponin, alkaloids, and phenol. Also, it has been reported that alkaloids, phenol, saponin, and flavonoids all have wound healing and hemostatic activity (Fetse *et al*., 2014; Sharma *et al*., 2021). In addition, the similarity between bioactive compounds present in this study and the ones that have been previously reported from leaves of *Jatropha multifida* (Chioma *et al*., 2021; Rampadarath *et al*., 2014) justifies the hypothesis that fungal endophytes and their host plants might have been producing similar bioactive compounds.

Wound contraction is the healing response that functions to reduce the size of the tissue defect and eventually decrease the size of damaged tissue that needs repair (Lesperance *et al*., 2006). Wound contraction involves three distinct phases i.e. inflammation, proliferation (fibroblastic), and tissue remodeling (maturation) (Fetse *et al*., 2014). In this study wound healing and coagulation test results revealed that *Phlebiopsis gigantea* (OK021602) extract had a high level of bioactivity compared to *Phyllosticta sp* (OK021603) extract, which had slight bioactivity. Animals in the FUCE 1 group treated with a crude fungal extract of 30 µg/mL had slightly higher bioactivity with 53% wound contraction compared to animals in the control group which had 42% wound contraction. This indicates that the presence of bioactive compounds in the crude fungal extract promoted wound healing even in a small concentration. On the other hand, FUCE 1 group treated with 50 µg/mL and 70 µg/mL had even higher bioactivity with 98% and 99% wound contractions respectively compared to the control group. Results indicated that as the concentration of bioactive compounds increases the wound contraction percentage increases as well hence, promote fast wound healing in mice. Animals in FUCE 2 groups treated with crude fungal extracts with 30 µg/mL, 50 µg/mL and 70 µg/mL respectively, had less wound contraction percentages compared to animals treated with FUCE 1 extracts and had almost similar wound contraction percentage of animals in the control group.

In the evaluation of mice whole blood coagulation, the *Phlebiopsis gigantea* (OK021602) extract had a high level of bioactivity compared to that of *Phyllosticta sp* (OK021603) extract. The blood treated with FUCE 1 of 30 µg/mL coagulated in 42.6 s, two times faster than blood in the control group. This result indicates that bioactive compounds with coagulant activity in FUCE 1 extract speed up the blood coagulation process. On the other hand, FUCE 1 extracts with 50 µg/mL and 70 µg/mL coagulated whole blood in 32.33 s, three times faster than the blood coagulation in the control group and 14.6 s which is seven times faster than blood coagulation in the control group, respectively. These results indicate that the presence of bioactive compounds in high concentrations promotes high bioactivities. In this case, high

bioactive compounds with coagulant activity promoted or sped up mice's whole blood coagulation process. Surprisingly, FUCE 2 extracts with 30 µg/mL, 50 µg/mL, and 70 µg/mL coagulated mice while blood in 986 s, 2.67×10^3 s, and 3.04×10^3 s, which is 10, 27, and 30 times slower than blood coagulation in the control group respectively. These results could be due to the presence of blood thinner compounds (anticoagulants) that prevent blood from clotting, increasing the mice's whole blood coagulation time.

Results from the present study clearly indicate that *Jatropha multifida* leaves harbor endophytic fungi that produce pharmacologically important bioactive metabolites that can be used as an alternative in the treatment of wounds and prevention of bleeding (hemorrhage). Further studies on functional analysis of individual compounds are highly recommended.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s). The experiment was conducted in compliance with the Guidelines on the Humane Treatment of Laboratory Animals as stipulated in the Tanzania Animal Welfare Act, 2008.

Availability of Data

The data in the study is available to other research upon request.

Authorship Contribution Statement

Fulgence Ntangere Mpenda: Envision of study, data analysis, supervision, revision, original draft, proof reading; **George Madaha:** Envision of study, data collection, data analysis; **Fortunatus Jacob:** Envision of the study, original draft, revision of manuscript

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Research Article

Chemical profiling and bioactivity studies on aerial parts *Ammoides atlantica* (Coss. et Durieu) H. Wolff

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Abstract: The Algerian endemic plant *Ammoides atlantica* (Coss. et Durieu) H. Wolff was studied for the chemical profiling and biological activities of its essential oil (EO) and ethanolic extract (EE). The chemical analysis by GC/MS and HPLC/DAD/UV revealed, respectively, the major compounds thymol (39.46%), γ-terpinene (31.74%), and p-cymene (19.01%) in the EO, and apigenin (33.58%), luteolin 7-*O*-glucoside (20.09%), and luteolin (14.39%) in the EE. The EO exhibited strong antioxidant activity, with a significant $ABTS^{\bullet+}$ scavenging capacity (IC_{50} = 2.79 µg/mL) compared to EE, Trolox, and BHT. The EE showed comparable effects to BHT in DPPH scavenging and reducing power tests. Moreover, the EO demonstrated noteworthy antibacterial activity against *S. aureus, E. coli,* and *P. aeruginosa*, with inhibition zone diameters ranging from 32.1 to 70 mm and MICs below 0.3 to 5 mg/mL. Furthermore, the EE exhibited strong anti-inflammatory activity by inhibiting hemolysis of red blood cells >70% at a concentration of 20 µg/mL.

1. INTRODUCTION

In recent years, the world has witnessed a tremendous development of effective synthetic drugs and new generations of antibiotics that have saved the lives of millions of people. Synthetic drugs, on the other hand, are experiencing difficulties in the treatment of some diseases caused by organisms or cells being resistant to drugs, such as multidrug-resistant bacteria and some chemotherapy-resistant tumor cells. Furthermore, drugs designed to cure certain ailments seem to be toxic or harmful to some other body organs. As a result, there is currently a strong trend toward researching natural sources for biologically active extracts that can be combined with synthetic drugs to create more effective and safer medications (Kieliszek *et al.*, 2020). Statistics on the sources of new drugs from 1981 to 2007 indicate that almost half of the drugs approved since 1994 are based on active metabolites from natural sources (Akoto *et al.*, 2021). Medicinal and aromatic plants contain the chemical constituents first used by humans as medicines for healing, as flavoring agents for food and drink, and as mental stimulants for mystic interactions with super natural gods. These plant materials continue to play positive roles in human life, as

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sources of modern pharmaceuticals to treat medical problems, as herbs and spices to tempt the palate, and in a multitude of other applications (Inoue & Craker, 2014).

Moreover, herbal medicines have, nowadays, received great scientific interest because they provide both important biomolecules, which are used in the treatment of several diseases, as well as a broad spectrum of long-term use and safety. Nevertheless, plant secondary metabolites are excellent candidates for developing new phytopharmaceuticals with various biological activities, including antioxidant, antimicrobial, and anti-inflammatory (Toiu *et al.*, 2018; Gunathilake *et al.*, 2018).

The genus *Ammoides* belonging to the Apiaceae family (Umbelliferae) is represented in Algeria by two species: *A. pusilla* (Brot.) Breistr. and *A. atlantica* (Coss. et Dur.) Wolf. *Ammoides atlantica* is an endemic species of Algeria found in mountain grasslands above 1000 m (Quezel & Santa, 1962). In Algerian folk medicine, the species is widely used as an infusion to treat headaches, fever, and diarrhea. It is also used in compresses, alone or soaked in alcohol or vinegar and mixed with henna, to treat children with mental deficiency. This plant also has interesting digestive properties and is used as a spice in certain recipes (Laouer *et al.*, 2003; Laouer *et al.*, 2008). Most of the previous studies on this plant were focused on volatile fraction while the non-volatile fraction and their biological effects have not yet been fully investigated, especially their anti-inflammatory activity. The purpose of this study is to assess the antibacterial, antioxidant, and anti-inflammatory properties of the essential oil and ethanol extract of *A. atlantica.*

2. MATERIAL and METHODS

2.1. Plant Material

The aerial parts of *A. atlantica*, were harvested in the region of "Amira Arrès" located in the Mila province (east of Algeria, 36° 32′ 15″ N 6° 03′ 55″ E) at 1000 m of altitude, during the flowering period (March 2021). The taxonomic identity of the plant was confirmed Prof. H. Abdelkrim by comparing it with specimens of known identity (ID: 052_64) already deposited in the herbarium of the National Superior School of Agronomy (ENSA), Algiers.

2.2. Isolation of Essential Oil

The essential oil was isolated by hydrodistillation in a Clevenger-type apparatus according to the protocol of the European Pharmacopoeia (2007). The traces of water in the collected oil were removed using anhydrous sodium sulfate (Na_2SO_4). The oil was then kept at 4 °C in dark vials hermetically sealed until analysis.

2.3. Gas Chromatography/Mass Spectrometry (GC/MS) Analysis of the Essential Oil

The essential oil was analyzed with a Perkin-Elmer CLARUS 500 model GC/MS device. Analyzes were performed on an Elite Series 5-MS gas chromatograph fitted with a fused silica capillary column with an apolar stationary phase HP 5MS (30 m x 0.25 mm x 0.25 µm film thickness), with a stationary phase nonpolar, directly connected to a Hewlett-Packard 6890 quadrupole mass spectrometer. The chromatographic conditions were as follows: a heated oven with a temperature setting of 70 to 220°C for 15 min at a rate of 4°C/min and maintenance of the final temperature for 56.5 min. Essential oil diluted in hexane (1/10, v/v) was injected by splitting (split ratio 1/25). The injector and transfer line were maintained at a temperature of 250°C. For each sample, the analysis was carried out by electron impact (EI) at 70 eV or by chemical ionization (CI) with methane as the pressurized gas used. The temperature of the ion source was maintained at 250°C. The carrier gas is helium (He). The gas chromatograph was operated in scan mode between 20 and 550 atomic mass units

2.3. Identification of the Essential Oil Compounds

The identification of the compounds was carried out by comparing the mass spectra of the EO compounds with those of computerized commercial libraries (NIST, PFLEGER, NBS and WILEY) and those of the database developed by the laboratory from authentic substances. The

modified Van den Dool and Kratz formula (Tranchant et *al.*, 1995) was used to calculate the retention indices. The column and analysis conditions used for the determination of the retention indices are those described above in the section on gas chromatography. Confirmation was carried out by comparing the retention indices of the separate products with those described in the Adams Library (Adams, 2001).

2.4. Preparation of Ethanolic Extract

The ethanolic extract was prepared using ethanol as a solvent by submitting 20 g of dried powdered plant to extraction with 200 mL of absolute ethanol in a Soxhlet apparatus. The ethanolic solution obtained was concentrated using a rotary evaporator under vacuum, lyophilized to obtain a dry extract (4.6 g), and then stored at 4 °C until analysis.

2.5. HPLC/UV/DAD Analysis and Identification of Ethanolic Extract Compounds

The ethanolic extract was analysed using an Agilent 1100 series HPLC device equipped with a UV/DAD detector, a quaternary pump, an in-line degasser, and an automatic injector. The diode array detector (DAD) with several wavelengths of maximum absorption (chosen according to the maximum absorbance (ʎmax) of the researched molecules), is fixed with the analytical column Hypersil (BDS-C18, 5 µm, 250 × 4.6 mm) constituting the stationary phase. The mobile phase used is a mixture of two solvents, the first is a mixture of water and acetic acid (0.2%) at pH = 3.1 (solvent A) and the second is acetonitrile (solvent B). The two solvents were used in a linear gradient elution for 30 min at 1 mL/min, starting with 95% of solvent A and ending with 100% of solvent B. The flow rate is 1.5 mL/min, the injection volume of 20 µL and the detector wavelength is fixed at 220 nm, 255 nm, 280 nm, 300 nm, and 355 nm, chosen according to the maximum absorbance of the molecules identified. The identification of the compounds was carried out by comparing the retention times and the UV spectra of the peaks of the sample's chromatogram with those of the standards previously analysed under the same operating conditions and recorded in the database of the HPLC chromatograph (Tzima *et al.*, 2018).

2.6. Total Phenolic and Flavonoid Content Determination

The total phenolic content (TPC) was determined according to the Folin-Ciocalteu colorimetric method (Singleton *et al.*, 1999). 1 mL of the Folin-Ciocalteau reagent solution diluted 10 times was added to 0.25 mL of ethanolic extract. Then, 1 mL of 7.5% sodium carbonate solution (75 g/L) was added after 3 minutes of reaction. Afterward, the reaction mixture was incubated for 30 minutes in the dark at room temperature. Finally, the absorbance was measured using a spectrophotometer at 765 nm against a blank, which was prepared under the same conditions by replacing the extract with methanol. The samples were analyzed in triplicate. A calibration curve established with gallic acid was used to determine the amount of total phenols (TP) in mg of gallic acid equivalent per gram of dry weight (mg GAE/g DW) ($Y = 0.0111X - 0.0067$, where X is the absorbance and Y is the GAE (mg/g), R^2=0.99). The total flavonoid content (TFC) was determined according to the aluminium chloride method (Lamaison & Carnet, 1990). 1 mL of $AlCl_3$ was added to 1 mL of the extract solution dissolved in methanol. After incubation for 60 minutes in the dark at room temperature, the absorbance of the reaction mixture was measured using a spectrophotometer at 510 nm against a blank prepared under the same conditions by replacing the extract with ethanol. The content of total flavonoids (TF) was estimated using a calibration curve of quercetin and the result was expressed in mg quercetin equivalent per gram of dry weight (mg QE/g DW) ($Y = 0.0344X + 0.008$, where X is the absorbance and Y is the QE (mg/g), R^2=0.99).

2.7. Antibacterial Activity

2.7.1. *Bacterial strains*

The bacterial strains used in this study were: a Gram-positive bacterium: *Staphylococcus aureus* (ATCC 25923) and two Gram-negative bacteria: *Escherichia coli* (ATCC 25922),

Pseudomonas aeruginosa (ATCC 27853). All the strains were grown on Mueller-Hinton agar (MHA) at 37 °C.

2.7.2. *Susceptibility test by agar disk diffusion method*

The agar disk diffusion method was employed to determine the susceptibility of bacteria according to CLSI standards (2012a). Briefly, isolated colonies of each strain from an 18–24-h agar plate were suspended in 5 mL of sterile saline to achieve a turbidity equal to the 0.5 McFarland standard ((1–2)×10^8 CFU/mL). Then, the inoculum suspension was spread on the solid media plates using a sterile cotton swab. Filter paper discs (6 mm in diameter) individually impregnated with 15 µL of the diluted oil (or extract) (with DMSO at 10% w/v) were placed on the incubated plates. The plates were placed at 4°C for 2 h and then incubated at 37°C for 16–18 h. The diameters of the inhibition zones were measured and expressed in millimeters. DMSO and gentamicin (8 µg/mL) were used as negative and positive controls, respectively.

2.7.3. *Determination of Minimal Inhibition and Bactericidal Concentration (MIC & MBC)*

The minimum inhibition concentration was determined using the agar dilution method according to CLSI standards (2012b). Mueller-Hinton agar plates containing serial twofold dilutions of each extract from 0.3 to 20 mg/mL and gentamicin from 0.125 to 512 µg/mL were prepared. Then inoculation of the medium with a standardized suspension adjusted to 0.5 McFarland standard containing a concentration of 5×10^8 CFU/mL was conducted. The bacteria are diluted to around 10^7 CFU/mL (0.5 McFarland suspension 1:10 in sterile saline), and 2 µL are spotted with a multi-point into agar plates that contain approximately 10^4 CFU/spot. One agar plate was used as a control and seeded without an antibacterial agent. The inoculated plates were allowed to stand at room temperature until the moisture in the inoculum spots had been absorbed into the agar, and then incubated at 37 °C for 16 to 20 h. The MIC was defined as the lowest concentration of an antimicrobial that inhibited the visible growth of a microorganism after overnight incubation. Minimal Bactericidal Concentration (MBC) was determined by taking streaks from the plates exhibiting invisible growth and subcultured onto sterile MHA plates. The plates were incubated at 37 °C for 16 to 18 h and then examined for bacterial growth. MBC was defined as the concentration of an antimicrobial that did not exhibit any bacterial growth on the freshly inoculated agar.

2.8. Antioxidant Activity

2.8.1. *DPPH radical scavenging activity*

The scavenging activity of DPPH radicals was determined according to the method described by Hazzit *et al*. (2009). Briefly, 975 µL of ethanolic solution of 2,2-diphenyl-1- picrylhydrazyl (DPPH) (0.0024%) and 25 µL of a sample at final concentrations were mixed and the resulting solution was left at room temperature for 30 min. The absorbance was measured at 517 nm against ethanol as blank. BHT and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were used as positive controls. The scavenging activity of the $DPPH^{\bullet}$ radical expressed as percentage inhibition was calculated as follows:

$$\text{DPPH scavenging (\%)} = \left[\frac{\text{Ab - As}}{\text{Ab}}\right] x100$$

Where, Ab is the absorbance of the control reaction; As is the absorbance of the test compound. The concentration providing 50% inhibition of the $DPPH^{\bullet}$ radical or IC_{50} was calculated from the graph giving the percentage of inhibition in relation to the concentration of the sample. A low IC_{50} value indicates high antioxidant activity.

2.8.2. *$ABTS^{\bullet+}$ radical scavenging activity*

The scavenging activity of $ABTS^{\bullet+}$ radicals was evaluated following the method of Re *et al.* (1990). The $ABTS^{\bullet+}$ cationic radical was produced by reacting 7 mM of $ABTS^{\bullet+}$ solution with 2.45 mM of potassium persulfate ($K_2S_2O_8$), then the reaction mixture was kept in the dark and at room temperature for 18 h before use. Aliquots of 25 µL of each sample at different

concentrations were mixed with 975 µL of diluted $ABTS^{\bullet+}$ in ethanol whose absorbance should be equal to 0.7 ± 0.02 at 734 nm. After 7 min of reaction in the dark, the absorbance was measured using a spectrophotometer at 734 nm against ethanol as blank. BHT and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were used as positive controls. The percentage of inhibition and IC_{50} were calculated as described in the DPPH assay.

2.8.3. *Ferric reducing power*

The ferric reducing power (FRP) was evaluated using the method of Oyaizu (1986). A volume of different concentrations of the samples (0.125 mL) was added to 2.5 mL of phosphate buffer (0.1 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1.0%, w/v). Each mixture was incubated at 50 °C for 20 min, and afterwards 2.5 mL of trichloroacetic acid (10%) was added. The mixture was shaken vigorously, and the solution (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of $FeCl_3$ (0.1%, w/v). After 30 min of incubation at 50 °C, absorbance was read at 700 nm. BHT was used as a positive control. Increased absorbance of the reaction mixture indicates stronger reducing power. The IC_{50} values corresponding to the concentration of the reducing agent that allows obtaining an absorbance of 0.5 were calculated.

2.9. Anti-inflammatory Activity

The method of membrane stabilization of erythrocytes was used whose principle is based on the ability of the ethanolic extract of *A. atlantica* to prevent hemolysis of human red blood cells (HRBC) induced by hypotonia and heat and therefore prevent the release of hemoglobin (Thenmozhi *et al.,* 1989; Oyedapo *et al.,* 2004). The erythrocyte suspension was prepared according to the method described by Shinde *et al.* (1999) with some modifications. Whole human blood was collected from a healthy human subject. Blood in heparinized tubes was centrifuged at 3000 rpm for 5 min, and washed three times with an equal volume of normal saline (0.9% NaCl). After centrifugation, blood volume was measured and reconstituted as a 10% (v/v) suspension with an isotonic buffer solution (10 mM sodium phosphate buffer pH 7.4). The composition of the buffer solution (g/L) used was NaH_2PO_4 (0.2), Na_2HPO_4 (1.15) and NaCl (9.0). An Alsever solution is prepared by dissolving 2% dextrose, 0.8% sodium citrate, 0.05% citric acid, and 0.42% sodium chloride in distilled water, then sterilized. A volume of 1 mL of ethanol extract or aspirin at different concentrations (10-200 µg/mL) was mixed with 1 mL of phosphate buffer (pH-7.4), 2 mL of hypo saline (0.45%), and 0.5 mL of red cell suspension. The reaction mixture (4.5 mL) was incubated in a water bath at 54°C for 20 min and centrifuged again at 2500 rpm for 5 min and the absorbance of the released hemoglobin was measured at 560 nm. In parallel, the control was carried out under the same conditions, replacing the extract with distilled water (positive control corresponding to 100% hemolysis) and the phosphate buffer as blank. The level of hemolysis was calculated using the following equation (Okoli *et al.,* 2008):

$$\text{Percentage of inhibition of hemolysis (\%)} = \left[\frac{1 - A_2}{A_1}\right] x100$$

Where A_1 = absorbance of the control and A_2 = absorbance of test sample mixture

2.10. Statistical Analysis

In this study, three analyses of each sample were carried out and each experiment was carried out in triplicate (n = 3). The mean value and the standard deviation were calculated. The bioassay data was analyzed by ANOVA using IBM SPSS Statistics version 26.0 software, followed by the Tukey test. The significance level was set at $p < 0.05$.

3. RESULTS

3.1. Essential Oil and Ethanolic Extract Chemical Profiling

GC-MS analysis of *A. atlantica* essential oil revealed the presence of 14 constituents. In total, six compounds were identified accounting for 96.02% of the overall composition, most of

which are monoterpenes (Table 1). Thymol represented the major compound with a content of 39.46% followed by γ-terpinene and *p* -cymene with contents of 31.74 and 19.01% respectively. This composition is in agreement with those reported by some authors. In fact, Laouer *et al.* (2008) identified thymol (53.2%), γ-terpinene (19.4%), and *p*-cymene (10.6%) as main constituents in the EO of *A. atlantica* from the Djebel Megress (Setif region). Similarly, Latreche-Douar (2019) identified, in addition to thymol (48.5%), p-cymene (20.4%) and γ-terpinene (6.5%) as major compounds, limonene (8.9 %) and carvacrol (8.5%). However, Boudiar et al. (2011) reported a very different composition of the essential oil of *A. atlantica* collected in the Jijel region where safranal (17.9%), endoborneol (17.6%), chrysanthenone (15.5%), filifolone (12.1%) and camphor (11. 8%) represented the main constituents.

Table 1. Chemical composition (%) of the essential oil of *A. atlantica.*

N°	Compound[a]	RT[b]	CRI[c]	LRI[d]	(%)
	Monoterpene hydrocarbons				50.75
1	p-cymene	8.40	1022	1020	19.01
2	γ-terpinene	9.690	1060	1059	31.74
	Oxygenated monoterpenes				46.97
3	Thymol methylether	15.352	1229	1232	6.61
4	Thymol	17.98	1290	1289	39.46
5	Carvacrol	20.484	1296	1298	0.68
6	Methyleugenol	23.41	1405	1403	0.22
	Total (%)				97.72

[a] Compounds listed in order of elution from HP5MS column
[b] Retention time (min)
[c] Calculated retention indices relative to *n*-alkanes C6–C19
[d] Literature retention indices.

HPLC-DAD-UV analysis of the ethanolic extract revealed seven phenolic compounds (Table 2) including 4 phenolic acids (isovanillic, caffeic, sinapic and 3-hydroxy-4-methoxycinamic), and 3 flavonoids (luteolin 7-*O*-glucoside, apiginin and luteolin). Apiginin represented the most abundant phenolic compound with a percentage of 33.58 followed by luteolin 7-*O*-glucoside and luteolin (20.09 and 14.39% respectively). These compounds were previously reported. In fact, Louaar *et al.* (2008) isolated and identified 4 compounds from the extract of *Ammoides atlantica* namely apigenin, luteolin, luteolin 7-*O*-glucoside and apigenin 7-*O*-glucoside. Recently, Benteldjoune *et al.* (2019) characterized 45 constituents in the ethanolic extract of *A. atlantica* by RP-UHPLC-ESI-QTOF-MS including luteolin, apigenin, luteolin *O*-glucoside and caffeic acid.

The polyphenols and flavonoids contents for the ethanolic extract were 148.89 mg GAE/ g DW and 33.95 mg QE/g DW respectively. These values are more or less comparable to previously reported: 371.57 - 141.74 ± 0.44 mg GAE/g DW (Benteldjoune *et al.*, 2019) and 41.02 - 61.87 ± 6.7 mg QE/g DW (Loucif *et al.*, 2020).

Table 2. HPLC-DAD-UV chromatographic profile of *A. atlantica* ethanolic extract.

N°	Compound	Retention time (min)	Wavelenght (nm)	%
1	Isovanilic acid	6.989	255	2.35
2	Caffeic acid	7.626	300	2.73
3	Sinapic acid	8.706	230	2.12
4	Luteolin 7-*O*-glucoside	9.194	355	20.09
5	3-hydroxy-4-methoxycinamic acid	9.679	300	5.05
6	Apigenin	10.321	355	33.58
7	Luteolin	12.761	355	14.39

3.2. Antibacterial Activity

From the results summarized in Table 3, EO was found to be more active than EE. *E. coli* and *Staphylococcus aureus* were the most sensitive to the essential oil (70 and 30 mm diameter, respectively) compared to *Pseudomonas aeruginosa* (48 mm diameter). Unlike the EO, the strains tested were much less sensitive to the EE (13.5 - 16.5 diameters) (Figure 1). Moreover, the lowest MICs and MBCs and therefore the most important inhibitory activities were noted for EO against *S. aureus* (MIC < 0.3 mg/mL). While the *E. coli* and *P. auroginosa* strains were found to be less sensitive (MIC= 1.25 and 5 mg/mL, respectively). In addition, the EO were bactericidal against *S. aureus* and *E. coli* at 10 mg/mL. Contrarily, *P. aeruginosa* seemed to be more resistant. It should be noted that the strains tested were resistant to the EE (MBC>20 mg/mL). These findings are superior to those obtained by Laouer *et al.* (2008), who showed that the oil of *A. atlantica* possessed significant antibacterial activity against all Gram-positive and Gram-negative strains with MIC>25 mg/mL.

Table 3. Antibacterial activity expressed in DZI (mm), MIC and MBC (mg/mL).

	Microoganism	DZI[a] (mm)	MIC[b] (mg/mL)	MBC[c] (mg/mL)
Essential oil	*S. aureus*	70.0 ± 0.7	<0.3	10
	E. coli	48.2 ± 0.28	1.25	10
	P. aeruginosa	32.1 ± 0.14	5	> 20
Ethanolic extract	*S. aureus*	13.5 ± 0.5	20	> 20
	E. coli	15 ± 0.56	20	> 20
	P. aeruginosa	16.5 ± 0.28	> 20	> 20
Gentamicin	*S. aureus*	31 ± 0.0	4*	8*
	E. coli	24.1 ± 0.1	16*	64*
	P. aeruginosa	22 ± 0.0	16*	64*

[a] Diameter of zone of inhibition
[b] Minimal inhibitory concentration
[c] Minimal bactericidal concentration
*µg/mL

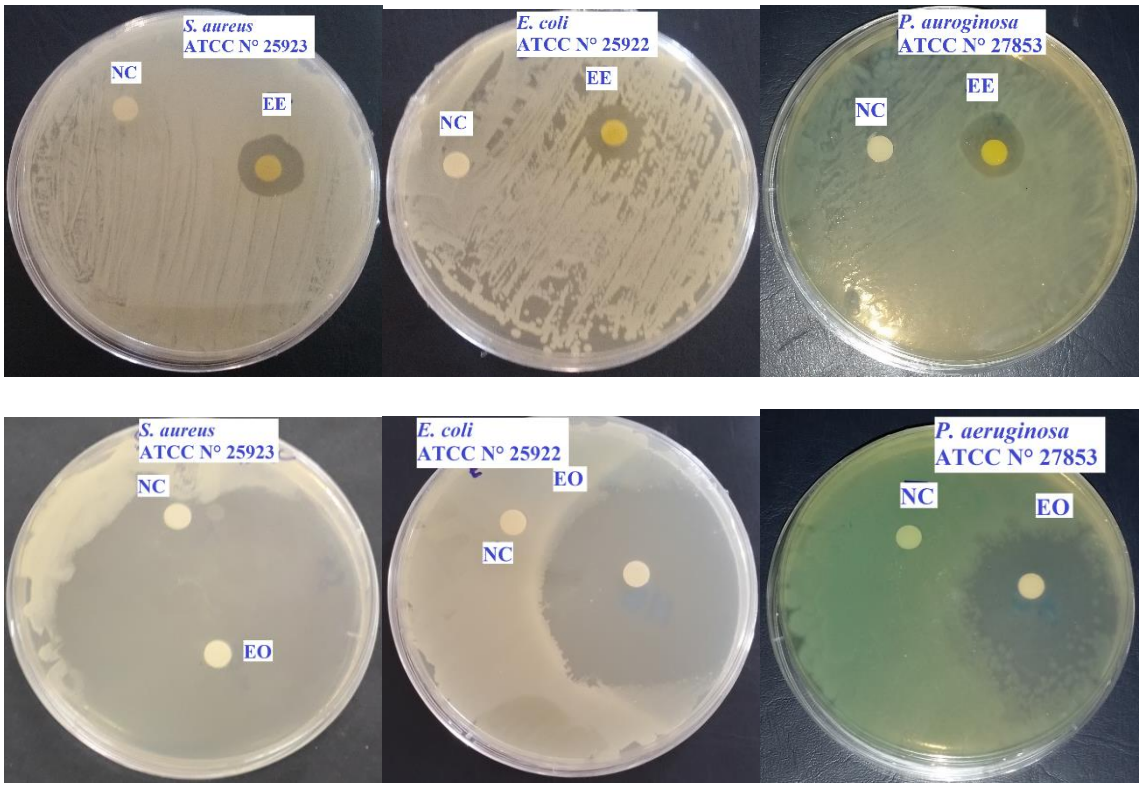

Figure 1. Petri plates showing the antibacterial activity expressed in zones of inhibition.

3.3. Antioxidant Activity

In the $ABTS^{\bullet+}$ test, the essential oil showed the highest scavenging activity with an IC_{50} of 2.79 µg/mL. This activity is superior to those of standard Trolox and EE whose IC_{50} are respectively 3.69 and 6.9 µg/mL (Table 4). However, in the DPPH test, the ethanolic extract and the essential oil showed a scavenging activity comparable to that of BHT with IC_{50} values of 33.78 and vs 22.32 µg/ml respectively, on the other hand, a very low activity for the essential oil (IC_{50} >1000 µg/mL). In addition, EO and EE demonstrated moderate iron-reducing capacities with IC50 values of 145.72 and 152.45 µg/mL respectively compared to 62.83 for BHT and 9.0 µg/mL for ascorbic acid. The latter is known as a very powerful reducing antioxidant of natural origin. These results are relatively in agreement with those previously reported on the hydro-alcoholic extract of the same species (Benteldjoune *et al.*, 2019). These authors indicated BHT/extract IC_{50} ratios of 0.55 versus 0.66 for this study in the DPPH.

Table 4. Antioxidant activity of *A. atlantica* EO and EE expressed in IC_{50} (µg/mL).

Sample	$ABTS^{+\bullet}$	$DPPH^{\bullet}$	FRP
EE	6.9 ± 0.11^{c}	33.78 ± 1.28^{a}	73.45 ± 4.51^{c}
EO	2.79 ± 0.04^{a}	150.0 ± 2.7^{b}	145.72 ± 6.82^{d}
BHT	13.01 ± 0.21	24.23 ± 1.28^{a}	65.38 ± 1.65^{b}
Trolox	3.81 ± 0.1^{b}	511.43 ± 2.51^{d}	–

Values in the same column followed by the same letter are not significantly different by Tukey's multiple range test (p<0.05)

3.4. Anti-inflammatory activity

Table 5 shows the effect of *Ammoides atlantica* and Aspirin on HRBC membrane stabilization expressed in percentage of inhibition of hemolysis (%). The highest inhibition percentages were recorded for the standard aspirin, a non-steroidal anti-inflammatory known for its activity, compared to the ethanolic extract of *A. atlantica*. A moderate activity was noted at a low concentration of 10 µg/mL with an inhibition of 38% against 87.5% for aspirin. This activity increased significantly to give an inhibition of 71.25% at 20µg/mL up to 87.5% at 200µg/mL, which ensures better protection of the human erythrocyte membrane against the lysis induced by the hypotonic solution and therefore against inflammation.

Table 5. Effect of *A. atlantica* and Aspirin on HRBC[a] membrane stabilization.

Concentration (µg/mL)	HRBC membrane stabilization (%)	
	AAEE	Aspirin
10	38.54 ± 1.47	87.5 ± 0.2
20	71.25 ± 1.18	88.3 ± 0.05
50	75.01 ± 1.16	90.42 ± 0.05
100	78.96 ± 0.88	90.83 ± 0.1
200	87.5 ± 0.4	95.83 ± 1.2

[a] Human Red Blood Cells

4. DISCUSSION and CONCLUSION

Considering EO composition, strong antibacterial activity was expected since monoterpenes like thymol, γ-terpinene and *p*-cymene dominated. Several studies concluded that, as lipophilic agents, these compounds execute their action at the level of the membrane and membrane-embedded enzymes (Sikkema *et al.* 1994). Juven *et al.* (1994) and Lambert *et al.* (2001) explained the action of thymol by the fact that it binds to the membrane protein and increases the permeability of the bacterial cell membrane. In addition, *p*-cymene causes swelling of the cytoplasmic membrane (Ultee *et al.*, 2002).

In the antioxidant activity, $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging reaction and ferric reducing power (FRP) assays mainly attributed to hydrogen atom donation and single electron transfer

(SET) reaction were used in the present study (Bondet *et al.,* 1997; Baczek *et al.,* 2017). In the DPPH and ABTS assays, such reaction results in the decrease in the absorbance of free radical species, visible as the change of color from purple-blue to yellow-transparent, respectively. In turn, FRP assay relies on the ability of antioxidants to reduce Fe^{3+} to Fe^{2+} in the presence of ferricyanide, which results in the formation of an intense prussian blue complex. From the results found, it seems that the antioxidant potential of the oil could be attributed mainly to the high content of thymol, while luteolin, apigenin, luteolin 7-*O*-glycoside are responsible for that of the extract. Several authors reported that the ABTS scavenging activity of EO was generally significantly higher than for DPPH (Özgen *et al.,* 2006; Bendjabeur *et al.,* 2018; Aebisher *et al.,* 2021). In this sense, Salamone *et al.* (2012) and Özturk (2012) explain that despite the fact that the initial reaction of antioxidants with DPPH is, in fact, much slower than $ABTS^{\bullet+}$ reactions due to reduced access to phenols like thymol at the site of the $DPPH^{\bullet}$ radical due to steric hindrance. Moreover, the high activity of the essential oil compared to the extract could be due to their lipophilic nature and their ability to access the $ABTS^{\bullet+}$ radical.

Inflammation is a part of the complex biological response of vascular tissues to harmful stimuli, which is frequently linked with pain and involves many biological occurrences, such as an increase of vascular permeability, an increase of protein denaturation, and membrane alteration (Ferrero-Millani *et al.,* 2007). Non-steroidal anti-inflammatory drugs (NSAIDs) are used for the treatment and management of inflammation, pain and fever. However, adverse effects associated with NSAIDs can lead to ulcers and hemorrhage (Hajhashemi *et al.,* 2009 Gunathilake *et al.,* 2018). NSAID acts by inhibiting the function of prostaglandin, an autocoid that is released extracellularly and initiate pain, whose synthesis is blocked by anti-inflammatory agents via either inhibiting cyclooxygenase (COX) or protecting lysosomal membrane from breakdown (Yusuf *et al.,* 2009). According to Chippada *et al.* (2011), stabilization of the lysosomal membrane is vital in controlling the inflammatory response by inhibiting the release of lysosomal constituents from activated neutrophils, and given the similarities between the 2 membranes, the effect of the extract on the stabilization of red blood cells could be extrapolated to the stabilization of the lysosomal membrane and this could be considered as a factor of anti-inflammatory activity (Murugasan *et al.,* 1981; Kumar *et al.,* 2011). The protective effect against heat-induced erythrocyte lysis can be explained by the interaction of the extract with membrane proteins, thus inhibiting their denaturation (Lepock *et al.,* 1989). Phenolic compounds, in particular flavonoids, in the membrane of erythrocytes improve their stability against hypotonic lysis. This would be due to the increase in the volume/surface ratio of the cells either by the expansion of the membrane or the shrinkage of the cell. In addition, the deformability and cell volume of erythrocytes are closely related to the intracellular calcium content. Therefore, we can think that the protective effect of the extract would be due to its ability to modify the influx of calcium into erythrocytes (Chopade *et al.,* 2012).

Our results showed that extracts (EO and EE) from *A. atlantica* aerial parts possess interesting antioxidant properties acting as free radical scavengers and reducing agents. Moreover, the EO exhibited a potent antibacterial effect against ATCC Gram positive and negative bacteria due to its high content in monoterpenes. In addition, the anti-inflammatory effect of the EE has also been demonstrated *in vitro* by its protective role against hemolysis of human lysozyme-like erythrocyte membranes. These findings revealed the medicinal and pharmacological potential of this plant, which could be an excellent candidate for the treatment of inflammation and pain-related illnesses as well as infections or as a food preservative. Therefore, this medicinal plant could be potentially exploited for the development of new drugs and have potential applications in pharmaceuticals, nutraceuticals and cosmetics. As it is the first contribution which concerns both the evaluation and the comparison of the bioactivities of EO and EE, this study opens the way to investigate more deeply the highlighted activities and to treat other aspects as well as explore other biological properties and the chemical biodiversity of the compounds of this plant.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

The first author carried out the biological activities, compound identification of HPLC analysis, and writing of the article. The second author contributed to the GC/MS analysis and provided laboratory assistance for antioxidant activity as well as a review of the article.

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Research Article

Cytotoxic effect of *Trigonella coerulescens* subsp. *ayvalikensis* Erdoğan, Selvi & Tümen in prostate and colon cancer cell lines

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Abstract: *Trigonella coerulescens* subsp. *ayvalikensis* is an annual local endemic taxon distributed only in Ayvalık/Balıkesir district and evaluated in the Vulnerable Category (VU). *Trigonella* L. is an important genus with medicinal and economic value in the Fabaceae family. Seeds of the genus *Trigonella* are known to contain several groups of secondary metabolites, the most abundant compounds being steroidal saponins, as well as flavones, isoflavones, and polysaccharides. In our study, the cytotoxic effect on two different cancer cell lines namely, PC-3 (prostate) and SW480 (colon), was investigated by extracting *Trigonella coerulescens* subsp. *ayvalikensis* with different solvents. The cytotoxic effects of extracts obtained from plant seeds with different solvents (hexane, methanol, ethanol, acetone) were investigated. The MTT test was used to examine the cytotoxic effect, which was studied with PC-3 and SW480 cancer cell lines. The different concentrations (23.45 µg/µL, 46.875 µg/µL, 93.75 µg/µL, 187.5 µg/µL, 375 µg/µL) of seed extracts were applied to the cells at different times points (24h, 48h and 72h) and absorbance was taken at 550 nm in the spectrophotometer. As a result of cytotoxic studies, it was observed that hexane extract had the most reducing effect on PC-3 compared to the control groups. In the SW480 cell line, a proliferative effect was observed in extracts prepared with methanol, hexane, and acetone in the early period of 24 hours. In the later period (72 hours), the extract prepared with hexane and acetone showed the most cytotoxic effect on SW480 cells.

1. INTRODUCTION

Trigonella L. is an important genus with medicinal and economic value in the Fabaceae family. Members of the genus *Trigonella* are distributed in areas such as the Eastern Mediterranean, Western Asia, Southern Europe, North and South Africa, Near East, India, and Ethiopia (Akan *et al*., 2020; Koç, 2002). This genus, known by vernacular names such as Çemenotu, Boyotu, Poyotu, Pıtlan, Pıltan, or Hulbe in Turkey, is represented by 106 species in the world and 32 species (34 taxa), 11 of which are endemic in Turkey (Güner *et al*., 2012; POWO, 2024). The seeds of species belonging to the genus *Trigonella* (fenugreek) are popularly used as a breast

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softener, expectorant, and laxative. They have also been used since ancient times as a tonic and sexual stimulant (Baytop, 1999).

Seeds of the genus *Trigonella* are known to contain several groups of secondary metabolites, the most abundant compounds being steroidal saponins, as well as flavones, isoflavones, and polysaccharides (Krol-Kogus *et al*., 2020). In recent years, herbal foods and plant-derived supplements have become more preferred in traditional medicine and health services. Some herbal products with rich bioactive and nutritional content have been used in traditional medicine to prevent various human diseases. The effectiveness of *Trigonella* species on different cancer cells has been demonstrated in several studies. The methanol and ethyl acetate extracts of *Trigonella foenum-graecum* L. seeds inhibited neurodegeneration in the hippocampus and cortex regions of the brain compared to the control group (Ahmed *et al*., 2017). The extracts exhibited anticancer activity against Hep2 and MCF-7 cells, low cytotoxicity against HCEC, and protected healthy cells in vitro. The hydroalcoholic extract of *T. foenum-graecum* seed had cytotoxic and anti-angiogenic effects *in vitro* (HUVEC and 3T3) and *in vivo* chick chorioallantoic membrane (CAM) (Iranmanesh *et al*., 2018).

The cytotoxicity, antitumor, antimetastatic, and antiangiogenic effects of the steroidal compound, ethyl iso-allocholate, isolated from *T. foenum-graecum* seeds were studied on A549 lung cancer cells in vitro and in vivo. The steroidal derivative isolated from *T. foenum-graecum* seeds induces caspase-dependent apoptosis in cancer cells, reduces tumor growth, metastasis, and angiogenesis in vivo, and is safe on normal tissues (Thakur & Ahirwar, 2019). The two protein hydrolysates of *T. foenum-graecum* (Purafect and Esperase) were studied in treating and progressing colorectal cancer. It has been suggested that hydrolysates of *T. foenum-graecum* protein could be used as nutraceutical molecules in treating colorectal cancer (Allaoui *et al*., 2019). In another study, in which two different aqueous extracts from the dry seed and germinated seed of *T. foenum-graecum* were used, evaluated the anticancer activity, its growth inhibitory effect on MCF7 human breast and pancreas (AsPC-1) cells was detected Abas & Naguib (2019); Mahapatra *et al*. (2020) also reported that the Fe (II) Schiff base complex MCF-7 in *T. foenum-graecum* induces cytotoxicity and DNA fragmentation through intracellular ROS production. They found that Fe-complex treatment also inhibited tumor growth in the solid tumor model without any side effects.

Stefanowicz-Hajduk *et al*. (2021) evaluated the cytotoxic activities of SKOV-3, HeLa and MOLT-4 cancer cell lines by obtaining extracts from the compounds (sapogenins, flavone C-glycosides, alkaloid trigonelline) in the seeds of the *T. foenum-graecum*. The extract showed a strong cytotoxic effect on cancer cell lines and was observed to significantly increase ROS production and caspase activity in the studied cells. *T. coerulescens* (M. Bieb.) Halácsy. subsp. *ayvalikensis* Erdoğan, Selvi & Tümen was introduced to the scientific world in 2017 with an international article by Erdoğan *et al*. (2017). This taxon has been evaluated in the VU (Vulnerable) category because its population is only found on the Badavut coast of Ayvalık district and the coastal edges of Küçükköy and carries the risk of being exposed to anthropogenic pressures (Erdoğan *et al*., 2017).

This study investigated for the first time the cytotoxic effect of the seeds of *T. coerulescens* subsp. *ayvalikensis*, which is locally endemic in Türkiye. The cytotoxic effect was evaluated in prostate cancer (PC3) and colon cancer (SW480) cells by MTT test.

2. MATERIAL and METHODS

2.1. Plant Collection and Extract Preparation Process

Mature seeds of the *T. coerulescens* subsp. *ayvalikensis* were collected in May-2022 from the following locality. The collection point is also shown in Figure 1. Türkiye, B1 Balıkesir: Ayvalik, Küçükköy, west of Badavut beach, Sarımsaklı beach, sandy coast, 39°16′23″ N, 26°37′43″ E, 1 m, 7.04.2013, (GT 3102).

Figure 1. Collection point of the taxon (indicated with an asterisk).

The seeds were left to dry for two days in a cool and airy room without light. Then, they were thoroughly grounded into powder in a metal mortar. It was then thoroughly ground into powder in a metal mortar. 0.75 grams were weighed on a precision scale and transferred to 4 centrifuge tubes of 50 mL for each solvent (Methanol, Ethanol, Acetone Hexane), and 10 mL of each solvent was added as a solvent and kept in a shaking incubator at +4 °C for one night. At the end of the process, the upper liquid part was transferred to a separate tube, and 10 mL of each solvent was added to the solid part at the bottom and kept at room temperature until it settled to the bottom. The liquid that rose to the top was transferred back to the centrifuge tube. The solvent was added again to the solid part at the bottom and centrifuged at 4000 RCF for 10 minutes. The liquid parts were transferred back to the centrifuge tube. The resulting extract was placed in a 100 mL bottle, the solvents were removed in a rotary evaporator and the extract was collected. The extract taken from the evaporator was dissolved in DMSO to create a stock solution (Habib-Martin *et al.*, 2017).

2.2. Culturing of Cells

Dulbecco's Modified Eagle's Medium (DMEM) high glucose (EuroClone) medium was used to grow the SW480 (Human Colon Carcinoma) cells used in the cell culture study, and DMEMF12 Mix (EuroClone) medium was used for PC-3 (Human Prostate Carcinoma) cells. By adding 10 % FBS (Fetal Bovine Serum) to these media, the cells were grown by passage 2-3 times a week at 37 °C in a humid atmosphere containing 5 % CO_2 (Cömert *et al*., 2016).

2.3. Identification of Live Cells and Cell Counting

One of the staining methods, using a solution called Trypan Blue, was applied to detect and count cells that remained viable. Thoma (hemocytometer) Slide was used to determine the number of cells in one milliliter of the total cell suspension. Cells separated from the surface by trypsinization process were centrifuged at 1000 rpm for 5 minutes and dissolved in DMEM with 10 % FBS content. To determine live cells, 10 µL of cell suspension and 10 µL of Trypan blue were mixed in a 1:1 dilution ratio and incubated at room temperature for 3-5 minutes, and then homogenization was achieved by pipetting. 10 µL of this prepared mixture was placed on a Thoma slide and the cells were counted under the microscope.

2.4. MTT Assay

In the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) method, when the mitochondria of living cells create a reaction, the tetrazolium ring in the MTT solution is broken down by the dehydrogenase enzymes present in the cell mitochondria and formazan crystals appear. MTT is a yellow formazan salt. Proliferating cells convert MTT into purple-colored water-insoluble formazan crystals with increased mitochondrial dehydrogenase enzyme activity (Cömert *et al*., 2016).

Cells counted the previous day were seeded into 96-well plates. PC-3 cells were plated at a density of $1x10^4$ cells per well and SW480 cells at $5x10^3$ cells per well. Extracts prepared in different solvents (Methanol, Ethanol, Acetone, and Hexane) were administered to the cells at different doses: 23.45 µg/µL, 46.875 µg/µL, 93.75 µg/µL, 187.5 µg/µL, 375 µg/µL. It was

applied a day later. These doses were applied to each cell line separately in triplicate. After 24, 48, and 72 hours of application periods, stock MTT solution was added to each well with a final concentration of 0.5 mg/mL and kept in an incubator device containing 5% CO_2 for 4 hours at 37 °C. At the end of 4 hours, the medium was removed, the crystals were dissolved with isopropanol containing 0.004 M HCl, and the absorbance was measured at a wavelength of 550 nanometers (nm) in a spectrophotometer. The results were graphed using the GraphPad Prism 8 program. The results were evaluated statistically with One Way Anova in the program ($p<0.05$ * was considered significant). The experiment was carried out in 3 repetitions (Cömert *et al*., 2016).

3. RESULTS

The effect of *T. coerulescens* subsp. *ayvalikensis* extract on the viability of human colon cells, SW480, and human prostate cancer cells, PC-3 was evaluated in vitro. One of the important processes in the pharmaceutical and nutritional supplement industries is the extraction of active ingredients from herbal products. In our study, different solvents, such as methanol, ethanol, hexane, and acetone, were used in seed extraction. All of the extracts obtained were dissolved in DMSO and evaluated for their effect on cell proliferation in selected cancer cell lines. This study used the MTT assay, which measures cell growth in vitro using human cell-mediated tetrazolium reduction. The assay is commonly used to determine the cytotoxicity of potential anticancer agents. The quantity of viable cells produced directly correlates with the formazan concentration. Figure 2 show the effect of 5 different concentrations (23.45–345 µg/µL) of each extraction of the PC-3 cell line for 24, 48, and 72 h. In the PC3 cell line, an increasing effect was observed in *T. coerulescens* subsp. *ayvalikensis* methanol extract at the dose of 23.24 µg/µL applied at the 24th hour compared to the control groups, while a decreasing effect was observed at all doses applied at the 48th and 72nd hours (Figure 2A). subsp. *ayvalikensis* ethanol extract at doses of 187.5 µg/µL and 375 µg/µL applied at the 24th hour; A reducing effect was observed compared to the control group at all doses administered at the 48th and 72nd hours (Figure 2B). In the PC3 cell line, an increasing effect was observed in subsp. *ayvalikensis* acetone extract compared to the control group at doses of 23.45 µg/µL and 93.75 µg/µL applied at the 24th hour, while a decreasing effect was observed compared to the control group at all doses applied at the 48th and 72nd hours (Figure 2C). A reducing effect was observed at all doses administered in the hexane extract compared to the control group. It was observed that among the extracts applied to the PC-3 cell line, hexane extract had the most reducing effect compared to the control group (Figure 2D).

On the other hand, extracts show the differential effects on SW480 cells (Figure 3). In the methanol extract applied to SW480 cells, at doses of 46,875 µg/µL and 375 µg/µL applied at the 48 hours; While a reducing effect was observed in all doses at the 72 hours compared to the control group, an increasing effect was observed in other applied doses compared to the control group (Figure 3A). In the ethanol extract applied to SW480 cells, doses of 46.875 µg/µL, 187.5 µg/µL, and 375 µg/µL were applied at the 24th hour; While an enhancing effect was observed compared to the control group at the dose of 187.5 µg/µL applied at the 48th hour, at the doses of 23.45 µg/µL and 93.75 µg/µL applied at the 24th hour; At doses of 23.45 µg/µL, 46.875 µg/µL and 375 µg/µL applied at the 48th hour; A reducing effect was observed compared to the control groups at doses of 23.45 µg/µL, 46.875 µg/µL, 187.5 µg/µL and 375 µg/µL applied over 72 hours (Figure 3B). An increasing effect was observed in the acetone extract applied to SW480 cells compared to the control group at all doses applied at 24 hours, while a decreasing effect was observed compared to the control group at all doses applied at 48 and 72 hours (Figure 3C). In the hexane extract applied to SW480 cells, an increasing effect was observed compared to the control group at the doses of 23.45 µg/µL, 46.875 µg/µL, 187.5 µg/µL and 375 µg/µL applied at the 24th hour, while a decreasing effect was observed compared to the control groups at all other doses. It was observed that among the extracts applied to the SW480

cell line, hexane extract had the most reducing effect compared to the control group (Figure 3D).

In summary, *T. coerulescens* subsp. *ayvalikensis* extracts have different effects in different concentrations and at different time points in colon cancer cells (SW480) and prostate cancer cells (PC-3 cells). It is clear that the anti-cancer effect of the *T. coerulescens* subsp. *ayvalikensis* extracts must be detailed. The anti-tumoral effect should be tested cell-specifically and the underlying molecular mechanism should be explained.

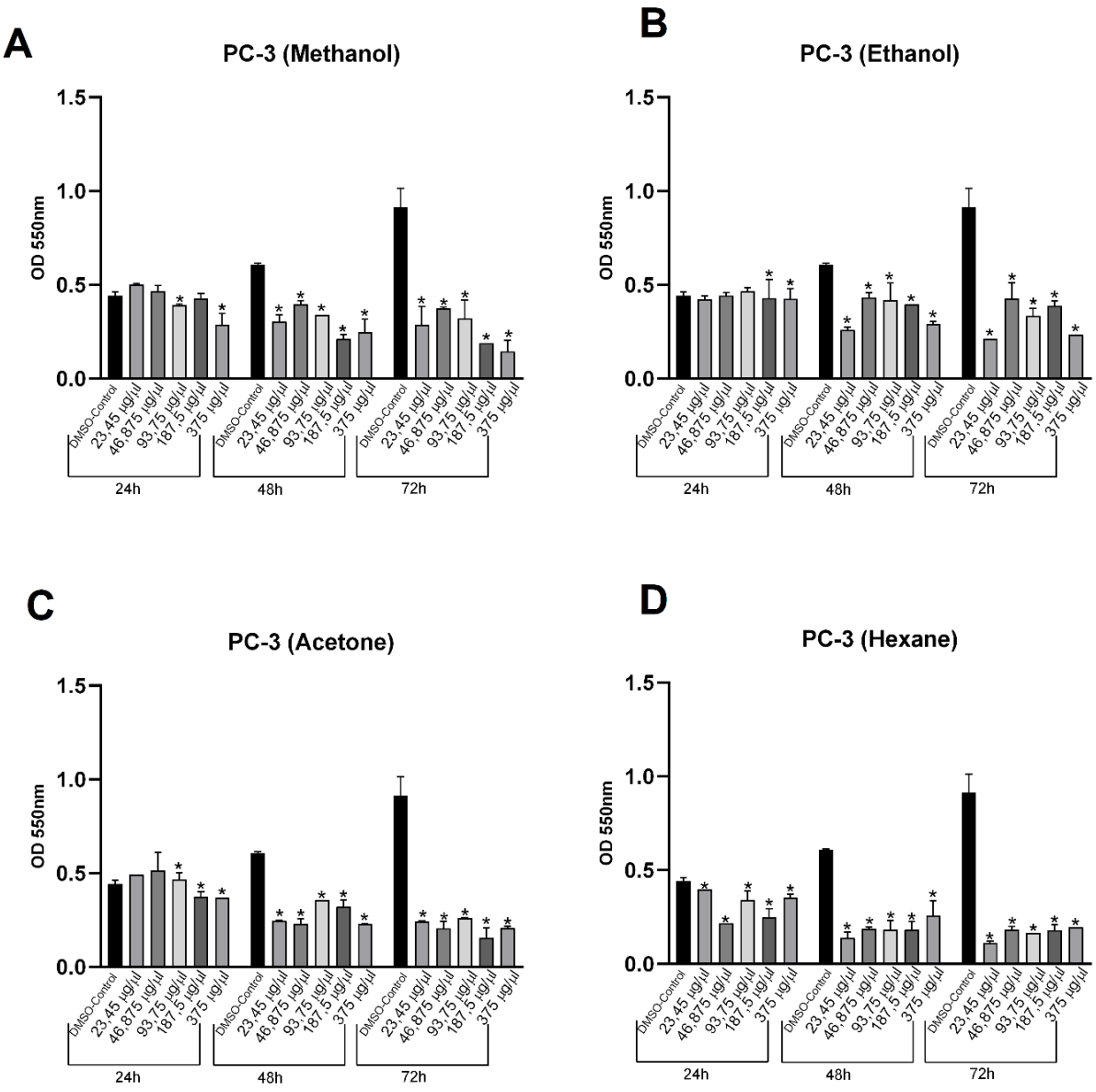

Figure 2. *In vitro* cytotoxicity *T. coerulescens* subsp. *ayvalikensis* extracts methanol (A), ethanol (B), acetone (C), and hexane (D) against malignant human prostate cells (PC-3). The cells were treated with DMSO vehicle or the indicated concentrations of *T. coerulescens* subsp. *ayvalikensis*. Cell viability was determined using MTT assay and p-value was obtained with ANOVA *$p < 0.05$ and was considered statistically significant.

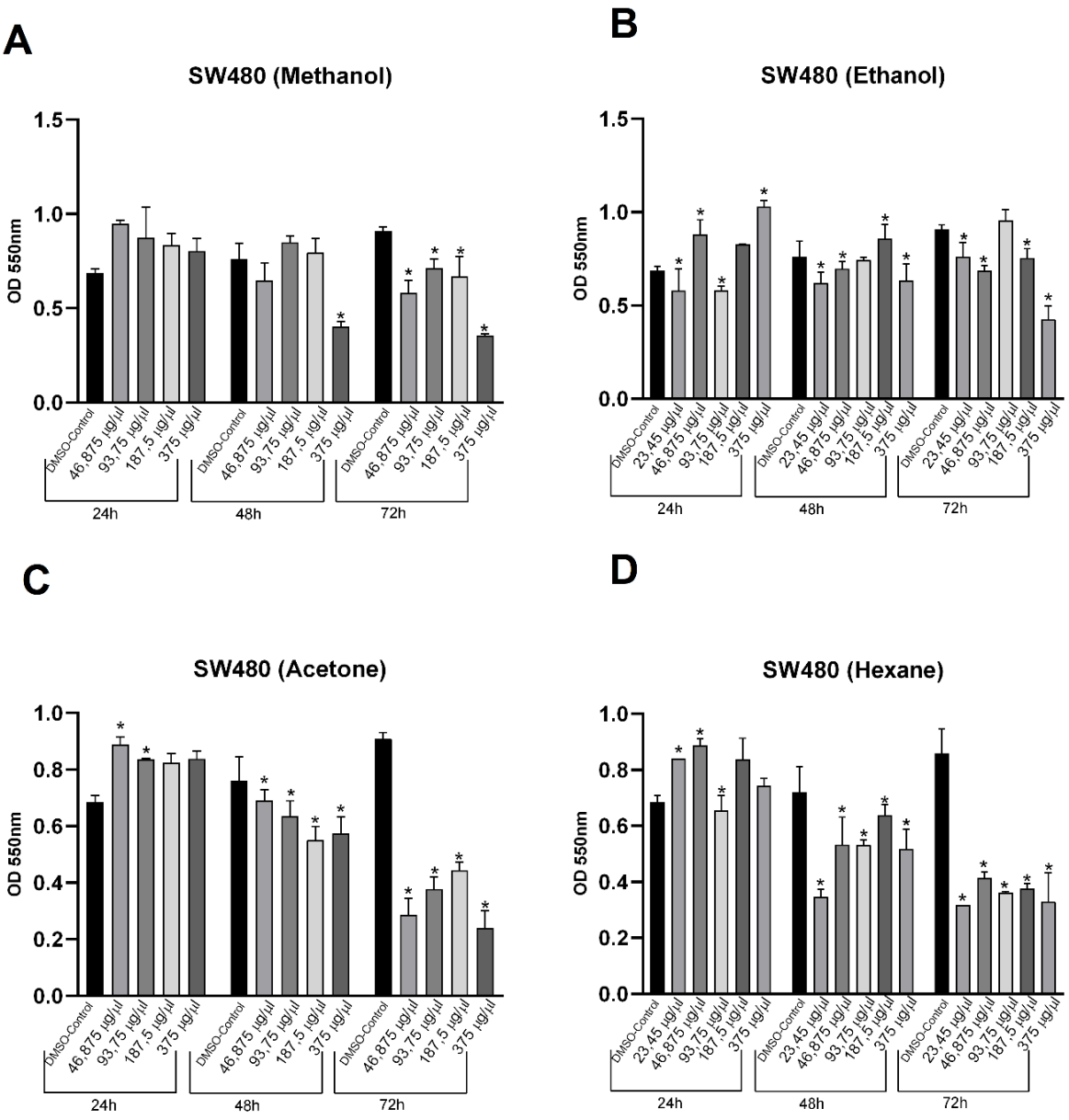

Figure 3. *In vitro* cytotoxicity *T. coerulescens* subsp. *ayvalikensis* extracts methanol (A), ethanol (B), acetone (C), and hexane (D) against malignant human colon cells (SW480). The cells were treated with DMSO vehicle or the indicated concentrations of *T. coerulescens* subsp. *ayvalikensis*. Cell viability was determined using MTT assay and *p*-value was obtained with ANOVA *$p < 0.05$ and was considered statistically significant.

4. DISCUSSION and CONCLUSION

Cytotoxicity refers to the rate of toxic effects on living cells. Cells may die due to autophagy, apoptosis, and necrosis. Cytotoxicity studies aim to determine the viability rates (amount of live/dead cells) of cells exposed to cytotoxic substances, depending on the dose and duration of effect(Tokur & Aksoy, 2017).

One of the methods used in cytotoxicity studies is the MTT method. MTT method; cytotoxic analysis is one of the preferred quantitative colorimetric methods for cell viability and proliferation. In this method, the tetrazolium ring in the MTT solution is broken down by the dehydrogenase enzymes in the cell mitochondria in the early stage of apoptosis or the reaction carried out by the mitochondria of living cells, creating colored formazan crystals. The MTT method is known as the method by which the mitochondria of healthy cells break down the tetrazolium ring of the MTT dye.

In this study, information on cytotoxicity studies of *T. coerulescens* subsp. *ayvalikensis* was presented for the first time. One of the important processes in the pharmaceutical and nutritional supplement industries is the extraction of active ingredients from herbal products. The solvent used in plant extraction can change the content of the products (Akinmoladun *et al*., 2022; Nawaz *et al*., 2019). Product content is affected by the time of plant collection, ambient temperature, extraction time, and solvent content (Dzah *et al*., 2020). In studies conducted with different plants, it has been reported that total phenol, flavonoid, and antioxidant activity values vary depending on the solvents, and it is necessary to use different solvents for each plant and the content to be obtained (Çoklar *et al*., 2016; Ribeiro *et al*., 2020; Salem *et al*., 2011). It is also clear that changing extract content depending on the solvent will affect biological activity. In our study, different solvents such as methanol, ethanol, hexane, and acetone were used in the extraction process from seeds. All of the extracts obtained were dissolved in DMSO and evaluated for their effect on cell proliferation in selected cancer cell lines.

Generally, when the phytochemical analysis of the seed content of the *Trigonella* genus is examined, it has been determined that flavonoid (vitexin, orientin, isoorientin, vicenin-1, vicenin-2, vicenin-3), saponin (yamogenin, tigogenin, diosgenin) and alkaloid (trigonelline) compounds are found in high amounts (Krol-Kogus *et al*., 2020; Krol-Kogus *et al*., 2021; Stefanowicz-Hajduk *et al*., 2021).

In the literature, in cytotoxicity studies conducted with extracts obtained from the seeds of the *Trigonella* genus, Stefanowicz-Hajduk et al. (2021), in SKOV-3, HeLa and MOLT-4 cell lines; Thakur and Ahirwar (2019) in the A549 (lung cancer) cell line; Abas and Naguib (2019), in MCF-7 and AsPC-1 cell lines; Goyal *et al*. (2018) in A431 (skin cancer) cell line; Al-Dabbagh *et al*. (2018) observed a cytotoxic effect on cancer cells in the HepG2 (human hepacellular cancer) cell line.

According to the cytotoxicity effect of *T. coerulescens* subsp. *ayvalikensis* hexane extract had the most reducing effect on PC-3 cells compared to the control groups. In the SW480 cell line, a proliferative effect was observed in extracts prepared with methanol, hexane, and acetone in the early period of 24 hours. In the later period of 72 hours, the extract prepared with hexane and acetone showed the most cytotoxic effect on SW480 cells. In this study, *T. coerulescens* subsp. *ayvalikensis* dose, time, and cell type-dependent effect was detected. Cell proliferation and antiproliferation occurred simultaneously, allowing the determination of the antitumoral and toxic effects for high doses of the extracts only at later periods (48, 72h). Based on the literature, at least two mechanisms can be put forth to explain this result. The first mechanism suggests that the drug's protective effects may be due to an anti-apoptotic mechanism; the second mechanism involves an antioxidant mechanism that increases the intracellular antioxidant enzymatic system's activity. Consequently, we propose that different solvents vad different extract concentrations of *T. coerulescens* subsp. *ayvalikensis* may impact the survival of human colon cancer cells through comparable anti-apoptotic and antioxidant mechanisms at early time points but apoptotic mechanisms may activated at late time points. More research is required to determine the precise cutoff value and concentration limits of *T. coerulescens* subsp. *ayvalikensis* extracts before their use as an anti-neoplastic agent, as well as to explain the mechanism(s) underlying this possible dual role of *T. coerulescens* subsp. *ayvalikensis* on the survival and growth of cancer cells.

As a result, we believe that the data we obtained will be an important resource for systematic studies to be conducted with similar genera or taxa in the future and for studies showing the effects of the extracts obtained for use in cytotoxicity experiments on different cancer cell lines. We think that the mechanism by which its effect on cancer cells occurs should be investigated in future studies.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s). Ethics Committee Approval and its number should be given by stating the institution name which gave the ethical approval.

Authorship Contribution Statement

Sümeyye Aydoğan Türkoğlu: Investigation, Resources, Visualization, Writing -original draft, Supervision, and Validation. **Selin Koç**: Investigation, Resources, Methodology. **Fatma Poyrazlı**: Investigation, Resources, Methodology. **Selami Selvi**: Plant collection and identification, Supervision, and Validation.

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Research Article

The isolation of bioactive compounds from *Warburgia ugandensis* bark: A report of albicanyl acetate, caseamemin and β-sitosterol from *Warburgia* species

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Abstract: *Warburgia ugandensis,* which is one of the indigenous species of Ethiopia, is known for its wide range of biological activities. A series of drimane sesquiterpenoids have been isolated from the stem bark of the plant. However, there is no report on the herbicidal potential of the plant against invasive weeds like *Parthenium hysterophorus*. In this study, the herbicidal potential of *W. ugandensis* against the *P. hysterophorus* weed was investigated. Following the bioassay protocol, muzigadial as powerful phytotoxic compound together with other eight compounds were isolated from the EtOAc soluble portion of the ethanol extract of the bark of the plant. These compounds were identified using different physical and spectroscopic methods. The isolated compounds are albicanyl acetate (35), caseamemin (36), β-sitosterol (37), muzigadial (38), cinnamolide-3β-acetate (39), ugandensidial (40), 11α-hydroxy muzigadiolide (41), polygodial (42) and 9-deoxymuzigadial (43). The first three compounds are new to the species *W. ugandensis.* Furthermore, two other compounds namely heptacosanol (44) and hentriacontane (45) were also isolated from this species. In summary, the purpose of this study, to the best of my knowledge, is to provide the three initially identified compounds from the plant material and provide information on the plant's potential utility in agricultural applications.

1. INTRODUCTION

Warburgia ugandensis Sprague (Canellaceae), one of Ethiopia's indigenous species, is an evergreen and aromatic perennial plant (Maroyi, 2014) with a characteristic aromatic and pungent bark. Traditional healers have used the plant to treat various ailments since antiquity, despite its multiple uses as timber, poles, charcoal, firewood, ornaments, shade, and resin (Kairu *et al*., 2013). The plant is a remedy for stomachache, headache, constipation, toothache, common cold, internal wound, and malaria (Kipkore *et al*., 2014; Maroyi, 2014). In addition to these, it is also reported that the plant is used to prevent diarrhea, cough, sexually transmitted diseases, snake bites, bronchial infections, fever, oral thrush, muscle pain, urinary tract infections, constipation, weak joints, and measles (Okello & Kang, 2021).

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In a study done by Wube *et al*, the antimicrobial activity of the plant was reported (Wube et al., 2005; Okello & Kang, 2021). The antileishmanial activity of different extracts of *W. ugandensis* was studied, and it was reported that the nonpolar extract of the plant displayed strong activity against *Leishmania major* promastigotes (IC_{50} value of 9.95) and amastigotes (IC_{50} value of 8.65) at minimum inhibition concentrations of 62.5 µg/ml (Ngure *et al*., 2009). *In vitro* pharmacological studies on this plant have revealed its insecticidal potential against maize weevils (Opiyo, 2021) and molluscicids (Maroyi, 2014).

Based on a number of biological activities reported, several Drimane-type sesquiterpenes and flavonoids (Figure 1) have been isolated from various parts of the plant (Arot Manguro, Ugi, Hermann, *et al*., 2003; Wube *et al.*, 2005). Widely applicable dialdehydic compounds: warburganal (**1**), muzigadial (**2**) and polygodial (**3**) and different sesquiterpene compounds: ugandenial A (**4**), 9α, 11α-dihydroxy,6β-acetyl-cinnamolide (**5**), mukaadial (**6**), 9α-hydroxycinnamolide (**7**), and dendocarbins A, L, and M (**8, 9** and **10**) were isolated using different chromatographic and spectroscopic methods.

The first phytochemical investigation of *W. ugandensis* led to the isolation of drimane-type sesquiterpenoids from the heartwood of the plant. These are warburgin (**11**), warburgiadione (**12**), ugandensolide (**13**) and ugandensidial (**14**) (Brooks, 1969). Another study on the stem bark of *W. ugandensis* conducted by Gonfa, T. led to the isolation of four sesquiterpenes, namely nerolidol (**15**), muzigadial (**2**), ugandensidial (**14**), and cinnamolide-3*β*-acetate (**18**) (Gonfa *et al.*, 2020).

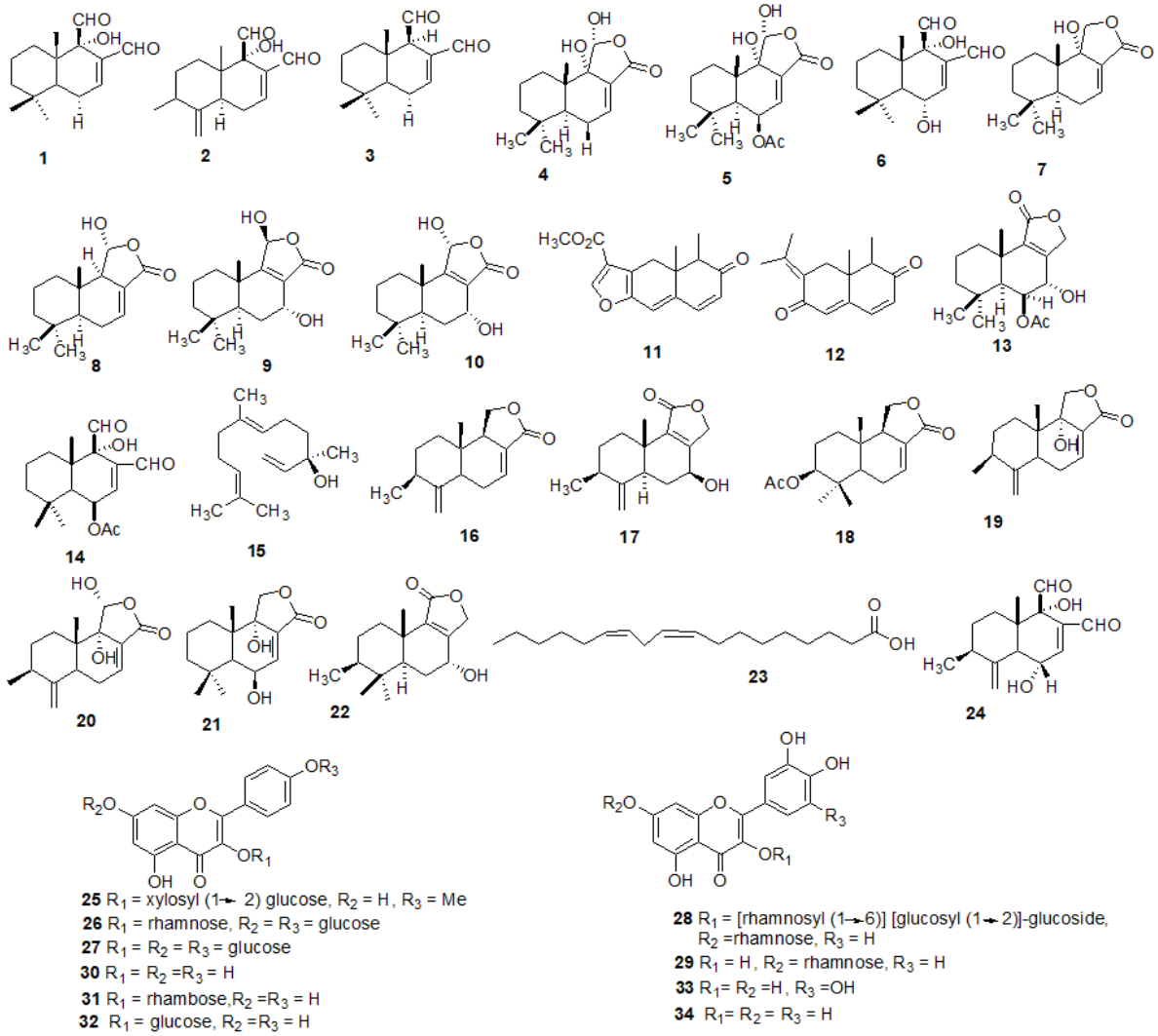

Figure 1. Previously isolated compounds from *Warburgia* species.

In the interest of searching for antibacterial sesquiterpenoids, numerous compounds were reported from *W. ugandensis.* The compounds isolated are 4(13),7-coloratadien-12,11-olide (**16**), and 7β-hydroxy-4(13),8 coloratadien-11,12-olide (**17**), together with nine known sesquiterpenes, i.e., cinnamolide-3β-acetate (**18**), muzigadial (**2**), muzigadiolide (**19**), 11α-hydroxy muzigadiolide (**20**), pereniporin-B (**21**), 7α-hydroxy-8-drimen-11,12-olide (**22**), 6α,9α-dihydroxy-4(13),7-coloratadien 11,12-dial (**23**), and linoleic acid (**24**) (Rabe & Staden, 2000). Flavonol glycosides (**25-29**) together with known flavonols like kaempferol (**30**), kaempferol-3-rhamnoside (**31**), kaempferol-3-glucoside (**32**), myricetin (**33**), and quercetin (**34**) were reported from the leaf methanol extracts of *W. stuhlmannii* and *W. ugandensis* (Arot Manguro, Ugi, Hermann, *et al*., 2003; Arot Manguro, Ugi, Lemmen, *et al*., 2003). Even though many bioactive components for various applications were reported from *W. ugandensis*, the scope is still there to identify bioactive compounds from this plant for different applications, including the agricultural sector. Thus, aiming to study the herbicidal potential of the plant led to the isolation of three previously unreported compounds from this plant.

2. MATERIAL and METHODS

2.1. Plant Material

Warburgia ugandensis bark (Figure 2) was collected from Bale Robe, Oromia region, south-central Ethiopia, which has a latitude of 6° 44' 59.99" N and a longitude of 40° 14' 60.00" E. It is the area found at an altitude of 2,492 meters and 430 kilometers far away by road from Ethiopia's capital, Addis Ababa. The specimen of the plant was deposited at the National Herbarium Department of Biology, Addis Ababa University Herbarium, with voucher number 97-41A, and its identity was determined by a plant taxonomist, Dr. Melaku.

Figure 2. Stem bark of *W. ugandensis* tree.

2.2. Materials Used for Purification and Spectroscopic Analysis

The compounds reported in this study were isolated using two sizes of column chromatography on silica gel and aluminum oxide (neutral), medium size and small size, which can carry 80 g and 12 g of silica gel, respectively. Fractions collected from CC were purified by Sephadex LH-20. Preparative thin-layer chromatography was run on a 1.0 mm thick layer of silica gel. The silica gel used for the CC is 60-120 mesh particle size. TLC was performed on precoated plates (Silica gel 60 F254, 230-400 mesh, Merck) and aluminum oxide plates; melting points are uncorrected; detection by UV light at 254 and 366 nm and spray reagents vanillin-H_2SO_4; IR: KBr disk or neat and measured on a Perkin Elmer 1600 and Pye Unicam Infrared spectrophotometer SP3-300. UV spectra were measured on a Shimadzu UV-VIS recording spectrophotometer, a UV-160 spectronic genesys spectrophotometer; ^1H and ^{13}C NMR were recorded, in $CDCl_3$, DMSO, $(CD_3)_2CO$, and CD_3OD using the solvent peak as reference (chloroform: δ_H 7.2 and δ_C 77.2, DMSO, δ_H 2.5 and δ_C 39.5, deuterated acetone: δ_H 2.05 and δ_C 29.5 and 205.5 methanol: δ_H 3.3 and δ_C 49.0). Chemical shift values were reported in δ (ppm) units, the solvent signals as internal references; ^1H, ^{13}C, and 2D-NMR spectra were

obtained on a Jeol F X 90 Ω spectrophotometer at 90 and 22.5 MHz; a Jeol JNM-EX400 instrument at 400 MHz and 100 MHz; and a Bruker Ultrashield TM 400 spectrometer at 400 and 100 MHz with TMS and solvents as internal standard, and δ values are given in ppm relative to TMS internal standard. EIMS was obtained on a Finnigan MAT 95Q and VG Quattro quadrupole mass spectrometer (70 eV).

2.3. Extraction Procedure

The bark of the plant was ground with a manual grinder into a fine powder. The powder (150 g) was extracted with ethanol (500 mL) to afford 15 g of red-like jelly material. It was then partitioned with EtOAc and methanol. The EtOAc soluble part (4 g) was adsorbed on silica gel (60-120 mesh) and subjected to silica gel (80 g) column chromatography. The column was eluted with an n-hexane: EtOAc solvent system by increasing polarity to afford 12 combined fractions.

Fraction 5 was collected from CC using n-hexane: EtOAc (9:1) and white powder was precipitated on the surface of the vial and labeled as compound **35** (27 mg). The remaining part was concentrated using rotary vapor, and 800 mg was obtained. From this fraction, 155 mg was taken and applied on PTLC using n-hexane: EtOAc (9:1) as a mobile phase, which gave compound **36** (15 mg). From the remaining part of fraction 5, 400 mg was applied on small-size silica gel (10 g) column chromatography, and eight subfractions were collected. Subfraction 2 (60 mg) was further purified by PTLC to give compound **37** (20 mg).

2.3.1. *Physical and spectral data for compound 35*

White solid (27 mg); soluble in $CHCl_3$; mp 130-136°C; R_f 0.62 (mobile phase hexane: EtOAc, 2:1); UV (EtOH) λmax nm: no absorbance; IR υ_{cm-1}: 3453 (OH stretching), 1736, and 1680 (*α, β* unsaturated C=O stretching), 1736 (acetate group), 1370 (geminal dimethyl stretching), 1230 and 1024 (C-O stretching and bending); ^{1}H-NMR (400 MHz, $CDCl_3$), chemical shift δ in ppm, coupling constant *J* in Hz: δ_H 3.53 (1H, *m*, H-3), 5.37 (1H, *t*, *J* = 2 Hz, H-6), 0.93 (3H, *d*, *J* = 6.8 Hz, H-19), 0.84 (3H, *d*, *J* = 2 Hz, H-24), 0.84 (3H, *d*, *J* = 2 Hz, H-26), 0.82 (3H, *d*, H-27), 0.70 (3H, *s*, H-28), and 1.03 (3H, *s*, H-29); ^{13}C NMR (100 MHz, CDC_3): δ_C 37.26 (C-1), 31.68 (C-2), 71.84 (C-3), 42.32 (C-4), 140.76 (C-5), 121.75 (C-6), 31.92 (C-7), 31.98 (C-8), 50.14 (C-9), 36.53 (C-10), 21.11 (C-11), 39.78 (C-12), 42.34 (C-13), 56.78 (C-14), 26.05 (C-15), 28.28 (C-16), 56.05 (C-17), 36.17 (C-18), 19.43 C-19), 33.95 (C-20), 24.33 (C-21), 45.84 (C-22), 23.07 (C-23), 12.01 (C-24), 29.15 (C-25), 19.86 (C-26), 19.05 (C-27), 18.80 (C-28), and 11.89 (C-29).

2.3.2. *Physical and spectral data for compound 36*

Jelly material (15 mg); soluble in $CHCl_3$; R_f 0.50 (mobile phase hexane: EtOAc, (5:1); no absorption in the UV-Vis region; IR υ_{cm-1}: 2923 (C-H stretching), 1735 (acetate unit), 1641 and 1461 cm-1 (exocyclic double bond stretching and bending), 1230 (C-O stretching), 1376 cm-1 (geminal dimethyl stretching); ^{1}H-NMR (400 MHz, $CDCl_3$), chemical shift δ in ppm, coupling constant *J* in Hz: δ_H 1.25, 1.68 (2H, *m*, H-1), 1.45, 1.52 (2H, m, *qt*, H-2), 1.09 (1H, *m*, H-5), 2.06, 2.41 (2H, *m*, H-7), 2.09 (1H, *m*, H-9), 4.53 (1H, *s*, H_a-12), 4.87 (1H, *d*, H_b-12), 4.2 (1H, *dd*, *J*= 11.2, 9.2 Hz, H_a-11), 4.3 (1H, *dd*, *J*= 11.2, 3.6 *Hz*, H_b-11), 0.84 (3H, *s*, H-13), 0.77 (3H, *s*, H-14), 0.71 (3H, *s*, H-15), and 2.07 (3H, *s*, H-17); ^{13}C NMR (100 MHz, CDC3): δ_C 39.03 (C-1), 19.17 (C-2), 41.92 (C-3), 33.94 (C-4), 55.06 (C-5), 23.90 (C-6), 37.60 (C-7), 146.83 (C-8), 54.73 (C-9), 38.97 (C-10), 107.15 (C-11), 61.58 (C-12), 33.64 (C-13), 21.76 (C-14), 15.11(C-15), 171.41 (C-16), 21.12 (C-17).

2.3.3. *Physical and spectral data for compound 37*

Brown jelly material (20 mg); soluble in $CHCl_3$; R_f 0.5 (mobile phase hexane: EtOAc, (5:1); UV (EtOH) λmax nm: 298; IR υ_{cm-1}: 2916 and 1478 cm^{-1} are due to the C-H stretching and bending, 1376 cm^{-1} geminal dimethyl stretching, 1205 cm^{-1} C-O stretching; ^{1}H-NMR (400 MHz, $CDCl_3$), chemical shift *δ* in ppm, coupling constant J in Hz: δ_H 5.12 (1H, *m*, H-3), 2.14

(2H, *m*, H-4), 6.40 (1H, *d*, *J* =2.8Hz, H-5), 6.49 (1H, *d*, *J* = 2.8 Hz, H-7), 1.62 (3H, *s*, H-9), 2.14 (3H, *s*, H-10), 2.71 (2H, *t*, *J* = 6.4 Hz, H-1'), 1.74 &1.81 (2H, *td*, H-2'), 2.01 (2H, *m*, H-5'), 1.75 (2H, *m*, H-6'), 5.12 (1H, *m*, H-7'), 1.78 (2H, *m*, H-9'), 2.09 (2H, *m*, H-10'), 5.12 (1H, *m*, H-11'), 1.61 (3H, *s*, H-13'), 1.28 (3H, *s*, H-14'), 1.62 (3H, *d*, *J* = 3.6 Hz, H-15'), 1.70 (3H, *s*, H-16'); ^{13}C NMR (100 MHz, CDC_3, δ in ppm: δc 147.89 (C-2), 124.21 (C-3), 22.20 (C-4), 134.95 (C-4a), 112.63 (C-5), 121.20 (C-6), 115.71 (C-7), 127.30 (C-8), 145.92 (C-8a), 16.0 (C-9), 15.87 (C-10), 22.51 (C-1'), 31.42 (C-2'), 75.31 (C-3'), 39.71 (C-4'), 26.78 (C-5'), 39.71 (C-6'), 124.33 (C-7'), 135.10 (C-8'), 39.71 (C-9'), 26.62 (C-10'), 124.43 (C-11'), 131.31 (C-12'), 25.69 (C-13'), 24.0 (C-14'), 16.0 (C-15'), and 16.68, (C-16').

3. RESULTS and DISCUSSION

Generally, the EtOAc soluble portion of the EtOH extract of the bark of *W. ugandensis* resulted in the first report of the isolation of albicanyl acetate, caseamemin, and *β*-sitosterol together with other 6 known dialdehydic and drimane-type compounds (Figure 3) (Gizachew, 2019). These are muzigadial (38), cinnamolide-3β-acetate (39), ugandensidial (40), 11α-hydroxy muzigadiolide (41), polygodial (42), and 9-deoxymuzigadial (43). In the course, 1-heptacosanol (44) and Hentriacontane (45) were also isolated.

Different physical and spectroscopic examinations, along with a comparison of the results with related compounds in the literature, were carried out to elucidate the structures of the compounds. The NMR data of each compound is depicted as supporting material for this manuscript.

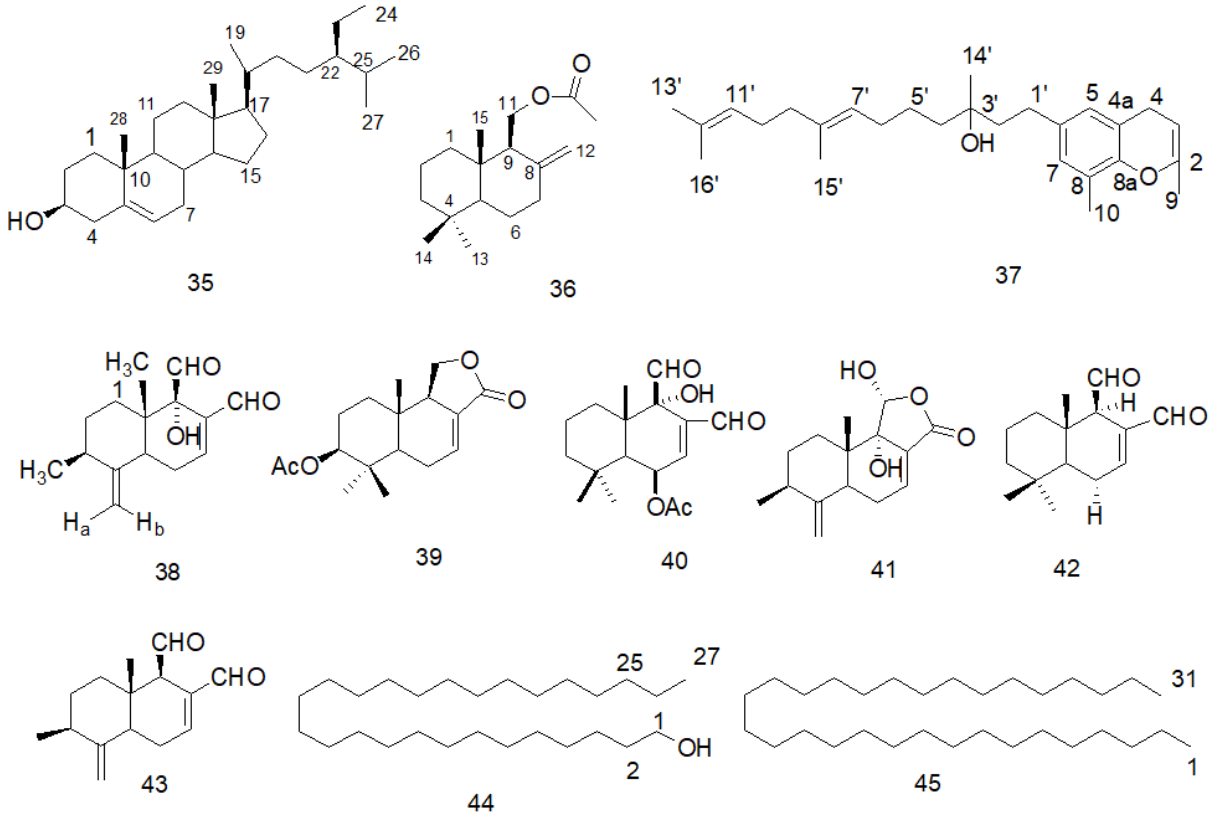

Figure 3. Compounds isolated from the stem bark of *W. ugandensis*.

3.1. Characterization of Compound 35

Compound **35** was isolated as a white solid (27 mg) from fraction five. The TLC profile developed using a hexane: EtOAc (9:1) solvent system and vanillin as a spraying agent showed a purple single spot (Rf 0.62). Compound **35** melts at 130-136ºC (lit. 134-136ºC) (Chaturvedula & Prakash, 2012). The UV-Vis spectrum (in ethanol) showed no absorption band from the 600-200 nm wavelength region.

The ^{1}H-NMR spectrum showed the most downfield signal for one olefinic proton at δ_H 5.36 and the other signals appeared at 3.53 as a multiplet for a proton corresponding to the proton connected to the C-3 hydroxyl group. The ^{1}H-NMR spectrum revealed the presence of 6 methyl groups, of which three are methyl singlets that appeared at δ_H 0.82 (3H, *s*), 0.70 (3H, *s*), 1.03 (3H, *s*), and the other three are observed as methyl doublets at δ_H 0.84 (3H, *d*), 0.86 (3H, *d*), and 0.93 (3H, *d*).

From the ^{13}C-NMR spectrum, the downfield signals that appeared at δ_C 140.7 and 121.7 were interpreted as the marker signals for olefinic carbons of *β*-sitosterol. The signal that appeared at δ_C 71.8 was assigned to the oxygenated carbon of the sterol (C-3). The remaining signals of both the ^{1}H and ^{13}C-NMR data of compound **35** were compared with literature values of *β*-sitosterol (Table 3) and found a good agreement (Chaturvedula & Prakash, 2012).

Table 1. ^{1}H and ^{13}C-NMR spectral data comparison of compound **35** with literature values of β-sitosterol.

^{13}C & ^{1}H-NMR data of compound **35**			Lit. value of β-sitosterol (Chaturvedula & Prakash, 2012)	
1	37.2		37..5	
2	31.6		31.9	
3	71.8	3.53 (1H, *m*)	72.0	3.53 (tdd, 1H, J = 4.5, 4.2, 3.8 Hz)
4	42.3		42.5	
5	140.7		140.9	
6	121.7	5.36 (1H, *t*, 2.8)	121.9	5.36 (t, 1H, J = 6.4 Hz)
7	31.9		32.1	
8	31.9		32.1	
9	50.1		50.3	
10	36.5		36.7	
11	21.1		21.3	
12	39.7		39.9	
13	42.3		42.6	
14	56.7		56.9	
15	26.0		26.3	
16	28.2		28.5	
17	56.0		56.3	
18	36.1		36.3	
19	19.4	0.93 (3H, *d*, 6.8)	19.2	0.93 (d, 3H, J = 6.5 Hz)
20	33.5		34.2	
21	24.3		26.3	
22	45.8		46.1	
23	23.0		23.3	
24	12.0	0.84 3H, *t*)	12.2	0.84 (t, 3H, J = 7.2 Hz)
25	29.1		29.4	
26	19.8	0.84 (3H, *d*, 6.8)	20.1	0.83 (d, 3H, J = 6.4 Hz)
27	19.0	0.82 (3H, *d*, 6.8)	19.1	0.81 (d, 3H, J = 6.4 Hz)
28	18.8	0.70 (3H, *s*)	19.0	0.68 (s, 3H)
29	11.8	1.03 (3H, *s*)	12.0	1.01 (s, 3H)

Chemical shifts are reported in parts per million ($CDCI_3$), and ***J*** values are in Hertz.

The data generated from the characterization of compound **35** agreed well with the literature report on *β*-sitosterol. To my knowledge, there has been no report made on the isolation of β-sitosterol from Warbirgia species previously. The compound is known for its antinociceptive, anxiolytic & sedative effects, analgesic, immunomodulatory, antimicrobial, anticancer, anti-inflammatory, and hepatoprotective (Babu & Jayaraman, 2020).

3.2. Characterization of Compound 36

This compound was isolated from fraction five using hexane: EtOAc (9:1) as eluent. Fraction five (100 mg) was applied on PTLC, which led to the isolation of compound **36** (15 mg) as a jelly-like material. The TLC profile of this compound was developed using a hexane: EtOAc (5:1) solvent system and 1% vanillin as a spraying agent and showed a single purple spot (R_f 0.5). The UV-Vis spectrum of the compound showed no absorption band from 600-200 nm, indicative of the absence of conjugated chromophore. The IR spectrum of compound **36** exhibited bands at 2923 cm^{-1}(C-H stretching), and 1735 (acetate unit). The other band observed at 1641 cm^{-1} is due to the presence of C-C stretching of the exocyclic double bond. Geminal dimethyl stretching is observed at 1376 cm^{-1}. The signal that appeared at 1230 cm^{-1} is due to C-O stretching in the molecule.

The ^{1}H-NMR spectrum of compound **36** displayed four terminal methyl protons, each appearing at δ_H 0.71 (3H, *s*), 0.77 (3H, *s*), 0.84 (3H, *s*), and 2.07 (3H, *s*). The two most downfield singlet signals that appeared at δ_H 4.87 (1H, *s*), and 4.53 (1H, *s*) were indicative of the presence of an exocyclic double bond (H-12). The other downfield signals that appeared as a doublet of a doublet at δ_H 4.20 (1H, *dd*, *J* = 11.2, 9.2 Hz), and 4.34 (1H, *dd*, *J* = 11.2, 3.6 Hz) were due to the diastereotopic protons on oxygenated carbons (H-11). The HH-COSY experiment revealed the correlation of protons of exocyclic double bonds with H-7 and H-9. The protons were assigned to the carbon with the help of HSQC and HMBC. The oxygenated protons were only correlated with protons that appeared at 2.07 (H-9).

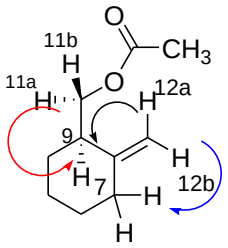

Figure 4. Showed the HH-COSY correlation.

The ^{13}C-NMR spectrum together with DEPT-135 revealed the presence of four methyls, seven methylenes, two methines, and four quaternary carbon atoms. The downfield signal that appeared at δ_C 171.4 together with a methyl signal that appeared at δ_C 21.1 was evident for the presence of an ester functional group. The signals observed at δ_C 146.8, and 107.1 in consistency with the proton NMR experiment were suggestive of the presence of an exocyclic double bond. The methylene signal that appeared at δ_C 61.5 was evident for the presence of one oxygenated carbon. The remaining methylene signals appeared at δ_C 39.0, 19.1, 41.9, 23.9, and 37.6. The two methine carbons appeared at δ_C 55.0 and 54.7. Both ^{1}H and ^{13}C spectral data of this compound were found in good agreement with the literature report on albicanyl acetate (Dumdei, E.J., *et al*, 1997).

This compound was previously reported from the skin extracts of *Cadlina luteomarginata* (Dumdei, *et al*, 1997). However, this is the first report from *W. ugandensis*. Albicanyl acetate is known as a potent fish antifeedant biological activity compound (Barrero *et al*., 1995).

Table 2. NMR data comparison of compound **36** with literature values of albicanyl acetate.

	Experimental data of compound **36**		Literature data of albicanyl acetate (Dumdei, E.J., *et al* 1997)	
	^{13}C-NMR	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR
1	39.0	1.25 (1H, *m*), 1.68 (1H, *m*)	39.0	1.25 (1H, *m*, H-eq), 1.68 (1H, *m*)
2	19.1	1.45, 1.52 (2H, m, *qt*)	19.2	1.45, 1.52 (2H, *m*, *qt*)
3	41.9		41.9	
4	33.9		33.5	
5	55.0	1.09 (1H, *m*)	55.1	1.09 (1H, *m*)
6	23.9		23.9	
7	37.6	2.06 (1H, *m*), 2.41 (1H, *qd*)	37.8	2.06 (1H, *m*), 2.36 (1H, *m*)
8	146.8		146.8	
9	54.7	2.09 (1H, *m*)	54.7	2.09 (1H, *m*)
10	38.9		38.9	
11	61.5	4.2 (1H, *dd*), 4.3 (1H, *dd*)	61.6	4.14 (1H, *dd*), 4.29 (1H, *dd*)
12	107.1	4.53 (1H, *s*), 4.87 (1H, *d*)	107.1	4.47 (1H, *s*), 4.81 (1H, *s*)
13	33.6	0.84 (3H, *s*)	33.6	0.84 (3H, s)
14	21.7	0.77 (3H, *s*)	21.7	0.77 (3H, s)
15	15.1	0.71 (3H, *s*)	15.1	0.71 (3H, s)
16	171.4		171.4	
17	21.1	2.07 (3H, s)	21.1	2.07 (3H, s)

s = singlet, *dd* = doublet of doublet, *qd* = quartet of doublet, *m* = multiplet

3.3. Characterization of Compound 37

Compound **37** (20 mg) was obtained as a jelly material from fraction 5. The TLC profile of the compound developed using the hexane: EtOAc (4:1) solvent system showed a single spot at *Rf* values of 0.5 visualized after spaying with 1% vanillin in sulfuric acid. The UV-Vis spectral analysis showed the presence of a conjugated chromophore with λmax 298 nm. In the IR spectrum of compound **37**, the broadband observed at 3429 cm^{-1} is suggestive of the presence of the OH group in the molecule. The sharp peak that appeared at 2916 is due to the C-H stretching. The peaks at 1607 and 1478 cm^{-1} are indicative of the presence of the aromatic ring. The other band displayed at 1376 cm^{-1} is a characteristic of the presence of geminal dimethyl stretching. The band at 1205 cm^{-1} is due to the C-O stretching.

The ^{1}H-NMR spectrum is suggestive of the presence of aromatic groups in the molecule due to the two signals that appeared at δ_H 6.49 (1H, *d, J= 3.2*), and 6.40 (1H, *d, J= 2.8*). The presence of other olefinic protons is confirmed by the proton signals observed at δ_H 5.08-5.14 (3H, *m*). The triplet signal at δ_H 2.71 integrated for two protons is due to two methylene protons of the chain appended to the aromatic ring., Six terminal methyl protons are observed in the ^{1}H-NMR spectrum, of which three methyl protons appear to be overlapped at δ_H 1.61 (9H, *s*). The remaining terminal methyl protons are shown at δ_H 1.70 (3H, s), 1.28 (3H, *s*), and 2.14 (3H, *s*).

Two-band carbon signals are observed in the olefinic (aromatic) and aliphatic regions in the ^{13}C-NMR spectrum. There are twelve carbon signals observed in the olefinic region (from δ_C 112.6 up to 147.8). Only five of them (δ_C 112.6, 115.7, 124.2, 124.3, and 124.4) are methine carbons, and the rest seven (δ_C 121.1, 127.2, 131.2, 134.9, 135.1, 145.9, and 147.8) are quaternary carbons. The ^{13}C-NMR spectrum together with Dept-135 showed one oxygenated quaternary carbon at δ_C 75.3. All NMR data of compound **37** are compared with the literature values of caseamemin previously isolated from the stem of *Casearia membranacea*, and found to be in close agreement (Chang *et al*., 2003).

Table 3. NMR spectral data comparison of compound **37** with literature values.

	Experimental data of compound **37**		Literature data of caseamemin (Chang *et al*., 2003)	
	^{13}C-NMR	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR
2	147.8		147.7	
3	124.2	5.12 (1H, *m*)	124.2	5.13 (1H, *m*)
4	22.2	2.14 (2H, *m*)	22.1	2.14 (2H, *m*)
4a	134.9		135.0	
5	112.6	6.40 (1H, *d*, *J* =2.8Hz)	112.6	6.38 (1H, *d*, *J* = 3 Hz)
6	121.2		121.2	
7	115.7	6.49 (1H, *d*, *J* = 2.8 Hz)	115.6	6.48 (1H, *d*, *J* = 3 Hz)
8	127.3		127.4	
8a	145.9		146.0	
9	16.0	1.62 (3H, *s*)	16.0	1.60 (3H, *s*)
10	15.9	2.14 (3H, *s*)	15.9	2.13 (3H, *s*)
1’	22.5	2.71 (2H, *t*, *J* = 6.4 Hz)	22.5	2.69 (2H, *t*, *J* = 6.6 Hz)
2’	31.4	1.74 &1.81 (2H, *td*)	31.3	1.74 &1.81 (2H, *td*)
3’	75.3		75.3	
4’	39.7		39.7	1.53 & 1.64 (2H, *m*)
5’	26.7	2.01 (2H, *m*)	26.7	2.04 (2H, *m*)
6’	39.7	1.75 (2H, *m*)	39.7	1.69 (2H, *m*)
7’	124.3	5.12 (1H, *m*)	124.3	5.08 (1H, *m*)
8’	135.1		135.1	
9’	39.7	1.78 (2H, *m*)	39.7	1.69 (2H, *m*)
10’	26.6	2.09 (2H, *m*)	26.6	2.08 (2H, *m*)
11’	124.4	5.12 (1H, *m*)	124.4	5.10 (1H, *m*)
12’	131.3		131.3	
13’	25.7	1.61 (3H, *s*)	25.7	1.59 (3H, *d*, *J* = 0.8 Hz)
14’	24.0	1.28 (3H, *s*)	24.0	1.26 (3H, *s*)
15’	16.0	1.62 (3H, *d*)	16.0	1.60 (3H, *s*)
16’	16.7	1.70 (3H, *s*)	17.7	1.68 (3H, *d*, *J* = 0.8 Hz)

Chemical shifts are reported in parts per million ($CDCI_3$), and ***J*** values are in Hertz.

The previous report showed that this compound was isolated from the stem of *Casearia membranacea* and exhibited cytotoxicity activity (Chang *et al*., 2003). This is the first time to been reported from the Warburgia species.

3.4. Phytotoxic Activity of Isolated Compounds

Parthenium hysterophorus weed seeds were prevented from germinating when exposed to a 0.05 mg/mL concentration of the crude ethanol extract of *Warburgia ugandensis* bark (Gizachew, 2019). The crude ethanol extract was further partitioned with hexane, chloroform, and methanol. Each fractionation was screened for its phytotoxic activity. The hexane and chloroform soluble portions showed impressive phytotoxic activity with 100% parthenium seed germination inhibition at 0.05 mg/mL concentration. However, the methanol-soluble part showed minimum seed germination inhibition (20%) as it is shown in Figure 5.

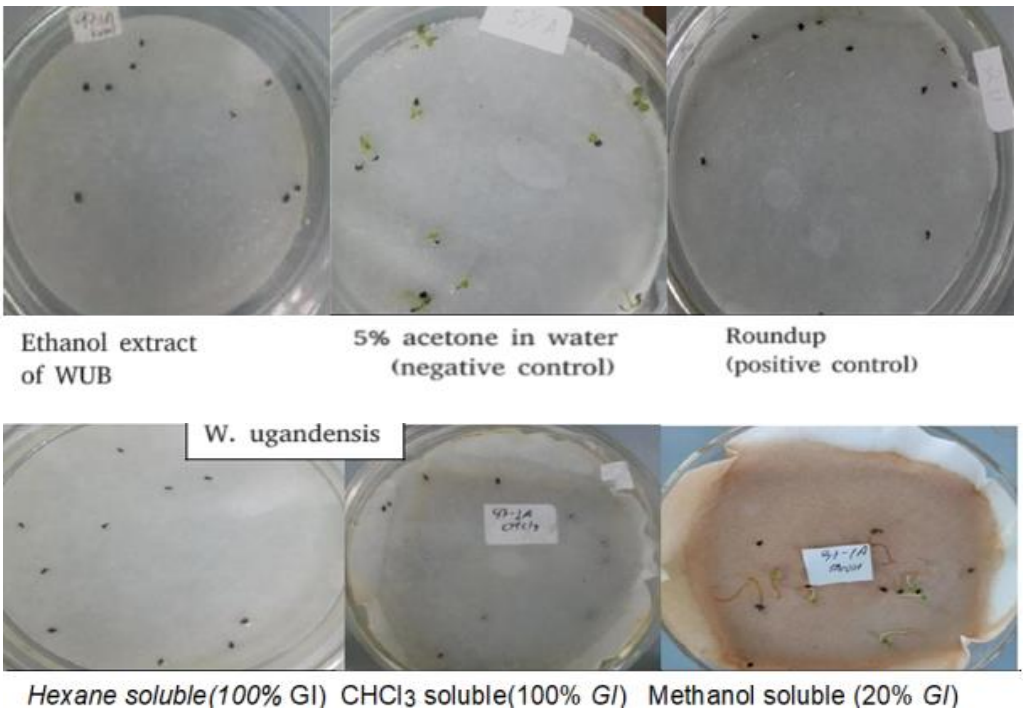

Figure 4. Results of the *in vitro* phytotoxicity of the ethanol extract and its soluble component in hexane, chloroform, and methanol against the germination of parthenium seeds.

So, from the medium polar fraction of the ethanol extract, a total of 11 compounds were isolated. The NMR spectra of these compounds are depicted in the supporting material of this manuscript. Six of the isolated compounds were subjected for their phytotoxic activities both *in vitro* and *in vivo*. The phytotoxic study of other compounds was not studied due to their amount for the bioassay. From the compounds screened for their herbicidal activity, muzigadial showed the highest activity with 100 and 95% germination inhibition (*in vitro*) and seedling growth inhibition (*in vivo*), respectively. Cinnamolide-3*β*-acetate is the second bioactive compound against the target weed, with 96 and 91% seed germination and seedling growth inhibition, respectively. For reference, the commercial herbicide Roundup® used as a positive control also achieved 100% seed germination inhibition at the same concentration as muzigadial and Cinnamolide-3*β*-acetate. Hence, these two compounds could be considered to be used as future generations of organic-based herbicides. However, additional herbicide parameters and field validation have to be studied.

Table 4. Summary of *In vitro* and *in vivo* results of pure compounds from *W. ugandensis.*

Compound name	*% GI (In vitro) 0.05 mg/mL*	*%GI (In vivo) 1 mg/mL*
Muzigadial	100	95
Cinnamolide-3*β*-acetate	96	91
Ugandensidial	100	87
11α-hydroxy muzigadiolide	66	40
Heptacosanol	59	66
Hentriacontane	55	18
Roundup® (+ve control)	100	97
5%acetonein water (-ve control)	0	0

4. CONCLUSION

Generally, the EtOAc soluble portion of the EtOH extract of the bark of *W. ugandensis* resulted in the first report of the isolation of albicanyl acetate, caseamemin, and *β*-sitosterol from this species together with other 6 known dialdehydic and drimane-type compounds that indicate the plant is not exhaustively studied and there is still a possibility to find other new compounds from it.

Muzigadial is isolated as the most phytotoxic compound against Parthenium weed. This compound may be utilized as an organic herbicide to fight the weed if other herbicide requirements are investigated and satisfied.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Zelalem Gizachew: Investigation, carrying out both the chemistry and bioassay experiments, data analysis, and writing the original draft manuscript. **Chiristopher Suh:** Focused on Manuscript editing and analyze the data

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Research Article

Comparative assessment of antioxidant activity in red apricot (*Prunus armeniaca* L.) and Fig fruits (*Ficus carica* L.) cultivated in Nakhchivan AR, Azerbaijan

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Abstract: Red apricot (*Prunus* spp.) and Black Fig (*Ficus carica* L.) are two of the most commonly grown summer fruits in the Nakhchivan region. In this preliminary study, ethanol fruit extracts were compared based on their total phenolic content (TPC) and antioxidant properties. According to the Folin-Ciocalteu method, the TPC was found to be 176.20 mg GAE/100 g in red apricot and 45.30 mg GAE/100 g in fig. Their antioxidant capacities were assessed using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. The scavenging activity, identified as the concentration of ethanol extract needed to scavenge half of the radicals (SC50), was determined to be 3.52 mg/mL for red apricot and 6.46 mg/mL for fig. Based on these two biological activity assays, red apricot exhibited higher phenolic composition and radical scavenging activity. However, further studies are required to understand whether these differences are attributed to secondary metabolites in these fruits.

1. INTRODUCTION

Nutrition plays a crucial role not only in promoting natural, high-quality, and balanced diets but also in preventing or treating certain diseases. Foods contain varying proportions of proteins, fats, carbohydrates, minerals, and vitamins, along with one or more antioxidants. Food antioxidants are defined as substances that can mitigate the adverse effects of reactive oxygen species (ROS) or reactive nitrogen species (RNS) produced under physiological conditions in humans. Maintaining a balance between oxidants and antioxidants is essential for health. The term "antioxidant" is broad and varies in content and bioavailability depending on factors such as the type of food product, harvest time and methods, climate, temperature, humidity, storage conditions, and food preparation methods, influencing individuals' and communities' product choices and consumption habits (Akin *et al*., 2008; Kasnak & Palamutoğlu, 2015; Malkoç *et al*., 2019).

Apricot, considered a valuable natural gift, is rich in essential nutrients such as vitamins A, B, C, and P, along with significant amounts of iron. Additionally, apricots are abundant with calcium, phosphorus, sulfur, chromium, and magnesium. The high potassium content further underscores its nutritional benefits for human health. Apricots are noted for their nourishing

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and appetizing effects, and their substantial iron content aids in combating anemia. They contribute to detoxification processes in the body and possess protective properties against various types of cancer. Apricots enhance overall body resistance, particularly supporting the physiological development of adolescents. Furthermore, they promote nerve calming and facilitate sleep induction (Caliskan *et al*., 2012). Research indicates that apricot seeds are purported to aid in the treatment of several conditions including bronchitis, bronchial asthma, laryngitis, tracheitis, and upper respiratory tract infections. However, it is cautioned against consuming apricots on an empty stomach or following meals containing meat and rice, as these combinations can hinder the digestion process and weaken metabolism. Consumption of apricots during gastritis may elevate stomach acidity. Moreover, individuals with liver and pancreas diseases are advised to limit apricot intake (Campbell *et al*., 2011; Fratianni *et al*., 2018). Apricots aid in digestion, enhance hemoglobin levels, and support cardiovascular and nervous system health. The fruit's pectin content accelerates metabolism and lowers cholesterol. Apricot kernels are recommended for treating anemia, while apricot oil, rich in linolenic acid, is used in medicinal preparations. In traditional medicine, apricots are employed for respiratory ailments (Calıskan *et al*., 2012; Fan *et al*., 2018; Göttingerová *et al*., 2021).

The fig tree (*Ficus carica* L.) thrives in diverse soil types and is resilient to frost damage in the Autonomous Republic of Azerbaijan. Female flowers form in newly developed pods after above-ground frost, whereas one-year branches with preserved bushes produce both female and male flowers, ensuring effective pollination and high yield. Figs are cultivated extensively throughout Azerbaijan, and historical Azerbaijani texts document their medicinal benefits extensively. According to Muhammad Momin, figs are effective against high fever, chills, thirst, and diarrhea, while also possessing choleretic and liver-strengthening properties (Mawa *et al*., 2013). They are recommended for conditions like urinary retention, heart palpitations, asthma, cough, lung pain, and wheezing. Consumption guidelines suggest up to 300 grams of fresh figs or 120 grams of dried figs, preferably accompanied by cumin and other spices to aid digestion. Ibn Sina notes that fig milk has diuretic effects and aids in kidney stone removal. Figs are considered beneficial for kidney and bladder health. According to Muhammad Huseyn Khan, combining figs with almonds prevents thinness, and consuming figs before meals aids in weight gain and soothes the stomach (Rahimova, 2023).

In folk medicine, fig leaves are also utilized for therapeutic purposes. Leaf decoctions and extracts are administered for cough, bronchial asthma, kidney ailments, and applied topically to boils. Young branch leaf decoctions are used against scurvy. Fig milk serves as a pain reliever for injuries, rheumatism, and gout. Fresh figs contain 23% sugar (glucose, fructose), pectin, dietary fiber, organic acids, vitamins C, B1, B2, B6, PP, carotene (provitamin A), pantothenic acid, folic acid, potassium, phosphorus, calcium, magnesium, and iron. Dried figs, with up to 71% sugar content, are calorically dense. Modern medicine recognizes figs as beneficial in treating cardiovascular disease and reducing blood clotting, particularly when prepared with milk for upper respiratory conditions (Guliyev *et al*., 2023). These two fruits are used variously fresh, dried or as pulp, especially as a bowel mobility enhancer (Erdem *et al*., 2024).

2. MATERIAL and METHODS

2.1. Chemicals

2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, methanol and ethanol were purchased from Sigma Chemical Co. St Louis, MO, USA.

2.2. Plant Samples and Extraction

The antioxidant activities of Red Nakhchivan apricot (*Prunus armeniaca* L.) and Black Nakhchivan fig (*Ficus carica*) varieties cultivated in the city of Nakhchivan were investigated in this study. Fresh fruits were harvested from the experimental area and subsequently dried in shaded conditions. Upon collection, the fresh fruits underwent initial washing with distilled water followed by drying with paper towels. They were then finely chopped and mixed with

methanol using a blender. Specifically, 100 chopped fruits were combined with 250 mL of 60% ethanol and stirred for 24 hours to facilitate extraction of antioxidant compounds. Following the extraction period, the mixture was filtered sequentially through Whatman No. 4 filter paper and then through Whatman No. 1 filter paper to obtain a clear extract. The resultant extract was then stored in a deep freezer at -18°C until further analysis (Bayrak *et al*., 2023; Kolayli *et al*., 2024).

2.3. Determination of Total Phenolic Content (TPC)

Total phenolic content was determined using the Folin-Ciocalteu assay, following the method described by Singleton *et al*. (1999). Initially, 680 µL of distilled water was combined with 400 µL of 0.2 N Folin reagent in a test tube. Subsequently, 20 µL of the ethanolic fruit extract was added to the mixture, followed by the addition of 400 µL of 10% Na2CO3 solution. The resulting solution was thoroughly mixed and then incubated in darkness for 2 hours to allow color development (Singleton *et al*.,1999).

After incubation, the absorbance of the reaction mixture was measured at 760 nm using a spectrophotometer. The total phenolic content of the extracts was quantified based on a calibration curve prepared using gallic acid standards at concentrations ranging from 0.50 to 0.0156 mg/mL. The results were expressed as milligrams of gallic acid equivalents (GAE) per gram of fresh fruit (mg GAE/g fresh fruit).

This analytical approach ensured accurate determination of the phenolic compounds present in Red Nakhchivan apricot and Black Nakhchivan fig extracts, providing valuable insights into their antioxidant potential and health-promoting properties.

2.4. Determination of Total Antioxidant Capacity

The radical scavenging activity of the samples was assessed using a 100 µM methanolic solution of 2,2-Diphenyl-1-picrylhydrazyl (DPPH). Initially, 3.94 mg of DPPH was accurately weighed and dissolved in methanol to achieve a final volume of 100 mL, ensuring a standardized concentration of DPPH radicals in the solution. To measure the DPPH radical scavenging activity of the sample components, various dilutions of the sample extracts were prepared. Each sample solution was mixed in equal parts with the constant concentration of DPPH radical solution. The reaction mixtures were then allowed to incubate for a specified time in darkness to facilitate the reaction between the antioxidants in the samples and the DPPH radicals. Subsequently, the absorbance of the reaction mixtures was measured spectrophotometrically at a wavelength of 517 nm.

The results were plotted on an exponential graph to determine the radical scavenging power of the antioxidant substances present in the samples. This methodological approach ensured precise evaluation of the antioxidant capacity of the ethanolic frutis extracts, providing valuable insights into their free radical scavenging abilities and potential health benefits (Kolaylı *et al*., 2016; Kolaylı *et al*., 2024).

To determine the SC50 value, which represents the concentration of the sample extract required to scavenge 50% of DPPH radicals, solutions were prepared at least in six different concentrations. A 100 µM methanolic solution of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was used as the radical source. Initially, 3.94 mg of DPPH was dissolved in methanol to a final volume of 100 mL, ensuring a consistent concentration of DPPH radicals. The sample solutions were prepared by serial dilution to achieve varying concentrations. Each sample concentration was then mixed in equal volume with the DPPH radical solution. These mixtures were incubated in darkness for a specific time to allow the reaction between the antioxidants in the samples and the DPPH radicals. Following incubation, the absorbance of each mixture was measured spectrophotometrically at a wavelength of 517 nm. A dose-response curve was plotted using the percentage inhibition values against the logarithm of the concentrations of the sample extracts. From this curve, the SC50 value, representing the concentration of the sample required to scavenge 50% of the DPPH radicals, was determined.

3. RESULTS AND DISCUSSION

In Nakhchivan Autonomous Republic, apricot varieties are categorized into two main groups based on their flowering times: (i) early blooming and (ii) late blooming types. The apricot tree, belonging to the Rosaceae family, typically reaches heights of 5-7 meters, with round or oval-shaped canopies. The fruits, which are whitish-yellow in color and weigh between 65-75 grams, are predominantly single-seeded. The common apricot (A. vulgaris Lam.) blooms in March-April with white or light pink flowers that appear before the leaves. Fruits ripen from June to August, varying in weight from 3-18 grams and exhibiting different shapes, ranging from white to yellow and red-orange hues.

The Red Nakhchivan apricot variety studied has pyramidal trees reaching heights of 6-7 meters, characterized by reddish-brown trunks and branches. On the other hand, black figs are mainly propagated through root cuttings. Apricots are consumed fresh and are also processed into compote, juice, jam, jelly, and dried apricots used widely in culinary applications. Additionally, apricot bark can be used for activated charcoal production, and resin from its branches can yield clay.

Wild apricot species are valued for breeding cultural varieties and are notable for their large kernels containing non-drying fats, proteins, and carbohydrates. Apricots thrive in temperate climates, blooming briefly in spring and showing resilience to cold weather conditions (Akin *et al*., 2008; Campbell *et al*., 2011; Bayrak *et al*., 2023; Dinç *et al*., 2024).

Based on the research conducted by Guliyev *et al*., wild figs are often found in the territory of Nakhchivan AR (Guliyev *et al*., 2023). In addition, figs are very common on the Mediterranean coast and especially in Greece. Many varieties are grown in Azerbaijan. Large-fruited varieties with white and black fruits are mainly grown in backyards in the plains of Nakhchivan AR. Brown figs can also be found wild in Sharur, Ordubad, and Julfa regions. According to scientific studies, it can be said that among the subtropical fruit plants that are distributed and cultivated in the world, there is no other fruit plant that is as nutritious, fragrant and sweet as figs. White, black, yellow, brown and Ordubad fig varieties are cultivated in the autonomous republic.

The amounts of total phenolic substances were summarised in Table 1. Fig fruits had an average of 45 mg GAE/100 g, while red apricot had 176,20 mg GAE/100 g. It was found that the amount of polyphenol substance in apricot was about 3 times higher. When we compare these values with the values in the literature, it is possible to say that these fruits have similar values. In a study conducted in Turkey, it was reported that the amount of TPC in 3 different fig fruits varied between 35 and 47 mg GAE/100g (Bayrak *et al*., 2023). In another study conducted on apricots collected from different regions, it was reported that the amount of TPC varied between 74 and 274 mg GAE/g (Göttingerová *et al*., 2021). According to Dinç *et al*. (2024), red apricot fruit exhibits a higher total phenolic content compared to flower fruit. However, its phenolic content was found to be lower than that of grapes, as reported by Kavgacı *et al*. (Kavgacı *et al*., 2023). Total phenolic content serves as a significant indicator of antioxidant potential and health benefits in fruits, influencing their nutritional value and potential applications in food and pharmaceutical industries.

Plants with unlimited synthesis capacity have the ability to synthesize many secondary metabolites they need. Polyphenols are the secondary metabolites of plants with the widest membership and serve both themselves and humanity with their biologically active properties. Due to their biological activities such as antioxidant, antimicrobial, antiviral and anti-inflammatory, herbal products are used more and more frequently in diet programs. They are recommended to be taken regularly because they strengthen the immune system (Akyüz & Ersus, 2022; Özen & Yılmaz, 2023).

Plant-derived foods rich in phenolic phytochemicals are known for their potent antioxidant properties, crucial in defending the body against oxidative damage. These compounds act as

effective scavengers of free radicals and metal chelators, thereby positively influencing metabolic processes (Can *et al*., 2015; Kolayli *et al*., 2016; Degirmenci *et al*., 2020; Akyüz & Ersus, 2022; Özen & Yılmaz, 2023). In the study, ethanol extracts of Red Nakhchivan apricot and black fig varieties exhibited significant antioxidant activity as measured by their ability to scavenge the DPPH radical. The SC50 values, representing the concentration of extract required to scavenge 50% of DPPH radicals, were 3.12 ± 0.043 mg/mL for Red Nakhchivan apricot and 6.46 ± 0.092 mg/mL for black fig (Table 1).

Table 1. Total phenolic content (TPC) and DPPH radical scavenging activities.

	TPC (mg GAE/100 g)	DPPH (SC_{50} mg/mL)
Fig fruits (n=5)	45.30 ± 2.30 mg /100 g	6.46 ± 0.092
Apricot fruits (n=5)	176.20 ± 5.66 mg /100 g	3.12 ± 0.043

In the study, it was found that extracts of fig and red apricot fruits possess the ability to scavenge the DPPH radical, with apricot fruit exhibiting a stronger antioxidant capacity. Research conducted on apricot and fig samples from different regions outside of Nakhchivan has consistently demonstrated these fruits to be rich sources of polyphenols, natural compounds known for their antioxidant properties. However, their antioxidant capacities vary depending on geographical regions and species (Ihns *et al*., 2011; Yiğit *et al*., 2009).

This preliminary study focused solely on total phenolic content analysis, while other studies have shown that apricots typically contain significant antioxidants such as chlorogenic acid, neochlorogenic acid, caffeic acid, and quercetin, which contribute to their health-promoting properties (Göttingerova *et al*., 2021). Studies have indicated that fig fruits, similar to apricots, are rich in chlorogenic acid. Additionally, they have been reported to contain high levels of flavonoids such as luteolin, apigenin, and rutin (Bayrak *et al*., 2023).

4. CONCLUSION

In this preliminary study conducted for the first time in the Nakhchivan region, apricots and figs, two fruit types known for promoting bowel movement and alleviating constipation, were compared in terms of their phenolic content and antioxidant properties. It was found that both fruits exhibited high levels of phenolic compounds, with the phenolic content in Red apricots being three times higher than in figs. Apricots were also determined to have a higher antioxidant value correlated with their phenolic component levels. Consequently, both fruits possess beneficial secondary metabolites for human health; however, further studies are needed to elucidate the specific nature of these metabolites.

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Declaration of Conflicting Interests and Ethics

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Authorship Contribution Statement

Elsevar Asadov: Research, investigation, analysis, systematization, visualization, editing. **Sura Rahimova:** Methodology, monitoring and evaluation, research, software, formal analysis. **Aziza Huseynova:** Collection of samples, research, editing

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Research Article

Comparison of matrix metalloproteinase levels in periodontal pockets treated with chlorhexidine and propolis

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Abstract: Natural antimicrobial products are attracting interest in the treatment of periodontal diseases due to their minimal or nonexistent side effects. Propolis, a complex resin content produced by bees, has drawn particular interest for its antibacterial effects, including inhibitory effects on periodontopathogenic bacterial species. This study compared the effects of propolis, a natural product, and chlorhexidine, the gold standard antimicrobial agent, on MMP-1 and MMP-9 levels in gingival crevicular fluid (GCF). Subgingival irrigations of chlorhexidine and propolis solutions were performed in selected deep periodontal pockets of periodontitis patients along with scaling and root planing. The treatment protocol was administered three times at one-month intervals starting from the initial day of the study. GCF MMP-1 and MMP-9 levels, along with clinical periodontal parameters, were evaluated before and after treatment. The study results showed that a statistically significant decrease in clinical periodontal parameters in both groups (p<0.001). Biochemical analysis revealed a statistically significant decrease in levels of GCF MMP-1 and MMP-9 in both groups (p<0.005). Additionally, the decrease in GCF MMP-1 levels was found to be significantly greater in the propolis group compared to the chlorhexidine group (p<0.001).

1. INTRODUCTION

Periodontitis is a bacterial infectious disease that may lead to destruction of gingival fibers, destruction of the alveolar bone, and, if untreated, eventual tooth loss. The condition arises from an inflammatory response triggered by enzymes produced by bacteria that accumulate in the dental and gingival sulcus, subsequently spreading to the surrounding tissues that support the tooth (Teles *et al*., 2018). Clinical attachment loss, radiographic alveolar bone loss, the presence of periodontal pockets and bleeding on probing are some of the key diagnostic indicators of of periodontitis (Papapanou *et al*., 2018). These conditions are caused by periodontopathogenic bacteria within the periodontal pocket and tissue-destructive enzymes secreted as part of the host's immune response (Vitkov *et al*., 2021). The subgingival plaque and calculus in the

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periodontal pocket are the most important local factors in the development and progression of periodontal disease. Therefore, the primary objective of non-surgical periodontal treatment is to eliminate microbial deposits and debris from the periodontal pocket and root surface (Yan *et al*., 2020). Scaling and root planing (SRP) to remove subgingival plaque and calculus is the gold standard for mechanical debridement of periodontal pockets (Shrivastava *et al*., 2021). However, SRP may not be effective in the removal of all periodontopathogenic bacteria in deep periodontal pockets (Nakao *et al.,* 2020). Antimicrobial agents can therefore be used in combination with mechanical treatment (van Winkelhoff *et al.,* 2000).

Chlorhexidine is the most effective broad-spectrum antimicrobial agent clinically used for oral hygiene maintenance and therapeutic chemical plaque control due to its substantivity and antibacterial properties (Jones, 1997; Sajjan *et al.,* 2016). Chlorhexidine is recognised as the gold standard because of its ability to bind to the dental pellicle and oral mucosa, thereby, enhances its anti-plaque effect (Jenkins *et al.,* 1988). It also has anti-gingivitis properties. Several clinical studies have shown that chlorhexidine inhibits the development of microbial plaque, calculus and gingivitis (Kolahi & Soolari, 2006; Paraskevas, 2005). Chlorhexidine is used for oral hygiene and professional prophylaxis and is available as a mouthwash, gel, tablet, varnish, chewing gum, toothpaste, spray, and sustained-release tablet (Paraskevas, 2005). Despite its many benefits, chlorhexidine has many reported side effects, including browning of tooth surfaces and restorations, altered taste, enlarged parotid glands, mucosal erosions, and allergic reactions (Addy, 1995; Mariotti&Rumpf, 1999). It is essential to investigate alternative approaches to avoid the potential adverse effects of chlorhexidine (Bush *et al.,* 2011). One of these alternative agents is propolis. Propolis is a product with a complex resin content produced by honeybees. The composition of propolis can be derived from a variety of plant sources. However, antibacterial activity has been reported for all types of propolis (Kujumgiev *et al.,* 1999; Khurshid *et al.,* 2017). Studies have reported that propolis has a strong inhibitory effect on periodontopathogenic bacterial species, some fungi, viruses, and protozoan species (Kosalec *et al.,* 2005; Yoshimasu *et al.,* 2018). The significant anti-inflammatory properties of propolis have made it a natural antimicrobial agent. In addition to its immunomodulatory and local anesthetic effects and its contribution to wound healing, propolis also reduces the prevalence of dental caries and pulpal inflammation (Rufatto *et al*., 2018; Eslami *et al.,* 2016). Studies have reported that the topical use of propolis contributes to the protection of periodontal tissue health (Sanghani *et al*., 2014; Nakao *et al*., 2020).

Matrix metalloproteinases (MMPs) are a group of enzymes responsible for degrading extracellular matrix proteins during organogenesis, growth, and normal tissue regeneration. There is a balance between MMP activity and their specific endogenous tissue inhibitors in maintaining physiological events in the organism. Shifting this balance towards MMP activity leads to the destruction of the matrix and the formation of pathological events. In healthy tissues, the synthesis and activity of matrix metalloproteinases (MMPs) are typically low. However, their levels are elevated in various pathological conditions, including inflammation, tumor formation, and metastasis, where they contribute to tissue destruction (Sekhon, 2010). In periodontitis, MMP levels are elevated due to gingival inflammation. Periodontal pathogens contribute to the imbalance between MMPs and MMP inhibitors. Studies have reported a significant correlation between MMP levels in saliva and gingival crevicular fluid (GCF) and clinical parameters of periodontal disease. Increased levels of GCF MMP-1, MMP-8, MMP-9, MMP-12, and MMP-13 have been observed in periodontitis, in particular (de Morais *et al.,* 2018; Séguier *et al.,* 2001; Romano *et al.,* 2019). Among these enzymes, MMP-1 and MMP-9, released by gingival fibroblasts and neutrophils during bacterial infection, cause the breakdown of collagen in periodontal tissue (Romano *et al.,* 2019).

Our study compared the effects of chlorhexidine, used as an antimicrobial and antiplaque agent, and natural propolis that is produced by bees through enzymatic processing of the substances collected from plants and trees with the enzymes secreted from the glands in their

heads, on GCF MMP-1 and MMP-9 levels in periodontal pockets. It was therefore investigated whether natural propolis, which does not have the side effects of chlorhexidine such as staining of oral tissues, could be an alternative for the protection and treatment of periodontal tissues.

2. MATERIAL and METHODS

2.1. Study Population and Ethical Approval

Our study was designed in accordance with the principles of the Declaration of Helsinki. Ethical approval was obtained from Muğla Sıtkı Koçman University Clinical Research Ethics Committee with decision number 12/VI dated 01.06.2023. Informed consent was obtained from all subjects included in the study. Clinical and radiographic examinations were performed at the Department of Periodontology, Faculty of Dentistry, Muğla Sıtkı Koçman University. The study sample consists of 14 patients diagnosed with periodontitis, including 7 female and 7 male patients. For each patient, two teeth were selected, with one tooth randomly assigned to the chlorhexidine group and the other to the propolis group. In total, 28 teeth from 14 patients were included in the study.

Patients with periodontitis were selected for this study based on the following criteria: 1) patients aged between 25 and 40 years; (2) systemically healthy patients confirmed by health checkup results; (3) patients who do not take any regular medication; (4) patients who had not used antibiotics in the previous three months; (5) patients who had not received any periodontal treatment in the prior six months; (6) non-smokers; and (7) patients who had at least two deep periodontal pockets of 5 mm or more. Patients with allergies to any of the study components were excluded.

Each patient's two randomly assigned periodontal pockets were divided into two treatment groups. Subgingival irrigation with two different bioactive agents was performed during periodontal treatment: 0.12% chlorhexidine gluconate subgingival irrigation (control group, n=14) and propolis extract subgingival irrigation (test group, n=14). Thus, from the same patient, one periodontal pocket was assigned to the control group and the other to the test group. In total, 28 periodontal pockets from 14 patients constituted the study groups.

2.2. Study Design

The study used a randomized, double-blind, controlled design. A randomisation programme was used to prevent bias in sample collection and analysis. A total of 28 samples were randomly assigned to one of two different treatment groups, the chlorhexidine group and the propolis group.

The study protocol included four visits for each patient over a three-month period. Clinical periodontal parameters were obtained at the first periodontal assessment. Gingival crevicular fluid (GCF) samples were collected from patients diagnosed with periodontitis using blinded fashion. Subgingival irrigation was then applied to the periodontal pockets of patients undergoing initial scaling and root planing (SRP). Patients who received two additional sessions of scaling and root planing (SRP) and subgingival irrigation at one-month intervals were invited for a control assessment at the third month of treatment. For post-treatment evaluation, clinical periodontal parameters and GCF samples were collected from selected teeth at this session.

2.3. Gingival Crevicular Fluid Sample Collection

Gingival crevicular fluid (GCF) samples were collected from an identified tooth site with a probing pocket depth (PPD) ≥ 5 mm in all participants. GCF samples were collected at baseline (month 0) and at the final follow-up visit (month 3). Before collecting the GCF samples, supragingival plaque was removed with a sterile scaler without touching the gingival margin. The tooth was isolated with cotton roll tampons and saliva absorbents and dried with sterile 2x2 gauze. Paper strips (Proflow Inc., Amityville, NY, USA) of standard size and absorbency (2 x 14 mm) were used for the collection of GCF. Special strips of paper were placed in the periodontal pocket and held in place for 30 seconds. Paper strips contaminated with saliva or

blood were discarded. The paper strips were then placed in sterile Eppendorf tubes containing 500 µL of PBS. Paper strips, samples were centrifuged at 10,000xg for 10 min (Hettich Mikro 200, Andreas Hettich Co., Tuttlingen, Germany). The supernatants obtained from the gingival crevicular fluid were isolated for analysis in the study. The samples were mixed thoroughly and stored at -80°C until the day of the study.

2.4. Subgingival Irrigation

For subgingival irrigation, the two deepest periodontal pockets of each participant were selected. After mechanical periodontal treatment, subgingival irrigation was performed with 0.12% chlorhexidine gluconate (Kloroben, Drogsan, Turkey) in the control group, and propolis extract solution in the test group. 2 mL Chlorhexidine and propolis doses were applied to the periodontal pockets by irrigation using 2 mL syringes. These procedures were performed to the periodontal pockets on three occasions at intervals of one month (month 0, month 1 and month 2).

2.5. Preparation of Propolis Extracts

Propolis can be stored in its raw form at a room temperature of less than 25°C. However, it is preferable to keep it refrigerated at 4°C to avoid mottling. The raw propolis was stored at +4°C until extraction. Propolis samples were first frozen using liquid nitrogen. They were then ground using a mill. Propolis extracts were prepared following the established protocols from previous literature, with modifications when necessary (Bankova *et al.*, 2021). 1 kg of the propolis powder sample was mixed with 4 kg of 99.5 % ethanol in a glass extraction vessel using a mechanical mixer for 21 days, and a 20 % extract of propolis was obtained. The ethanol extracts obtained were filtered through Whatman filter paper and combined. A low-pressure evaporator was then used to remove the ethyl alcohol in the combined solution. The 100% propolis extracts obtained were stored at -18^{o} C until use. To prepare the subgingival irrigation solution, 2 g of propolis extract was dissolved in 10 mL of 80% ethanol.

2.6. Clinical Parameters

Clinical and radiological examinations of all volunteers were performed. The periodontal examination included plaque index (PI), gingival index (GI), bleeding on probing (BOP), probing pocket depth (PPD), and clinical attachment level (CAL). Measurements were taken at six sites per tooth. During these measurements, a Williams periodontal probe (Hu-Friedy, Chicago, IL, USA) was used. All measurements were performed by a single examiner who was blinded to the type of treatment. The examiner was calibrated against an expert periodontist. Calibration was validated if 90% of the recordings could be reproduced within a 1 mm difference. Diagnosis of periodontal diseases was done according to the 2017 American Academy of Periodontology and European Federation of Periodontology classification (Papapanou *et al*., 2018). Participants were diagnosed with periodontitis based on the following criteria: interdental CAL is detectable at ≥2 non-adjacent teeth, or buccal or oral CAL ≥3 mm with pocketing >3 mm is detectable at ≥2 teeth (Tonetti *et al.*, 2018).

2.7. Biochemical Evaluation

The GCF concentrations of MMP-1 and MMP-9 were quantified by ELISA technique (BT-laboratory, Shanghai, China; Cat No: E0916Hu and E0936Hu, respectively) at Muğla Sıtkı Koçman University Faculty of Medicine Medical Biochemistry Research Laboratory based on the manufacturer's instructions. Enzymatic reactions were quantified in an automated microplate photometer (absorbance at 450 nm using Thermo Scientific Multiskan GO, Thermo Fisher Scientific, USA). Sample concentrations were calculated from the standard curve generated using calibrators for each antimicrobial peptide. The kit sensitivity was 0.05 ng/mL, and the assay range was 0.1 ng/mL-10 ng/mL. Upper and lower outliers were examined in duplicate.

2.8. Statistical Analysis

The statistical analyses of the study were carried out using the SPSS software package (IBM Corp. Chicago, 2020. IBM SPSS Statistics for Windows, version 27.0. Armonk, NY: IBM Corp) and Minitab 21.1 software. The Shapiro-Wilk test was used to determine whether the quantitative variables were appropriate for a normal distribution. Independent groups were compared using the Mann-Whitney U test for non-normally distributed variables, and an independent samples t-test was employed for the analysis of normally distributed quantitative variables. Paired samples t-tests were used to compare related samples that conformed to a normal distribution, while the Wilcoxon signed-rank test was used for non-normally distributed variables. The Chi-Square test was used to compare study groups based on gender. Mean ± standard deviation, median (minimum - maximum) were used for descriptive statistics. Descriptive statistics for qualitative variables were expressed as frequencies (%). $p<0.05$ values were considered statistically significant.

3. RESULTS

The gender distribution in the chlorhexidine and propolis groups was homogeneous, with each group comprising 7 males and 7 females. This distribution was statistically non-significant ($p > 0.999$). The mean age of the patients was 36.79±1.81 years. There was no statistical difference ($p>0.05$) between the two groups in terms of age or gender. Demographics are shown in Table 1.

Table 1. Descriptive statistics and comparative outcomes of age variables in men, women, and the sample.

	Total (n=14)	Male (n=7)	Female (n=7)	*p*-value
Age	36.79±1.81	35.86±1.57	37.71±1.60	0.052[t]

t: Independent samples *t*-test

Descriptive statistics are shown as mean±standard deviation.

Table 2. Assessment of clinical periodontal parameters and biochemical parameters in the groups.

Groups	Parameters	Baseline	Post-treatment	*p*-value
Chlorhexidine group (n=14)	MMP-1	26.90±1.11	22.73±1.04	<0.001[s]
	MMP-9	2.96±0.14	2.61±0.16	<0.001[s]
	PI	2.22±0.44	1.11±0.27	<0.001[s]
	GI	1.98±0.60	0.73±0.29	<0.001[s]
	BOP (%)	0.53±0.28	0.16±0.14	<0.001[s]
	PPD (mm)	4.98±0.87	4.44±0.78	<0.001[s]
	CAL (mm)	5.92±0.90	5.45±0.89	<0.001[s]
Propolis group (n=14)	MMP-1	27.34±1.39	21.05±1.51	<0.001[s]
	MMP-9	3.07 (2.60-3.32)	2.47 (2.39-2.97)	0.001[w]
	PI	2.21±0.31	1.17±0.29	<0.001[s]
	GI	2.05±0.34	0.67±0.25	<0.001[s]
	BOP (%)	0.50 (0.17-0.67)	0.17 (0.00-0.17)	0.001[w]
	PPD (mm)	4.75±0.51	4.07±0.48	<0.001[s]
	CAL (mm)	5.46±0.53	5.05±0.54	<0.001[s]

[s]: Paired samples t-test, [w]: Wilcoxon signed-rank test.

Descriptive statistics are shown as median (minimum-maximum) and mean±standard deviation. Statistically significant *p* values are given in bold.

Chlorhexidine group, periodontal pockets treated with subgingival irrigation of chlorhexidine; propolis group, periodontal pockets treated with subgingival irrigation of propolis.

Abbreviations: MMP-1, matrix metalloproteinase-1; MMP-9, matrix metalloproteinase-1; PI, plaque index; GI, gingival index; PPD, probing pocket depth; CAL, clinical attachment loss; BOP, bleeding on probing.

The improvement in all clinical periodontal parameters (PI, GI, BOP, PPD, CAL) following the treatment was statistically significant in both groups (p<0.001) (Table 2). However, no statistically significant difference was found between the chlorhexidine and propolis groups (p>0.05) (Table 3). The decrease in MMP-1 levels after treatment was found to be statistically significant for both groups (p<0.001) (Table 2). The decrease in MMP-1 levels was found to be statistically significantly higher in the propolis group compared to the control group (p<0.001) (Table 3). The decrease in MMP-9 levels after treatment was statistically significant in both groups (p<0.01) (Table 2), this decrease however showed no statistical difference in the comparison between groups (p=0.051) (Table 3).

Table 3. Comparison of differences between groups in clinical periodontal parameters and biochemical parameters before and after treatment.

Parameters	Chlorhexidine group (n=14)	Propolis group (n=14)	*p*-value
MMP-1	-4.07 (-5.77- -2.61)	-6.34 (-8.43- -3.54)	**<0.001**[m]
MMP-9	-0.35±0.13	-0.47±0.18	0.051[t]
PI	-1.12±0.43	-1.05±0.40	0.683[t]
GI	-1.25±0.39	-1.38±0.31	0.330[t]
BOP (%)	-0.34 (-0.67- -0.17)	-0.34 (-0.50- -0.17)	0.839[m]
PPD (mm)	-0.53±0.25	-0.67±0.27	0.150[t]
CAL (mm)	-0.34 (--1.00- -0.17)	-0.34 (-0.67- -0.17)	0.946[m]

[m]: Mann Whitney U test, [t]: Independent samples t-test.

Descriptive statistics are shown as median (minimum-maximum) and mean±standard deviation. The statistically significant *p*-value is given bold.

Chlorhexidine group, periodontal pockets treated with subgingival irrigation of chlorhexidine; propolis group, periodontal pockets treated with subgingival irrigation of propolis.

Abbreviations: MMP-1, matrix metalloproteinase-1; MMP-9, matrix metalloproteinase-1; PI, plaque index; GI, gingival index; PPD, probing pocket depth; CAL, clinical attachment loss; BOP, bleeding on probing.

4. DISCUSSION and CONCLUSION

The treatment of periodontitis includes scaling and root planning (SRP). Mechanical treatment is the gold standard to eliminate periodontal pockets and to remove microbial plaque and calculus. However, antimicrobial agents are used to eliminate subgingival microflora (Nakao *et al.*, 2020; Zarch *et al*., 2021). Chlorhexidine mouthwash is one of the prominent agents in periodontal therapy and is considered the gold standard due to its effective anti-plaque properties. However, chlorhexidine mouthwash alone is not sufficient to eliminate subgingival microflora. Subgingival irrigation with chlorhexidine, on the other hand, is effective for microflora in the periodontal pocket (Sajjan, 2016) but the use of chlorhexidine has potential side effects (Poppolo & Ouanounou, 2022). For this reason, researchers have become increasingly interested in alternative agents that target inflammation in the periodontal pocket. The fact that propolis is a natural product and is biologically active has made it one of these alternatives (Kujumgiev *et al.*, 1999).

In our study, subgingival irrigation of periodontal pockets with chlorhexidine and propolis in combination with mechanical periodontal treatment resulted in a significant improvement in clinical periodontal parameters (PI, GI, BOP, PPD and CAL) at three months in both groups. Sanghani *et al.* (2014) reported a greater improvement in GI, PPD and CAL values as a result of subgingival propolis application after SRP than in the control group. In a similarly designed study by Coutinho *et al.* (2012), periodontal pockets treated with subgingival propolis showed greater BOP improvement and PPD reduction compared to control groups. Nakao *et al.* (2020) investigated the effect of various bioactive agents on periodontopathogens in selected deep periodontal pockets and reported an improvement in BOP, CAL and PPD levels as a result of propolis ointment application. In their study of patients with periodontitis, Zarch *et al*. (2021) used chlorhexidine mouthwash and propolis subgingival irrigation in addition to mechanical

treatment. As a result, both treatments demonstrated improvements in clinical periodontal parameters, including Bleeding on Probing (BOP), Probing Pocket Depth (PPD), Clinical Attachment Level (CAL), and Gingival Index (GI). Seth *et al.* (2022), in their study designed similarly to ours, reported a significant decrease in GI, PI and PPD values in the first month of initial treatment with subgingival irrigation with chlorhexidine and propolis solutions as an adjunct to SRP treatment. Studies on the topical effects of chlorhexidine and propolis consistent with those observed in our study.

MMP-1 and MMP-9 are important biomarkers of periodontitis (Ertugrul *et al.*, 2013). Kubota *et al*. (2008) reported that MMP levels increased in gingival tissue samples taken during the transition from healthy periodontium to periodontitis. Romano *et al.* (2019) reported that GCF MMP-1 and MMP-9 levels significantly increased during the active and progressive stages of periodontitis. Therefore, these markers were selected to assess the effect of the antimicrobials used in our study on GCF. Biochemical findings of this current study revealed a reduction in MMP-1 and MMP-9 levels in both groups after treatment. Vitt *et al.* (2020) observed a decrease in GCF MMP-8 levels as a result of chlorhexidine irrigation of periodontal pockets after SRP. Azmak *et al.* (2002) reported that GCF MMP-8 levels decreased more in periodontal pockets treated with chlorhexidine chips after SRP than in periodontal pockets treated with SRP alone. Yuan *et al.* (2022) reported that the addition of chlorhexidine subgingival irrigation to SRP resulted in a greater reduction of inflammatory mediators, specifically IL-6 and MMP-8, in GCF compared to SRP alone. In a study conducted by Zarch *et al*. (2021), salivary MMP-8 levels were reported to decrease in both groups following treatment. Kale *et al.* (2022) found that propolis reduced MMP-2 and MMP-9 levels in their study on stem cells isolated from human exfoliated deciduous teeth. In another review study, the possibility of using caffeic acid phenethyl ester (CAPE) from propolis as an alternative treatment for oral cancer was discussed. As a result of this study, CAPE was shown to reduce the protein expression and enzymatic activity of MMP-2 (Kuo *et al.*, 2015). In another experimental animal study conduted by Liberio *et al*. (2011), it was reported that the application of oral propolis gel led to an increase in anti-inflammatory serum levels of IL-4 and IL-10. The results of these studies are consistent with the results of our study.

When MMP levels were compared between the groups in our study, a more significant reduction in GCF MMP-1 levels was observed in the propolis group following treatment compared to the control group. Although there was no statistically significant difference in GCF MMP-9 levels, a greater decrease was observed in the propolis group after treatment. Zarch *et al*. (2021) reported that although the difference was not statistically significant, salivary MMP-8 levels decreased more in the propolis group than in the chlorhexidine group.

To the best of our knowledge, this is the first study in which chlorhexidine and propolis subgingival irrigation with SRP was performed in periodontitis patients and GCF MMP-1 and MMP-9 levels were biochemically assessed at the end of treatment. Comparison with other data in the literature is therefore difficult. A current limitation of the study may be the small sample size and short follow-up period. However, given that our study employed gingival crevicular fluid analysis instead of saliva and serum samples, we contend that this methodology has enabled a standardized approach for comparing the results across the treatment groups within the same patient cohort.

The results of our study showed that subgingival irrigation with propolis and chlorhexidine together with SRP led to a decrease in GCF MMP-1 and MMP-9 levels, which are clinical periodontal parameters and biomarkers of periodontal disease, in periodontal pockets. In addition, the effect of propolis on GCF MMP-1 was found to be greater than that of chlorhexidine. Further research with larger sample sizes and extended follow-up periods is required to establish the efficacy of propolis, which is devoid of known side effects, as a subgingival irrigation solution, especially as an alternative to chlorhexidine.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors. **Ethics Committee Number**: Muğla Sıtkı Koçman University, Clinical Research Ethics Committee, E-72855364-050.01.04-616130.

Authorship Contribution Statement

Ali Batuhan Bayırlı: Conception, Materials, Data collection and processing, Analysis and Interpretation, Writing the Original Draft, and Manuscript Review. **İbrahim Kıvrak:** Materials, Analysis and Interpretation, Supervision, and Writing. **Ercan Saruhan:** Design, Analysis and Interpretation and Writing. **Fulden Cantaş Türkiş:** Analysis and Interpretation and Writing.

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Research Article

Taxonomic characterization and secondary metabolite production of newly isolated *Streptomyces* sp. MC12

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Abstract: An actinobacterium newly isolated from soil during a screening study was identified as *Streptomyces* sp. MC12 (GenBank accession number: PP757795) based on 16S rRNA analysis. For secondary metabolite production, fermentation was carried out in ISP 2 broth at 30°C, pH 7.3, for seven days under shaking conditions at 180 rpm. As a result of fermentation studies, the antagonistic effect of the crude extract, obtained through ethyl acetate extraction, against various microorganisms was determined. The MIC values of the extract against *Staphylococcus aureus* and *Escherichia coli* were 101.3 µg/mL and 153.6 µg/mL, respectively. It was also found to exhibit strong antifungal activity against *Penicillium* spp. *Streptomyces* sp. MC12, which displays both antifungal and antibacterial properties, is considered a potential secondary metabolite producer for future studies, particularly in pharmacology and the biocontrol of fungal pathogens.

1. INTRODUCTION

Microbial secondary metabolites include antibiotics, pigments, toxins, substances involved in symbiosis and competition, enzyme inhibitors, pheromones, agents affecting the immune system, receptor antagonists and agonists, pesticides, antitumor agents, and plant and animal growth promoters (Barka *et al*., 2016; Cuozzo *et al*., 2023). Among the drugs used in treatment, antibiotics are some of the most studied secondary metabolites. They are used to treat various infections in humans and animals. In addition to their therapeutic or protective purposes, antibiotics are also used in animal nutrition by incorporating them into daily feed to stimulate growth, resulting in increased weight. This practice has raised concerns regarding antimicrobial resistance. Moreover, antibiotics are used in food preservation and to promote plant growth in agriculture (Wassenaar, 2005; Salwan and Sharma, 2020; Nazari *et al*., 2023). Although many antibiotics are currently used in treatments, new screening studies are necessary due to the development of resistance in many pathogens. Among these resistant bacteria, methicillin-resistant *Staphylococcus aureus* (MRSA) is responsible for most hospital-acquired infections. Vancomycin-resistant *Enterococcus*, which complicates abdominal surgeries, and multi-drug-resistant *Mycobacterium tuberculosis* also pose significant challenges to scientists. Additionally, Gram-negative respiratory pathogens are increasingly developing resistance to

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antibiotics (Gauba and Rahman, 2023). The need for new antibiotics is growing rapidly as pathogens continue to develop resistance, rendering existing treatments inadequate or toxic (Demain, 1998). Recent studies on the discovery of bioactive metabolites often result in the re-isolation of well-known compounds, which can lead to significant losses in time, effort, and cost. This has prompted researchers to develop more effective discovery strategies (Anderson and Wellington, 2001; Jose *et al*., 2021).

Actinobacteria are undoubtedly the most important producers of pharmacologically active metabolites, especially antibiotics. Most actinobacteria, particularly *Streptomycetes*, are saprophytic, soil-dwelling organisms that spend much of their life cycle in semi-spore form, especially under nutrient-limited conditions. However, this phylum can adapt to a wide range of ecological environments: they are found in soil, fresh and saltwater, as well as in the air. They make up a significant part of the microbial population in alkaline soils rich in organic matter compared to other environments (Hazarika and Thakur, 2020; Selim *et al*., 2021). Additionally, they are found extensively in composts, straw, aquatic environments, and humid buildings.

Among actinobacteria, members of the *Streptomyces* genus produce some of the most important metabolites, such as antibiotics and enzymes. The *Streptomyces* genus includes a large group of organisms found in various natural habitats, producing a wide array of bioactive compounds, including antibiotics. More than half of the commercially and medically useful antibiotics derived from microbes are produced by actinobacteria, and 85% of these are from members of the *Streptomyces* genus (Watve *et al*., 2001; Hui *et al*., 2021).

Members of the *Streptomyces* genus are renowned for producing numerous secondary metabolites with antibacterial, antifungal, antiparasitic, antitumoral, and immunosuppressive biological activities (Chinnathambi *et al*., 2023). Moreover, it is estimated that less than 10% of *Streptomyces* bioactive compounds have been identified so far. Approximately 3,000 species of this genus have been described since the 1970s, including those with unconfirmed validity, making *Streptomyces* a rich source of new bioactive and commercially important compounds (Watve *et al*., 2001).

This study aims to identify the taxonomic characterization of the MC12 isolate based on morphological features and 16S rRNA sequence analysis, which produces high levels of antimicrobial metabolites during screening studies, and to assess the effectiveness of its secondary metabolites against different pathogens.

2. MATERIAL and METHODS

2.1. Isolation and Identification of the Isolate MC12

The soil sample was collected from the campus of Manisa Celal Bayar University (38°67'92.6"N, 27°30'17.1"E) in 2017, and actinobacteria were isolated using the soil dilution plate technique (Balagurunathan *et al*., 2020) on Yeast Malt Extract Agar (ISP 2) (Shirling and Gottlieb, 1966). This medium was supplemented with nystatin and streptomycin at a final concentration of 50 µg/mL to minimize fungal and bacterial contamination, respectively. The prepared petri dishes were incubated at 27-30°C for 7-14 days. After incubation, *Streptomyces*-like bacterial colonies (filamentous, rough, tough, leathery, chalky, and dusty) that grew on the medium were selected, purified on the same medium, and subjected to further experiments (Oskay, 2009; Sapkota *et al*., 2020).

The identification of the MC12 strain (with MC representing Muradiye Campus) was performed by partially sequencing 16S rRNA genes using oligonucleotide primers: 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1525R (5'-AAGGAGGTGWTCCARCC-3') (Lane, 1991). DNA isolation of MC12 was carried out using the Genomic DNA Purification Kit (Promega, A1120) according to the manufacturer's protocol. The polymerase chain reaction (PCR) mixture (50 µL) contained 1 µL dNTPs (10 mM each), 4 µL $MgCl_2$ (25 mM), 1.5 µL forward primer (20 µM), 1.5 µL reverse primer (20 µM), 0.40 µL (5 U) Dream*Taq* DNA

polymerase (ThermoScientific, EP0702), 0.5 µL template DNA (approximately 50 ng), 5 µL 10× Dream*Taq* PCR buffer with $MgCl_2$, and 36.10 µL PCR-grade water. The sample was placed in a thermal cycler (Applied Biosystems Veriti Thermal Cycler, USA), and PCR was conducted with an initial denaturation at 96°C for 3 min, followed by 34 cycles of denaturation at 96°C for 45 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 2 minutes, with a final extension at 72°C for 10 minutes. The PCR product and molecular size marker (100 bp, Geneaid DL007) were subjected to electrophoresis in 1% agarose gel with TBE buffer, stained with safe DNA gel dye (Invitrogen), and examined using a gLite gel scanner. The single pure PCR product was commercially sequenced by GATC (Germany).

The sequence of MC12, along with related *Streptomyces* reference gene sequences obtained from GenBank (NCBI), was aligned using the MEGA XI (V 11.0.13) program (Tamura *et al*., 2021). The phylogenetic tree was constructed using the neighbour-joining method (Saitou and Nei, 1987) and Jukes-Cantor algorithms (Jukes and Cantor, 1969) with a bootstrap value of 1000 replicates (Felsenstein, 1985).

2.2. Secondary Metabolite Production by Fermentation

The seed culture of MC12 was prepared by inoculating a loopful of active culture into 250 mL Erlenmeyer flasks containing 50 mL of ISP2 broth (composition in g/L: yeast extract 4, malt extract 10, dextrose 4; pH 7.3) and incubated for three days at 28°C with shaking at 180 rpm. For fermentation, 2.5 mL (5%, v/v) of seed culture containing 1.0×10^6 spores/mL was inoculated into 50 mL of ISP2 broth. The fermentation was carried out at 28°C on a rotary incubator at 180 rpm for seven days. The fermentation broth was sampled at regular intervals (every 24 hours) and used in antimicrobial tests, and the process was stopped when optimal activity was observed. After determining the optimal production of secondary metabolites, the fermentation broth was centrifuged at 12,000 rpm at 4°C for 10 minutes to obtain the cell-free supernatant, which was then subjected to ethyl acetate extraction.

2.2.1. *Ethyl acetate extraction procedure of secondary metabolite*

Five different organic solvents—methanol, ethanol, ethyl acetate, hexane, and petroleum ether—were used for secondary metabolite extraction. Based on the antimicrobial test results of the obtained extracts, ethyl acetate was determined to be the most effective solvent and was subsequently used for all further extractions. Three hundred milliliters of cell-free supernatant, collected after centrifugation, was extracted twice with 300 mL of ethyl acetate at room temperature for 6 hours. After extraction, the ethyl acetate was evaporated using a rotary evaporator at 40°C, and the remaining extract was weighed. The extract was then dissolved in ethyl acetate at a concentration of 1 mg/mL and stored at -20°C for future experiments.

2.3. Determination of Antagonism

The primary antimicrobial activity of the identified *Streptomyces* sp. MC12 was assessed using the classical cross-streak method, following Oskay's protocol (Oskay, 2011) (data not shown). In secondary tests, the antimicrobial activity of the extracts obtained through fermentation was evaluated using the Well Diffusion method (Perez *et al.*, 1990) against *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Bacillus cereus* ATCC 7064, and *Penicillium* spp. (isolated from lemon). The test microorganisms were obtained from the Department of Biology, Manisa Celal Bayar University. In this method, 20-60 µL of the extract, prepared at a concentration of 1 mg/mL, was transferred into pre-prepared wells (5 mm), and activity was determined based on inhibition zones. Experiments were generally carried out using a final concentration of 40 µL/well (40 µg/well).

For the determination of minimum inhibitory concentration (MIC), the microtiter broth dilution technique was used according to CLSI standards (CLSI, 2003 and 2006). Serial dilutions of the extract and reference antibiotics were prepared at concentrations of 50-500 µg/mL and 10-150 µg/mL, respectively. All other experimental conditions followed Oskay's method (Oskay, 2011). In secondary experiments, real MIC values were determined by

conducting tests at intermediate concentrations, adjusting the extract concentration as necessary. Parallel experiments were conducted with ethyl acetate as a negative control and reference antimicrobial agents (streptomycin and nystatin) as positive controls for comparison.

2.4. Ultraviolet Spectroscopy of Secondary Metabolite

Ultraviolet (UV) measurements of the *Streptomyces* sp. MC12 extract, dissolved in ethyl acetate, were taken at a wavelength of 200-800 nm using a UV/Visible spectrophotometer (Varian Cary 50 UV-Visible Spectrophotometer) to determine the maximum absorbance of the active substance(s).

2.5. Statistical Analysis

All experimental data were collected in triplicate, and the results were presented as the mean (M) ± standard deviation (SD). Statistical analysis was performed using Minitab® 19 software.

3. RESULTS

3.1. Identification of *Streptomyces* sp. MC12

As a result of morphological examinations, it was observed that MC12, isolated from soil, was a typical Gram-positive filamentous *Streptomyces*-like bacterium. Its colony formed extensively branched aerial and substrate mycelium in ISP 2 medium, with colors ranging from white to cream. The temperature range for growth was 20 to 37°C, with an optimal temperature of 28°C; however, no growth was observed at 4°C or 45°C. Since MC12 exhibited high activity in screening studies (Figure 1), it was identified by 16S rRNA sequencing.

Figure 1. Antagonistic activity of *Streptomyces* sp. MC12 against *Penicillium* spp. by cross-streak method. Medium: Potato Dextrose Agar.

First, the DNA of the strain was isolated, and PCR was performed under the reaction conditions described in the methods section. The agarose gel image of the PCR amplification product is shown in Figure 2a. According to the phylogenetic tree (Figure 2b), created by comparing the 16S rRNA sequence data (1,145 bp) with the sequences of closely related *Streptomyces* species, the strain showed 97% similarity to *Streptomyces enissocaesilis* NBRC 100763 and *Streptomyces rochei* NBRC 12908. However, due to the relatively low similarity rate, the strain, which produces high levels of secondary metabolites, was registered in GenBank as *Streptomyces* sp. MC12, with the accession number PP757795.

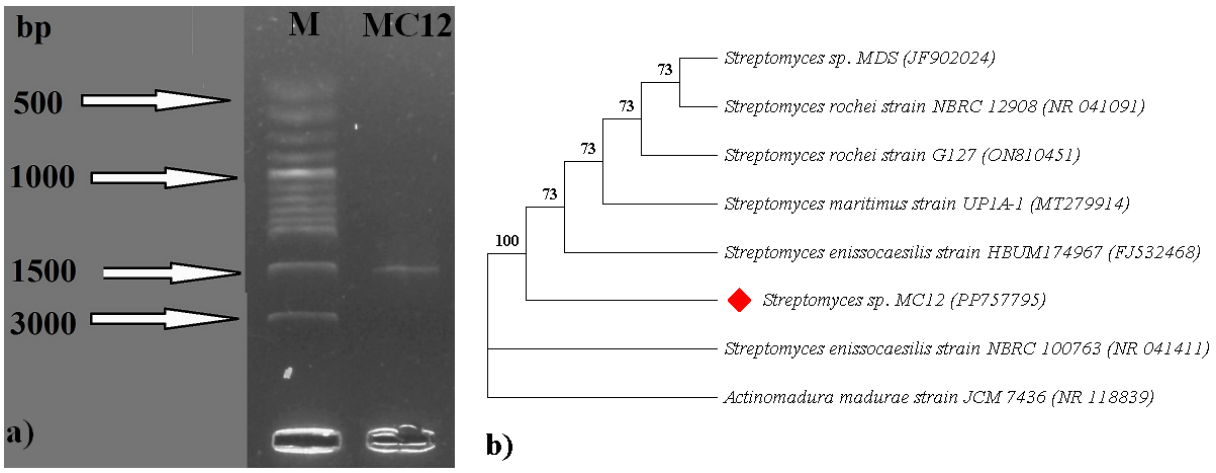

Figure 2. The agarose gel electrophoresis of the PCR product (a) and Comparative neighbour-joining phylogenetic tree based on 16S rRNA gene sequence of *Streptomyces* sp. MC12 with the sequences of closely related *Streptomyces* species (b). Bootstrap values calculated for 1000 replications are listed as percentages at branching points. Only bootstrap values >50% are shown.

3.2. Fermentation and Extraction of Secondary Metabolites

In this study, ISP 2 was selected as the fermentation medium, and only pH, temperature, and shaking speed were adjusted according to strain MC12 in preliminary experiments (data not shown). Secondary metabolite production by MC12 began at the 24th hour and reached its maximum on the 7th day. Since preliminary experiments determined that this isolate produced the highest yield of secondary metabolites on the 7th day, approximately 300 mL of cell-free culture supernatant was extracted twice with ethyl acetate in a 1:1 ratio. After evaporating the ethyl acetate from the extract, the remaining sample amount was approximately 5 mg. This extract was then dissolved to a concentration of 1 mg/mL and used in activity studies.

3.3. Antagonistic Effects of Secondary Metabolites against Different Microorganisms

The antagonistic effect of the ethyl acetate extract obtained at the end of fermentation was first tested using the Well Diffusion assay. As shown in Table 1, inhibition zones ranging from 10.6 to 20 mm were observed for the growth of the tested organisms at a concentration of 40 µg/well. The highest activity was observed against *E. coli* (20 mm), while the lowest activity was against *B. cereus* (10.6 mm). The activity against *Penicillium* sp. was also notable (14.6 mm).

Table 1. Well Diffusion assay and MIC results of the metabolite of *Streptomyces* sp. MC12 against test microorganisms.

Microorganisms[a]	Well diffusion assay (mm)[b]	MIC (µg/mL)	Streptomycin[c]	Nystatin
E. coli ATCC 25922	20.0±0.89[d]	153.6±2.87	20.3±1.3	NA
S. aureus ATCC 25923	18.6±0.51	101.3±2.73	8.5±0.44	NA
B. cereus ATCC 7064	10.6±1.03	ND	ND	NA
Penicillium sp.	14.6±1.03	ND	NA	27.0±0.89

[a] Bacteria tested in Mueller Hinton Agar, mould in PDA.
[b] Inhibition zone diameter in mm, not including well diameter (5 mm). The ethyl acetate extract concentration was 40 µg/well.
[c] MIC of the standard antimicrobial agents.
[d] Data presented as the M ± SD. ND - not determined, NA - not applicable.

Two microorganisms with high activity, *S. aureus* and *E.coli*, were selected and their MIC values were determined using the microdilution technique. MIC values against *S. aureus* and *E. coli* were determined as 101.3 and 153.6 µg/mL, respectively, and these values are comparable to MIC values of standard antimicrobial agents. The effect of ethyl acetate extract obtained from *Streptomyces* sp. MC12 at different concentrations against microorganisms is given in Figure 3.

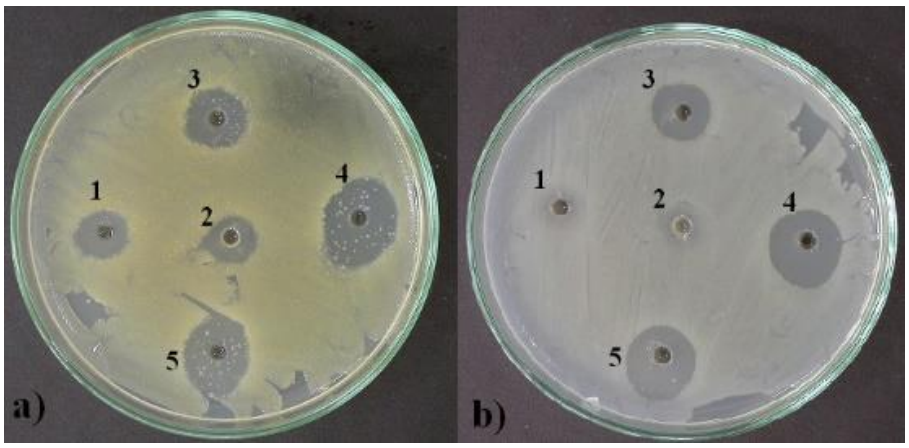

Figure 3. Antibacterial activity of the secondary metabolite of *Streptomyces* sp. MC12 against microorganisms; a) *S. aureus*, b) *E. coli*. Well diameter 5 mm, transferred volume 1) 20 µL, 2) 30 µL, 3) 40 µL, 4) 50 µL and 5) 60 µL/well at a concentration of 1000 µg/mL.

In the UV wavelength scanning of the ethyl acetate extract, the highest absorbance of the metabolite was observed between the 220-275 nm ranges (Figure 4). The UV absorbance value of the standard antibiotic streptomycin was in the range of 205-275 and was similar to the metabolite of *Streptomyces* sp. MC12.

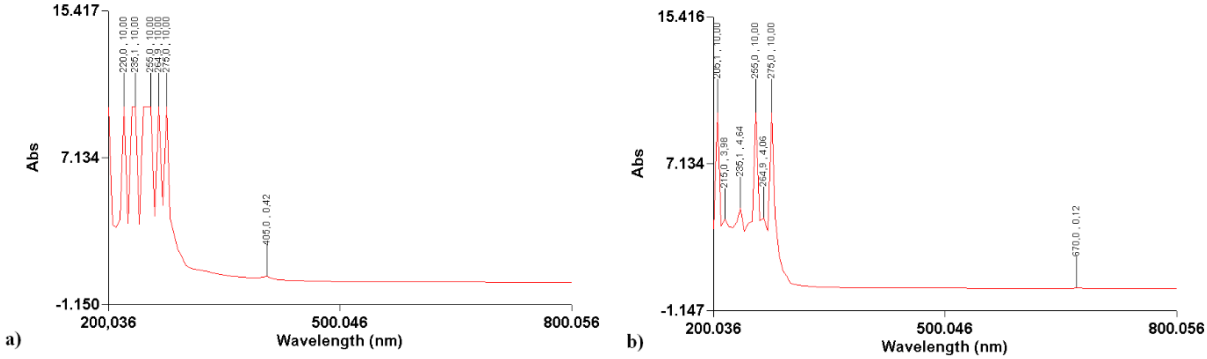

Figure 4. UV measurement at a wavelength of 200-800 nm; a) the secondary metabolites of *Streptomyces* sp. MC12 (30 µg /mL), b) standard antibiotic streptomycin (30 µg/mL).

4. DISCUSSION and CONCLUSION

In the current study, isolate MC12, which exhibited high inhibitory activity against certain pathogens during bioactive screening experiments, was characterized, and its secondary metabolite production was carried out through fermentation. Phylogenetic analysis of the 16S rRNA sequence, compared with known actinobacteria, placed MC12 within the genus *Streptomyces*. It showed 97% similarity to *Streptomyces enissocaesilis* NBRC 100763 and *Streptomyces rochei* NBRC 12908. However, due to the relatively low similarity for species identification, it was classified as *Streptomyces* sp. MC12 (PP757795) at the genus level. In numerous screening studies, secondary metabolite producers are generally identified as members of the *Streptomyces* genus.

ISP 2 is widely used and considered an ideal medium to produce secondary metabolites by fermentation of *Streptomyces* and related bacteria (Marzoug *et al*., 2023). Therefore, ISP 2, which proved to be suitable, was chosen for the fermentation of *Streptomyces* sp. MC12. Methanol, ethanol, and especially ethyl acetate are frequently used for metabolite extraction from fermentation supernatants (Al Farraj *et al*., 2020). In this research, ethyl acetate was selected as the appropriate organic solvent based on preliminary studies. The extract obtained (5 mg) was tested for activity against two Gram-positive bacteria (*S. aureus* and *B. cereus*), one

Gram-negative bacterium (*E. coli*), and one fungus (*Penicillium* spp.) from the culture collection of the Biology Department. The antibacterial activity varied with the concentration: *Streptomyces* sp. MC12 showed inhibition zones of 20 mm against *E. coli*, 18.6 mm against *S. aureus*, and 10.6 mm against *B. cereus*, using the Well Diffusion assay at a concentration of 40 µg/well. Almuhayawi *et al*. (2021) recorded the antibacterial activity of the Act19 isolate from desert habitat identified as *Streptomyces* sp. (MW240533) against *S. aureus*, *E. coli*, and *B. cereus* as 26 mm, 24 mm, and 28 mm, respectively. In another study, *Streptomyces albidoflavus* H12 showed strong activity against *Pseudomonas syringae*, *Pseudomonas corrugata*, and *Pectobacterium carotovorum* subsp. *carotovorum* (Djebaili *et al*., 2021). The isolate B3d, identified as *Streptomyces thermolilacinus*, demonstrated broad-spectrum antagonistic activity against multidrug-resistant bacterial pathogens such as *S. aureus, Salmonella* Typhi, *E. coli, Staphylococcus haemolyticus*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. (Talpur *et al*., 2020). *Streptomyces* sp. MC12 exhibited the highest activity against *S. aureus* and *E. coli*, with MIC values of 101.3 µg/mL and 153.6 µg/mL, respectively. These comparative results suggest that the secondary metabolites obtained from *Streptomyces* sp. MC12 may have a broad spectrum of antibiotic activity. For instance, *Streptomyces* sp. Kz-24 (KY000533) exhibited a MIC concentration of 0.024 µg/mL against methicillin-resistant *Staphylococcus aureus* ATCC 43300 and *Candida albicans* MTCC 227 (Sharma and Thakur, 2020). The findings of this research can be compared with other studies, where various *Streptomyces* species have been documented to possess significant antagonistic activity against fungal and bacterial pathogens (Sapkota *et al*., 2020; Chakraborty *et al*., 2022; Meena *et al*., 2023; Mirsonbol *et al*., 2023; Salehghamari *et al*., 2023).

Additionally, recent local research in Türkiye has focused on the production of secondary metabolites from actinobacteria, especially *Streptomyces*. Notable studies include those on actinobacteria isolated from various habitats, such as Karst Caves in the Eastern Black Sea Region (Tüfekci *et al*., 2023), Aras River (Seçkin *et al*., 2023), Kula Geopark (Bayraktar and Işık, 2024), Marmara and Avşa Islands (Topkara and Işık, 2023), symptomatic potatoes (Karagoz *et al*., 2024), lichen and orchid tissues (Ateş and Ay, 2023), legume nodules (Ay, 2020), and plant rhizosphere (Kum and İnce, 2021).

UV wavelength scanning of antibiotics provides valuable information about the active substances present in bacterial metabolites (Peris-Vicente *et al*., 2022). The UV absorbance value of the metabolite of *Streptomyces* sp. MC12 was in the 220-275 nm range and was similar to the absorbance peak of streptomycin (205-275 nm) (Figure 4). Most *Streptomyces*-derived antibiotics exhibit the highest absorbance values in the wavelength range of 200-400 nm. In a study, the UV peaks of cell-free filtrates from *Streptomyces* sp. HU2014 were found in the 291-352 nm range (Zhu *et al*., 2023).

Secondary metabolites are not essential for primary metabolic processes and growth in cells. Actinobacteria begin producing secondary metabolites in their natural environments, especially in soil, alongside morphological changes (Mehling *et al*., 1995; Mazumdar *et al*., 2023). Of over 23,000 known microbial secondary metabolites, 42% are produced by actinobacteria, 42% by fungi, and 16% by other bacteria. Approximately 45-55% of the 10,000 known antibiotics are produced by members of the *Streptomyces* genus (Watve *et al*., 2001; Hazarika and Thakur, 2020). The production mechanism of secondary metabolites is not fully understood but is thought to be related to sporulation (Barka *et al*., 2016; Zhu *et al*., 2023).

In the past 40 years, at least 15 million organisms have been isolated, and approximately ten thousand different antimicrobial substances have been identified in the search for new antibiotics. Each year, many of these are characterized, and 200-300 new ones are discovered. These studies have primarily focused on fungi, actinobacteria, and members of the *Bacillus* genus, which are known to be significant antibiotic producers. The genus *Streptomyces* is reported to be a major producer of bioactive secondary metabolites against various bacterial and fungal pathogens (Donald *et al*., 2023).

According to the current results, *Streptomyces* sp. MC12 exhibits broad-spectrum antagonistic activity against several microorganisms and could serve as a candidate for novel secondary metabolite discovery. Further studies should determine its species level, purify the bioactive metabolite, establish its structure, and conduct toxicity tests before it can be considered for therapeutic use.

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Declaration of Conflicting Interests and Ethics

The author declares no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author.

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Research Article

Preparation and characterization of chitosan nanoparticles with extracts of *Rheum ribes*, evaluation of biological activities of extracts and extract loaded nanoparticles

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Abstract: The biological activities of different parts of the *Rheum ribes* plant were evaluated comparatively. Extracts showing strong biological activity were identified and it was determined which of the extract-loaded nanoparticles showed stronger activity. Cytotoxic activity of *R. ribes* extracts was calculated on glial (C6) and fibroblast (NIH 3T3) cells using XTT assay. Spectrophotometry was used to evaluate the impact of these compounds on the enzyme activities of human carbonic anhydrase I and II (hCA I and hCA II). The findings showed that chitosan NPs with extracts loaded on them have a lower IC_{50} value and more cytotoxic activity in C6 cells than chitosan NPs with only extracts. *R. ribes* young shoots ultrasonic methanol extract (RYU) was shown to have the strongest antiproliferative efficacy against C6 cells. Results showed that RYU and ultrasonic methanol extract of *R. ribes* radix (RRU) were determined as the best carbonic anhydrase inhibitors. According to results of particle size, encapsulation efficiency, and release studies of chitosan NPs, it has been observed that they are suitable for application. At a concentration of 10 µg/mL, it was found that none of the *R. ribes* extracts exhibited cytotoxic action toward the NIH 3T3 cell line. According to results of particle size, encapsulation efficiency, and release studies of chitosan NPs, it has been observed that they are suitable for application. It was observed that none of the extracts of *R. ribes* at a concentration of 10 µg/mL showed cytotoxic activity in the NIH 3T3 cell line.

1. INTRODUCTION

Gliomas are complex malignancies with unknown biochemical and molecular detrimental effects. (Mamelak & Jacoby, 2007). In long-term chemotherapeutic drug treatment, chemical resistance occurs against the drug, and this causes the recurrence of glioma (Devasagayam & Sainis, 2002). Studies on natural substances with immunomodulatory and neuroprotective effects have become more important to remove or minimize these drawbacks associated with chemotherapy and long-term treatment. Phytochemicals with neuroprotective effects have gained value in studies by preventing or reducing the recurrence and growth of glioma. Extracts

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made from phytochemicals have been found to improve therapeutic effectiveness and lower the side-effect profile (Choudhari *et al*., 2020). Especially in cancer treatment, phytochemicals, which are natural herbal sources, are administered together with drug delivery systems and controlled release systems. Natural plant extracts can benefit from co-administration with a carrier system because it makes them more bioactive and makes them easier to apply, dose, and target. Applications of nanotechnology make it significantly easier to target, diagnose, and treat tumors in an effective and accurate manner. Important benefits of nanoparticular systems include optimal pharmacokinetic features, simple specific targeting, reduced adverse effects, and reduced drug resistance in the treatment of cancer (Dadwal *et al*., 2018; Palazzolo *et al*., 2018). The metalloenzyme CA (Carbonic anhydrase carbonate hydro-lyase, EC 4.2.1.1) works as a catalyst in the reversible hydration of carbon dioxide to bicarbonate and is present in many different tissues. It contains zinc (Zn2+) ions in its active site (Tutar *et al*., 2019; Koçyiğit *et al*., 2020; Huseynova *et al*., 2022). Based on where they are found in the body, carbonic anhydrases are divided into four groups. There are at least 16 different isoforms of these enzymes, spanning from CA I to CA XVI. In addition to being used as active ingredients in painkillers and diuretics to treat cancer, osteoporosis, and epilepsy, CA isoenzyme inhibitors (hCA I and hCA II) are also used to treat eye problems and hypertension (Koçyigit *et al*., 2017; Tutar *et al*., 2019; Baltacı *et al*., 2021; Gezegen *et al*., 2021).

Rheum ribes is a species of Polygonaceae family used by the public as food and medicine. The dried stem and root of this plant are used in traditional medicine to cure anemia, fatigue, anxiety, depression, diabetes, and to prevent cancer (Amiri *et al*., 2015). Different parts of the *R. ribes* contain bioactive substances such as tannin, anthracene derivatives, flavonoids quercetin, 5-desoxyquercetin, quercetin 3-0 rhamnoside, quercetin 3-0 galactose and auercetin 3-O-rutinoside (Amiri *et al*., 2015; Noori *et al*., 2022). Additionally, plants include minerals like Calcium, Potassium, Magnesium, and certain vitamins like A, B1, and C, as well as organic acids like citric and malic acids. In addition, it is known that the plant has a therapeutic effect against various ailments such as stomach upset, vomiting, hemorrhoids, diabetes, measles and smallpox symptoms and loss of appetite due to these bioactive compounds it contains (Amiri *et al*., 2015; Taskin & Bulut, 2019).

In this investigation, we sought to assess the effects of *R. ribes* extracts on the C6 and NIH 3T3 cell lines in terms of their enzyme, antibacterial, and cytotoxic properties. Additionally, mechanical, and in vitro characterization investigations were carried out, and the NPs of the most efficient extracts were synthesized. We investigated whether the antiproliferative activity of NPs containing the extracts increased compared to the extracts alone, while also examining their cytotoxic effects on the C6 and NIH 3T3 cell lines.

2. MATERIAL and METHODS

2.1. Plant Material And Preparation of Plant Extracts

The *R. ribes* was collected from Van-Gürpınar, Turkey. The voucher specimen was deposited in the Pharmacy Faculty of Marmara University Herbarium and the voucher specimen number was MARE 18817. The dried parts of the plant (young shoots, leaves, roots and flowers) were cut into small pieces and 10 g of each were weighed. Maceration of each part of the plant was carried out by adding methanol and keeping it in a dry and shaded place until the solvent became colorless. The solvent was processed in methanol using a Soxhlet apparatus until it became colorless, and in the ultrasonic bath extraction, the specified parts of the plant were kept in an ultrasonic bath with methanol solvent for a certain hour and prepared. Following extraction, the samples were filtered using filter paper, the solvents were vaporized using a rotary evaporator, and the unprocessed extracts were kept chilled at 4 $^{\circ}$C. The extracts used in the study are listed in Table 1.

Table 1. The extracts used in this study.

Rheum ribes parts	Methods of extraction	Extract name
Young shoots	Soxhlet	RYS
	Ultrasonic bath	RYU
	Maceration	RYM
Leaves	Soxhlet	RLS
	Ultrasonic bath	RLU
	Maceration	RLM
Flowers	Soxhlet	RFS
	Ultrasonic bath	RFU
	Maceration	RFM
Radix	Soxhlet	RRS
	Ultrasonic bath	RRU
	Maceration	RRM

2.2. Enzyme Studies

The enzyme activity of carbonic anhydrase was measured using the esterase activity technique. The technique is based on the esterase activity of CA. The method's underlying tenet is that the carbonic anhydrase enzyme's substrate, p-nitrophenyl acetate, is utilized. According to Baltac *et al*. (2021) and Verpoorte *et al*. (1967), p-nitrophenol or p-nitrophenol is hydrolyzed to produce absorbance at 348 nm. Armstrong, 1966) also support this theory. This method gives the same absorbance at 348 nm for p-nitrophenol and p-nitrophenolate. As a result, the measurement during the process is unaffected by phenol or phenolate generation (Armstrong, 1966; Verpoorte *et al*., 1967; Koçyigit *et al*., 2017; Baltac *et al*., 2021). The use of 348 nm p-nitrophenyl acetate is blind because of its extremely low absorption. By combining the reaction mixture in 3 mL quartz cuvettes, an activity determination approach was used in the measurements.

2.3. Cell Culture Studies

C6 cell line (ATCC CCL 107) and NIH 3T3 (ATCC CRL-1658) cell line were obtained from ATCC for the cytotoxic activity research. Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were procured from Merck Millipore. Phosphate buffer saline (PBS), tripolyphosphate (TPP), and chitosan (400 kDa, DD 92) were purchased from Sigma-Aldrich. The Sigma-Aldrich penicillin-streptomycin-L-glutamine solution was bought. Studies on cytotoxic action used the XTT reagent from Roche Diagnostic. In DMEM containing FBS (10%), penicillin (100 IU/mL), L-glutamine (1%), and streptomycin (10 mg/mL), C6 and NIH 3T3 cells were planted. Well plates with cells were cultured at 37 °C and 5% CO2 in an incubator. Cells have to be at least 80% confluent for the cytotoxic activity assays to be completed (Wolf *et al*., 2009).

2.4. Cytotoxicity Assay

On the C6 and NIH 3T3 cells, the XTT assay was used to determine the cytotoxic activity of *Rheum ribes* extracts. DMEM (100 L, 10% FBS) was first used to seed cells in two 96-well plates, where they were then left to incubate for the night (Wolf *et al*., 2009; Taskin *et al*., 2020). Twelve distinct *R. ribes* extracts, including those made using soxhlet methanol, ultrasonic methanol, and methanol maceration, were dissolved in DMSO (20% v/v) to test their ability to inhibit the proliferation of cell lines. The control group received an equivalent amount of DMSO while the extracts were added to each well after being pipetted and vortexed in DMEM at a concentration of 10 µg/mL. Incubation of plates containing cells and extracts for 24 hours followed. After this time, wells were cleaned with PBS (200 µL). Each well received 100 µL of colorless DMEM and 50 µL of the XTT reagent before the cells were given a 4-hour incubation period. The absorbance of XTT-formazan at 450 nm was measured using a micro plate ELISA reader. Calculations of *R. ribes'* cell viability in comparison to controls were made.

After reviewing the findings, the *R. ribes* extract with the best antiproliferative activity against the C6 cell line was made into nanoparticles (NPs). To determine the IC_{50} values on the C6 and NIH 3T3 cell lines, XTT experiments were conducted with and without NP.

2.5. Preparation of Chitosan NPs

NPs containing *Rheum ribes* were prepared using the ionic gelation technique. Based on information from the literature, the concentration of chitosan was dissolved in acetic acid (0.5% v/v) at 1000 rpm while being magnetically stirred. The pH of the chitosan solution should be between 4 and 5 to achieve good encapsulation and loading capacity of NPs. Using 5M sodium hydroxide, the pH of the chitosan solution was brought down to 4.3 (Calvo *et al*., 1997; Taskin *et al*., 2020). TPP was dissolved at a predetermined concentration (0.25% w/v) in sterile deionized water. Chitosan solution (0.5% w/v) with the extract dripped into the TPP solution. Centrifuging the NP expansion was done for 30 minutes at 10.000 rpm. To determine the encapsulation effectiveness of the NPs, the supernatant was taken out and a 1 mL sample was isolated from it. The pellet was afterwards cleaned with deionized water. The pellet received 30 mL of deionized water before being centrifuged for 15 minutes at 10.000 rpm. This procedure was carried out twice. NPs were then lyophilized and kept at +4 °C.

2.6. Encapsulation Efficiency (EE) and Loading Capacity (LC) Studies of NPs

The EE% and LC% of the *R. ribes* extract in NPs were determined using an ultraviolet visible (UV vis) spectrophotometer (Taskin *et al*., 2020). The extract's standard calibration curve and spectral line equation were established by measuring the extract's absorbance at various concentrations at a wavelength of 350 nm. The line equation was used to determine how much extract was present in the supernatant. The loading capacity and encapsulation effectiveness of the NPs were calculated using the following formulae.

$$EE\ (\%) = ((mo - ms)/mo) \times 100$$

$$LC\ (\%) = ((mo - ms)/wnp) \times 100$$

where, mo stands for the initial mass of natural extracts and ms stands for mass of natural the extracts in the supernatant and wnp = total weight of the naturally obtained extract of NPs (Purbowatiningrum and Ismiyarto, 2017) Each measurement was made three times, and the results were presented as mean SD (n = 3).

2.7. Measurement of Particle Size and Zeta (ζ) Potential

A Zetasizer Nano ZS equipment was used to assess the measurements of NPs' size and ζ potential. In this work, NPs were assessed while suspended in PBS (pH 7.4).

2.8. In vitro Release Study of Extract Loaded Nps

R. ribes young shoots ultrasonic methanol extract (RYU) and ultrasonic methanol extract of *R. ribes* radix (RRU) were released in vitro from chitosan nanoparticles in PBS (pH 7.4) using the same procedures as described in Keawchaoon & Yoksan (2011) and Mohammadi *et al*. (2015), with a few minor modifications. Initially, 2 mL of buffer solution was mixed with a certain quantity of *R. ribes* extract-loaded NPs, and then vortexed at room temperature. Samples were centrifuged at 10000 rpm for 10 min at 25 °C at preset intervals. To keep the total volume constant, 400 µL of the supernatant was removed for analysis and replaced with an equivalent volume of new buffer. Utilizing UV vis spectrophotometer, the amount of liberated *R. ribes* extract at a certain moment was calculated.

3. FINDINGS

3.1. Carbonic Anhydrases Inhibition Activity Results

The study also explored the potential of using these antiepileptic substances as safer alternatives to the currently available drugs, which may have various potentially harmful side effects. Spectrophotometric analysis was used to determine how these chemicals affected the enzyme

activity of carbonic anhydrase I and II isoenzymes. The inhibitory potentials of these medicines against two physiologically significant CA isoforms, the slower cytosolic isoform (hCA I) and the quicker cytosolic isoenzyme (hCA II), were investigated using an esterase assay approach. The inhibitory results of medicines against CA I and II isoforms are shown in Table 2 and Figure 1 (IC_{50} values expressed as µg/mL).

Table 2. The extracts that inhibit the enzymes carbonic anhydrase I and II.

Extracts	IC_{50} (µg/mL)			
	hCA I	r^2	hCA II	r^2
RYS	7.72	0.9551	6.73	0.9634
RYU	3.21	0.9966	4.76	0.9940
RYU NP	3.83	0.9407	2.70	0.9780
RYM	5.71	0.9538	5.31	0.9232
RLS	12.93	0.9027	3.66	0.8357
RLU	6.04	0.9396	8.13	0.9604
RLM	7.42	0.9170	10.69	0.8481
RFS	7.39	0.9429	7.30	0.9985
RFU	7.55	0.9552	12.96	0.9851
RFM	10.82	0.8372	6.52	0.9840
RRS	4.25	0.9515	5.07	0.9567
RRU	4.43	0.9787	3.44	0.9315
RRU NP	2.97	0.8049	5.37	0.9061
RRM	6.82	0.8773	4.57	0.9821

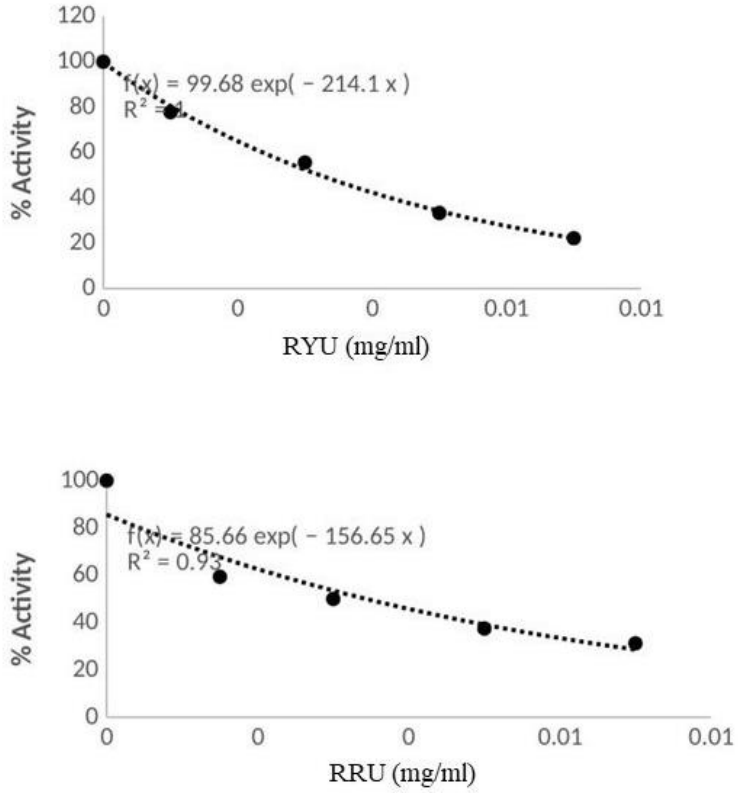

Figure 1. IC_{50} values for the hCA I and hCA II isoenzymes, with RYU (Rheum ribes young shoots ultrasonic bath extract) and RRU (*Rheum ribes* radix ultrasonic bath extract) respectively serving as the best inhibitors

All the compounds were remarkably inhibited both the cytosolic isoforms hCA I (IC_{50} ranging between 3.21 and 12.93 µg/mL) and hCA II (IC_{50} ranging between 3.44 and 12.96

µg/mL). We found that the most effective inhibitors for these isoforms were RYU and RRU (hCA I and hCA II) with IC_{50} values of 3.21 and 3.44 µg/mL, respectively. In addition, IC_{50} values on hCA I enzyme of NP samples including RYU and RRU were calculated as 3.83 µg/mL and 2.97 µg/mL. IC_{50} values on hCA II enzyme of NP samples including RYU and RRU were calculated as 2.70 µg/mL and 5.37 µg/mL (Table 2, Figure 1-2). These results indicated that NP containing RYU has more effective hCAII enzyme inhibitory activity compared to the extract. In addition, it can be said that RRU loaded NP has a higher hCAI enzyme inhibitory effect compared to only the extract.

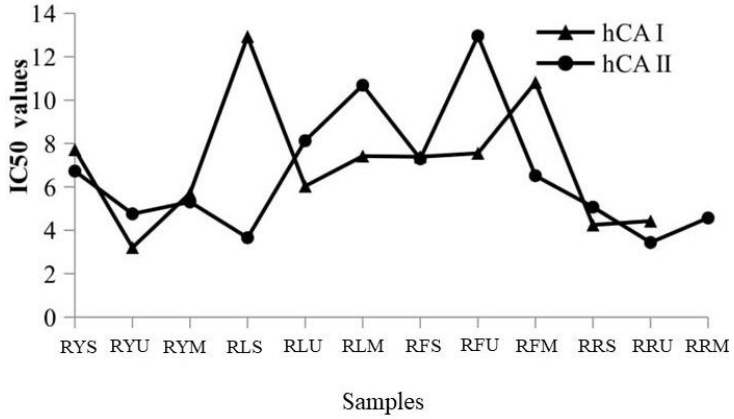

Figure 2. IC_{50} values for the isoenzymes hCA I and hCA II.

3.1. Assessment of Cytotoxic Activity Results

Rheum ribes extracts' cytotoxic activity was evaluated on C6 and NIH 3T3 cell lines using XTT assay. This method is based on the reduction of XTT, a tetrazolium salt, to orange formazan crystals by metabolically active cells (Taşkın *et al*., 2020). At the beginning of the study, DMEM + fetal bovine serum (FBS) + antibiotic-containing medium will be seeded into a 96-well microplate and incubated overnight for the cells to adhere. Then, the medium will be removed, the wells containing cells will be washed with PBS, and immediately after that, fresh medium and extracts will be seeded into the cells and incubated for 24 hours. Then, the medium will be removed and the cells will be washed three times with PBS. 100 µl of transparent DMEM and 50 µl of XTT solution will be added to each well and incubated for 4 hours in a CO2 oven. After incubation, the cell viability rate of the control group was calculated as % by reading at 450 nm in a microplate reader. Antiproliferative activity results of twelve extracts of *R. ribes* against C6 cells were indicated in Figure 3. When the extracts were given to C6 cells at a concentration of 10 g/mL, the soxhlet *R. ribes* rhizome methanol extract's cell viability was assessed to be 65.27±0.23% in the RYS, 59.13±0.25% in the RYU, 67.89±0.18% in the RYM, 69.05±0.32 % in the RLS, 71.92±0.24 % in the RLU, 76.86±0.22 % in the RLM, 73.02±0.24 % in the RFS, 64.99±0.22% in the RFU, 67.16±0.27% in the RFM, 72.23±0.19% in the RRS, 68.52±0.23 % in the RRU, and 78.96±0.32% in the RRM. The antiproliferative activity of R. ribes extracts RYU and RFU against the C6 cell line was found to be the most potent, as shown by the results of Figure 3. The best carbonic anhydrase inhibitors were found to be *R. ribes* extracts RYU and RRU. To do this, chitosan nanoparticle (NP) was prepared, and RYU and the NP containing the extract were applied to C6 and NIH 3T3 cells at varied concentrations.

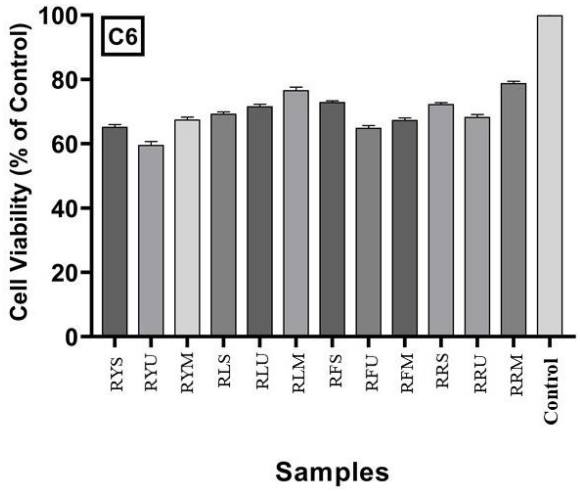

Figure 3. Cytotoxic effects of several *Rheum ribes* extracts on the C6 cell line at doses of 10 µg/mL.

Figure 4 displays the results of *R. ribes* extracts' cytotoxic efficacy against NIH 3T3 cells. The RYS was treated with NIH 3T3 cells at a preset concentration (10 µg/mL), and the cell viability was estimated to be 75.99±0.22%, 82.51±0.25% in the RYU, 85.16±0.19% in the RYM, 80.05±0.26 % in the RLS, 82.66±0.22 % in the RLU, 76.66±0.24% in the RLM, 85.06±0.19 % in the RFS, 78.66±0.28% in the RFU, 74.52±0.25% in the RFM, 84.10±0.22% in the RRS, 75.02±0.23% in the RRU and 80.86±0.27% in the RRM. According to the findings of Figure 4, none of the *R. ribes* extracts at a concentration of 10 µg/mL significantly reduced the vitality of NIH 3T3 cells, and they were also not cytotoxic because the viability of the cells reached 70%.

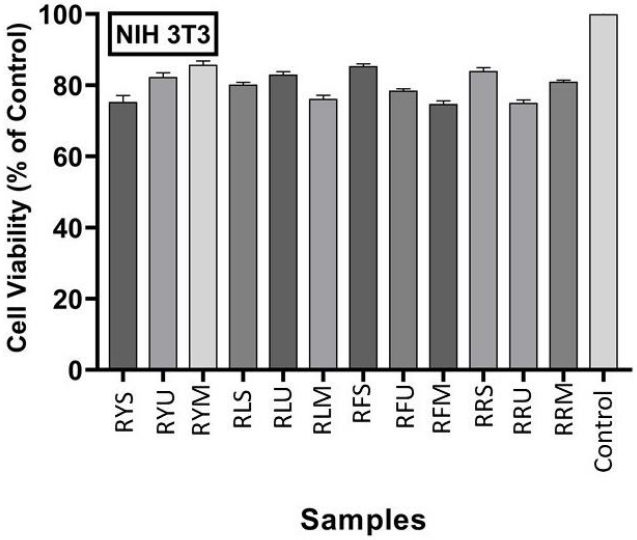

Figure 4. Different *Rheum ribes* extracts treated with doses at 10 µg/mL had anti-proliferative effects on the NIH 3T3 cell line.

To evaluate the IC_{50} values, C6 and NIH 3T3 cell lines were treated with NP containing RYU of *R. ribes* at predetermined doses. The C6 cell viability was severely decreased by both the RYU and the RYU loading NPs, according to the findings of the XTT investigation (Table 3), depending on the concentration. The cell viability rates were calculated as 66.16±0.24% and 68.28±0.31%, respectively, when the sole extract and the extract loading NP were fed to C6 cells at a concentration of 2.5 µg/mL. When C6 cells were exposed to extract and extract loading NP at a concentration of 5 µg/mL, the cell viability was estimated to be 63.28±0.29% and

63.44±0.21%, respectively. Additionally, the extract and NP containing extract were supplied to cells at a concentration of 10 g/ml, and the results showed that the cell viability was, respectively, 59.23 ±0.24% and 55.28 ±0.26%. Additionally, cells were treated with extract and extract loading NP at a concentration of 25 µg/mL, and the cell viability was estimated to be 50.16±0.22 and 47.24±0.32, respectively. The cell viability rates were calculated as 39.24±0.25% and 36.16±0.23%, respectively, when the extract and the extract loading NP were treated with C6 cells at the 50 µg/mL concentration. The greatest concentration (100 µg/mL) of the extract and extract loading NP on C6 cells resulted in cell viability rates of 28.22±0.23% and 25.16±0.21%, respectively. These results were used to calculate the IC_{50} values for RYU and RYU loading NP of *R. ribes*. On the C6 cell line, the IC_{50} values for RYU and NP containing RYU were 25.36±0.18 µg/mL and 18.23±0.21 µg/mL, respectively. The findings demonstrate that compared to RYU alone, RYU loaded NP has a lower IC_{50} value and greater cytotoxic effect in C6 cells. When C6 cells were exposed to simply RRU or RRU loading NP at a concentration of 2.5 µg/mL, the cell viability rates were calculated to be 78.12±0.27 % and 70.24±0.25 %, respectively. When C6 cells were treated with extract and extract loading NP at a concentration of 5 µg/mL, the cell viability was estimated to be 74.29±0.25 % and 67.75±0.17 %, respectively. Additionally, the extract and NP containing extract were fed to cells at a concentration of 10 µg/mL, and the estimated percentages of cell viability were 68.52±0.23 %, and 61.28±0.18 %, respectively. Additionally, cells were treated with extract and extract loading NP at a concentration of 25 µg/mL, and the cell viability was estimated to be 58.26±0.22 %, and 52.16±0.26 %, respectively. The cell viability rates were calculated as 44.52±0.11 % and 41.21±0.19 %, respectively, when the extract and the extract loading NP were treated with C6 cells at the 50 µg/mL concentration. The greatest concentration of the extract and extract loading NP (100 µg/mL) used to treat C6 cells resulted in cell viability rates of 30.16±0.13% and 24.98±0.14 %, respectively. These results were used to calculate the IC_{50} values for RRU and RRU loading NP of *R. ribes*. On the C6 cell line, the IC_{50} values for RRU and RRU loaded NPs were respectively 39.66±0.22 µg/mL and 29.53±0.14 µg/mL. According to the findings, compared to RRU, the RRU loading NP has a lower IC_{50} value and greater antiproliferative action in C6 cells. The solo RYU and the RYU loading NP were treated with NIH 3T3 cells at a concentration of 2.5 g/ml, and the cell survival rates were determined as 89.98±0.26 % and 87.48±0.33 %, respectively, according to the concentration-dependent XTT research results in the NIH 3T3 cell line (Table 4). NIH 3T3 cells were treated with the extract and extract loading NP at a concentration of 5 µg/mL, and the cell viability was estimated to be 86.420.27% and 83.240.27%, respectively. Additionally, cells were given the extract and NP containing the extract at a concentration of 10 µg/mL, and the cell viability was estimated to be 82.51±0.22 %, and 81.28±0.20 %, respectively. Additionally, cells were treated with extract and extract loading NP at a concentration of 25 µg/mL, and the estimated percentages of cell viability were 75.44±0.26 %, and 76.16±0.27 %, respectively.

Table 3. Results of extracts and NPs samples on the C6 cell line in terms of concentration-dependent cell viability.

	C6 cell viability (% of the control)			
Samples	RYU	NP loading RYU	RRU	NP loading RRU
2.5 µg/mL	66.16±0.24	68.28±0.31	78.12±0.27	70.24±0.25
5 µg/mL	63.28±0.29	63.44±0.21	74.29±0.25	67.75±0.17
10 µg/mL	59.23±0.24	55.28±0.26	68.52±0.23	61.28±0.18
25 µg/mL	50.16±0.22	47.24±0.32	58.26±0.22	52.16±0.26
50 µg/mL	39.24±0.25	36.16±0.23	44.52±0.11	41.21±0.19
100 µg/mL	28.22±0.23	25.16±0.21	30.16±0.13	24.98±0.14

Table 4. Results of extracts and NPs samples on the NIH 3T3 cell line in relation to concentration-dependent cell viability.

NIH 3T3 cell viability (% of the control)				
Samples	RYU	NP loading RYU	RRU	NP loading RRU
2.5 µg/mL	89.98±0.26	87.48±0.33	82.25±0.31	79.56±0.22
5 µg/mL	86.42±0.27	83.24±0.27	79.98±0.27	77.65±0.27
10 µg/mL	82.51±0.22	81.28±0.20	75.02±0.23	75.28±0.26
25 µg/mL	75.44±0.26	76.16±0.27	66.27±0.16	68.98±0.21
50 µg/mL	60.16±0.28	64.38±0.29	56.14±0.13	58.27±0.19
100 µg/mL	48.24±0.28	55.44±0.17	44.21±0.13	51.26±0.22

The cell viability rates were calculated as 60.16±0.28% and 64.38±0.29% respectively when the extract and the extract loading NP were administered to NIH 3T3 cells at the 50 µg/mL concentration. The cell viability rates were calculated as 48.24±0.28% and 55.44±0.17%, respectively, when the extract and the extract loading NP were treated with NIH 3T3 cells at the 100 µg/mL concentration. The IC_{50} value of the NP containing RYU at the indicated concentrations could not be calculated, and the IC_{50} value of RYU was determined to be 88.26±0.23 µg/mL. According to the findings, RYU loading NP has less antiproliferative action in NIH 3T3 cells than RYU alone. When NIH 3T3 cells were exposed to the single RRU and the NP containing RRU at a concentration of at least 2.5 µg/mL, the cell viability rates were calculated to be 82.25±0.31% and 79.56±0.22%, respectively. When NIH 3T3 cells were treated with RRU and RRU loading NP at the highest concentration (100 µg/mL), the cell viability was estimated to be 44.21±0.13% and 51.26±0.22%, respectively. The IC_{50} value of the NP containing RRU at the determined concentrations could not be estimated, and the IC_{50} value of RRU was calculated to be 73.28±0.18 µg/mL.

3.2. EE and LC of NP Including *Rheum ribes* Extract

The amount of RRU and RYU extract coated in chitosan nanoparticles is shown by the EE rate. The findings of the calculation used to determine the rate at which the encapsulated extract turned into chitosan NP are shown in Table 5. EE value of NPs containing RYU was determined at 78.27±0.03%, according to the results. Furthermore, the LC of NPs was determined to be 9.02±0.02%. The computed EE value of NP incorporating RRU was 69.36±0.04%. These findings demonstrated that chitosan nanoparticles effectively contained RYU and RRU. Furthermore, LC of NPs, comprising RRU, was discovered to be 8.62±0.01 %. The findings indicate that NPs containing *R. ribes* extracts were appropriate for cell culture investigations in terms of both EE and LC values.

Table 5. EE and LC of RYU and RRU loading NPs,

Parameter	RYU	RRU
Linear equation	y=0.2908 x ± 0.0197	y=0.3016 x ± 0.0221
Slope ± SD	0.2908 ± 0.027	0.3016 ± 0.022
Intercept	0.0197 ± 0.0012	0.0221 ± 0.0015
r	0.9903	0.9926
EE %	78.27 ± 0.03	69.36 ± 0.04
LC %	9.02 ± 0.02	8.62 ± 0.01

3.3. Characterization of Chitosan NPs

The findings of the evaluation of the NPs' particle size, ζ potential, and polydispersity index (PDI) are shown in Table 6. The dimensions of the NP1 and NP2 were determined to be respectively 384.28±2.40nm and 342.36±2.20 nm. The NP1 and NP2's respective potentials were measured at 2.65±0.03 and 2.42±0.02 mV. PDI values NP1 and NP2 were 0.278±0.06

and 0.306±0.03, respectively. The findings support the notion that NPs were homogeneous features devoid of any aggregation.

Table 6. Extract loading NPs' ζ potential, particle size, and PDI values.

Samples	ζ potential (mV) ± SD	Size (nm) ± SD	PDI ± SD
*NP1	2.65±0.03	384.28±2.40	0.278±0.06
*NP2	2.42±0.02	342.36±2.20	0.306±0.03

* NP1 and *NP2 include chitosan (MW: 400 kD, DD: 92 %), TPP, RYU and RRU respectively.

3.4. *In vitro* Release Kinetics Study Result of Extract Loading NP

At 37 ºC for 300 hours, the release profile of RYU and RRU from NPs was examined (Figure 5). 0.1 M PBS was utilized in this study to mimic physiological circumstances. The results of the controlled release of *R. ribes* extracts showed a quick initial release (50%) during the first 24 hours, followed by a constant and gradual release (75%) until 144 hours. The release characteristics of RYU and RRU containing NPs were comparable. The NPs released almost all the extracts (99.26% and 99.96%) in 288 hours. In a study for acetylsalicylic acid, this kind of continuous and slow release was seen (Ajun *et al*., 2009). The release of the extract from the NP is significantly influenced by diffusion and the breakdown of the extract's molecular matrix. Since the extract's size is less than that of the particle, it can easily diffuse from the NP's surface or pores (Zhou *et al*., 2001; Hu *et al*., 2008).

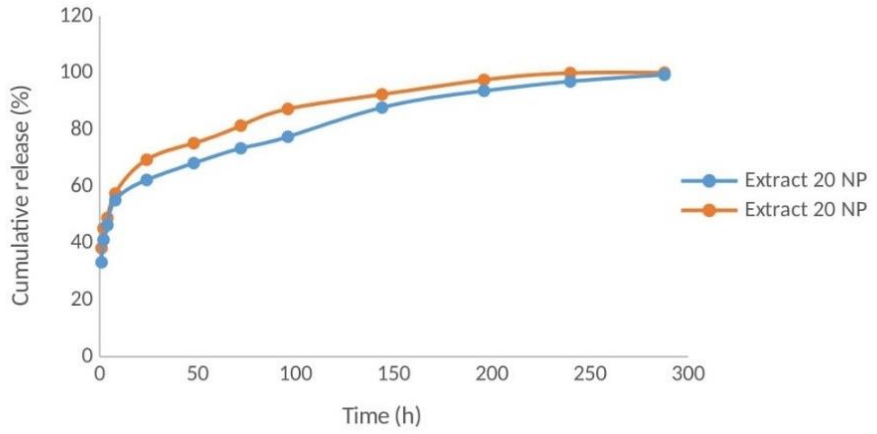

Figure 5. Release kinetics of NPs in 0.1 M PBS (pH 7.4), containing extracts RYU and RRU.

4. DISCUSSION and CONCLUSION

In Turkey, locals also use the *R. ribes* plant as an anti-cancer remedy. Furthermore, the abundant amounts of vitamins C, A, B1, B2, E, and K present in *R. ribes* are also significant. Moreover, antioxidant qualities are provided by the polyphenolic compounds. Additionally, it has been shown to be beneficial for conditions like diabetes, hypertension, asthma, colds, diarrhea, hemorrhoids, ulcers, influenza, and kidney disease (Aygun *et al*., 2020). Researchers investigated *Rheum emodi* root extracts from the *Rheum* genus. These investigations showed that *Rheum emodi* rhizome extracts, both methanolic and aqueous, demonstrated concentration-dependent cytotoxicity against cell lines that represent liver cancer (Hep3B) and breast cancer (MDA-MB-435S) (Rajkumar *et al*., 2011). Furthermore, extracts from various *Rheum* species, such as *R. ribes*, exhibited anti-proliferative properties against cell lines that were specifically related to breast, liver, and cervical cancer (Keser *et al*., 2011). The findings of this study support earlier findings in the literature and demonstrate that *R. ribes* extract nanoparticles at low concentrations can have encouraging effects on cancer cell death.

In conclusion, each *R. ribes* extract showed significant cytotoxic activity in the C6 cell line. The extracts decreased cell viability but did not show cytotoxic activity (cell viability up to 70

%) in the NIH 3T3 cell line. Both NP containing RYU and NP loaded RRU showed significant carbonic anhydrase enzyme inhibitory activity. The particle sizes of the NPs containing RYU and RRU were below 400 nm, making them applicable in both enzyme activity and cell culture studies. The high encapsulation rate of NPs ensured the suitability of the method used in the preparation phase and the minimum loss of extract. The fact that the extracts from the NPs are completely released in a period of more than 240 h showed that the NPs have the controlled release feature. While the extract-loaded NPs showed better cytotoxic activity in C6 cells, they did not show cytotoxic effects in healthy NIH 3T3 cells, while the high cell viability rates indicate that the study achieved the desired aim.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Murat Doğan: Investigation, Resources, Methodology, Experiment, Visualization, Formal Analysis, Writing. **Ümit Muhammet Koçyiğit:** Investigation, Resources, Experiment, Visualization, Formal Analysis, Writing. **Duygu Taşkın:** Investigation, Resources, Writing, Supervision. **Beyza Nur Yılmaz:** Investigation, Methodology, Experiment, Resources, Writing. **Turgut Taşkın:** Investigation, Methodology, Experiment, Resources, Supervision.

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Review Article

Review on the toxic effect of fluorine and lead on lichen metabolism

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Abstract: Thanks to their ability to absorb large amounts of trace elements from the atmosphere, lichens are widely used as bioaccumulators and bioindicators of air pollution. Among air pollutants, heavy metals represented by lead are the most important contributors to the deterioration of ecosystems. Fluorine is prevalent in a wide range of environmental matrices, even in trace amounts, and is one of the most phytotoxic halogens to plants. When lichens are exposed to air pollution, they frequently undergo structural, morphological and physiological alterations, and exhibit several coping strategies to combat and tolerate stressful situations. This manuscript presents general information about lichens, fluorine, and lead as well as the toxic effect of these two air pollutants on lichens, and the means of combat used by lichens to respond to fluorine and lead-induced stress.

1. INTRODUCTION

One of the major problems of current time is air pollution, the assessment of air quality through the use of lichens as bioindicators is a major concern at the moment. Lichens present a very important model of symbiotic organisms that include a fungus termed mycobiont, that captures fixed carbon from green algae and/or cyanobacteria called photobionts (Mitrović *et al.*, 2011; Calcott *et al.*, 2018). Because lichens have no roots and obtain their mineral nourishment from atmospheric inputs and moist deposits, they make good bioindicators of air pollution (Garty *et al.*, 2008; Gauslaa *et al.*, 2021). Lichens are also characterized by the presence of many original compounds, especially bioactive secondary metabolites (Mukemre *et al.*, 2021), which can be used for their antimicrobial (Maciąg *et al.*, 2014; Sargsyan *et al.*, 2021; Gandhi *et al.*, 2022) and antioxidant activity (Rodríguez *et al.*, 2016; Gessner *et al.*, 2017; Kandelinskaya *et al.*, 2021), the most important of which are polyphenols thanks to their pharmacological activity (Stromsnes et *al.*, 2021). Polyphenols have an important role in a plant's ability to withstand various stresses (Kiani *et al.*, 2021; Tuladhar *et al.*, 2021; Kołton *et al.*, 2022). They are necessary for plant's growth, nutrition, survival, and defenses (Singh *et al.*, 2021). Currently, lichens are being used for a variety of purposes, including the therapy of many human illnesses, especially as an anti-cancer activity (Nugraha *et al.*, 2019; Solárová *et al.*, 2020; Chae *et al.*, 2021; Šeklić *et al.*, 2022).

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Fluoride is one of the most phytotoxic chemical elements for plants and can be present in many different environmental matrices, even in small amounts (Banerjee & Roychoudhury, 2019). It affects the metabolic activity of plants by decreasing nutrient uptake, germination, photosynthesis, growth, and productivity (Sharma & Kaur, 2018). Fluoride toxicity causes the generation of reactive oxygen species (ROS), it has also a negative impact on enzyme activity, protein synthesis, and gene expression patterns (Choudhary *et al*., 2019). Heavy metals inhibit chlorophyll production in lichens (Rola *et al*., 2019). Abiotic stress has a major impact on photosynthesis in green plants at every stage (Ashraf & Harris, 2013). According to Nareshkumar *et al.* (2015), lead disrupts the prooxidant/antioxidant balance, resulting in a range of cell and tissue damage that eventually progresses to subcellular destruction.

When lichens are exposed to contaminants in the environment, they frequently undergo physiological, morphological, and structural alterations (Matos *et al.*, 2015). The most commonly used parameters to study the toxicity of atmospheric pollutants on lichens are chlorophyll degradation (Šujetovienė & Sliumpaitė, 2013; Balarinová *et al.*, 2014; Karakoti *et al*., 2014; Šujetovienė, 2015) and lipid peroxidation (Paoli *et al*., 2015; Šujetovienė *et al.*, 2019). Like all other plant components, including chlorophyll, polyphenols also respond differently to pollutants in the air. Plants use phenolic compounds to defend themselves from oxidative stress brought on by a variety of airborne pollutants (Nobile *et al.*, 2021). Plant polyphenols have the property of naturally acting as antioxidants and being only mildly harmful. Exposure to abiotic stress triggers the accumulation of amino acids and amines in different plant species. Soluble sugars play a crucial role in a variety of metabolic processes, acting as a signal to control gene expression in photosynthesis, osmolyte production, and sucrose metabolism (Khan *et al.*, 2020). Proline, on the other hand, is crucial for plants; it protects them from various stresses and helps their faster recovery from stress (Mundada *et al.*, 2021).

Plants manufacture many enzymatic components, such as catalase (Lei *et al.*, 2022), and non-enzymatic components, such as glutathione (Hasanuzzaman *et al*., 2020) to prevent ROS-induced damage, and change their protein composition (Amnan *et al*., 2022). In response to abiotic stress, plants also produce H_2O_2 as one of the ROS (Zhang *et al.*, 2022b). GSH levels grow in plants under stressful situations (Nahar *et al.*, 2017), and it is used as an adaptive mechanism for battling and tolerating stress (Gong *et al*., 2018). Catalase activity also increases in plant under fluoride stress correlating with high concentration (Sharma & Kaur, 2019) and with exposure time (Sharma *et al*., 2019).

Most studies showed the importance of the use of lichens as bioindicators of air pollution generated by heavy metals and the toxic effect generated follows the accumulation of these pollutants at the cellular level. However, few studies have conducted the toxic effect induced by fluoride on lichens, for this purpose the current review will shed some light on the fluoride-induced toxic effects in lichens and the adaptation mechanisms used by lichens to combat induced toxicity.

2. GENERAL DATA ON LICHENS

2.1. History and Definition

Lichenology is a branch of biology that is interested in the study of lichens. It is a discipline covering several aspects of these organisms: taxonomy, morphology, physiology, the algae-fungus relationship, ecology, and bio-indication (Kranner *et al.*, 2009). The term lichen is of Greek origin (hence its pronunciation likèn), it was found for the first time in the writings of THEOPHRASTE (IV century before our era) who also designates the plants growing on the trunks of trees (Ozenda & Glauzade, 1970). The use of lichens as bioindicators and bioaccumulators has been studied for several years, the first study was carried out by William Nylander (1822-1899) who published an article in 1866 (Les lichens du Jardins du Luxembourg) in which he studied the relationship between environmental pollution and the disappearance of several lichen species (Vitikainen, 2009).

Lichens have long been thought of as composite organisms made up of algae and/or cyanobacteria (phycosymbiont) hosted in a mutualistic relationship by a fungus (mycosymbiont): **(1)** Phycosymbiont (algae): it ensures the carbon supply of the whole by its assimilating pigments, **(2)** Mycosymbiont (The mushroom): thanks to the felting of its filaments, it provides support to the algae and prevents its dehydration. It represents more than 90 % of the lichen biomass. Within the lichen thalli, several algae species, yeasts, and even viruses have all been gradually found (Morillas *et al.*, 2022).

2.2. Lichen Morphology

Lichens are thallophytes, their vegetative apparatus is a thallus representing neither leaves, nor stems, nor conductive apparatus and carrying the reproductive organs, the thallus of lichens have very varied forms among which we distinguish several major morphological types (Ozenda, 2000). The fungus partner is principally responsible for determining the three main growth types of thallus structures: foliose, fruticose, or crustose. Foliose and fruticose lichens are only partially attached to the substrate through anchor-like structures like rhizines and hapters, whereas, crustose lichens, which lack the lower cortex, are entirely attached (Büdel & Scheidegger, 2008). The phytobiont and its close physical contact with the mycobiont have a significant impact on the shape of the lichenized thallus. There are five different types of lichen thalli (Figure 1).

2.2.1. *Principal type of lichen thalli*

Lichens are in the form of: **(1)** Crustose thalli: Phytobiont in a distinct layer below an upper mycrobiont cortical layer with no lower cortex, forming a crust closely adherent to the support (bark, rocks or earth), it is by far the most numerous (more than 4/5 of the lichens). The thallus is generally inseparable from the support. Example: *Lecanora*, *Pertusaria*, *Ochrolechia*; **(2)** Leprous thalli: Groups of phycobiont surrounded by mycobiont, result from a coherent association of granules (0.1-0.2 mm) each consisting of a bunch of hyphae associated with a few algal cells. Example: *Leparia*. **(3)** Foliose thalli: Phycobiont in a layer under an upper cortex with a discrete cortex underneath, in the form of lobes easily separable from the substrate to which they are sometimes attached by rhizines. Example: *Parmelia*, *Physia*, *Xanthoria*; **(4)** Filamentous thalli: Phycobiont surrounded by a sheath of mycobiont possessing the appearance of pads, they are in the form of very fine filaments, tangled and spread on the support. Example: *Usnea sp.*; **(5)** Fruticose thalli: Shrub; erect, vertical or dragging; radial structure, often attached to the base, with the phycobiont in a layer inside the outer cortex, either tufts hanging from the trunk or branches of trees, or tufts of stems. Example: *Usnea*, and *Ramalina* (Elkhateeb *et al.*, 2022).

2.2.2. *Other thalli*

Lichen thalli can be presented in three other forms (Figure 2): **(1)** Gelatinous thalli: These are the ones that contain cyanobacteria. The thallus is in the form of a simple structure with little differentiation. Example: *Collema*, *Ephebe*, *Lichina*, *Placodium*, *Leptogium*; **(2)** Squamulose thalli: They are intermediate thalli between crustose and foliose thalli, are found in the form of more or less close together or overlapping scales, on board not adhering to the support. Example: *Psoradecipien*, *Hypocenomycescalaris*, *Normaninapulchella*; **(3)** Composite thalli: The composite thallus is a component of two thalli: the first is crustose, squamulose thallus or foliose, more or less spread out on the substrate. The second of a fructicose nature, growing perpendicular to the substrate. Example: *Cladonia* (Büdel & Scheidegger, 1995).

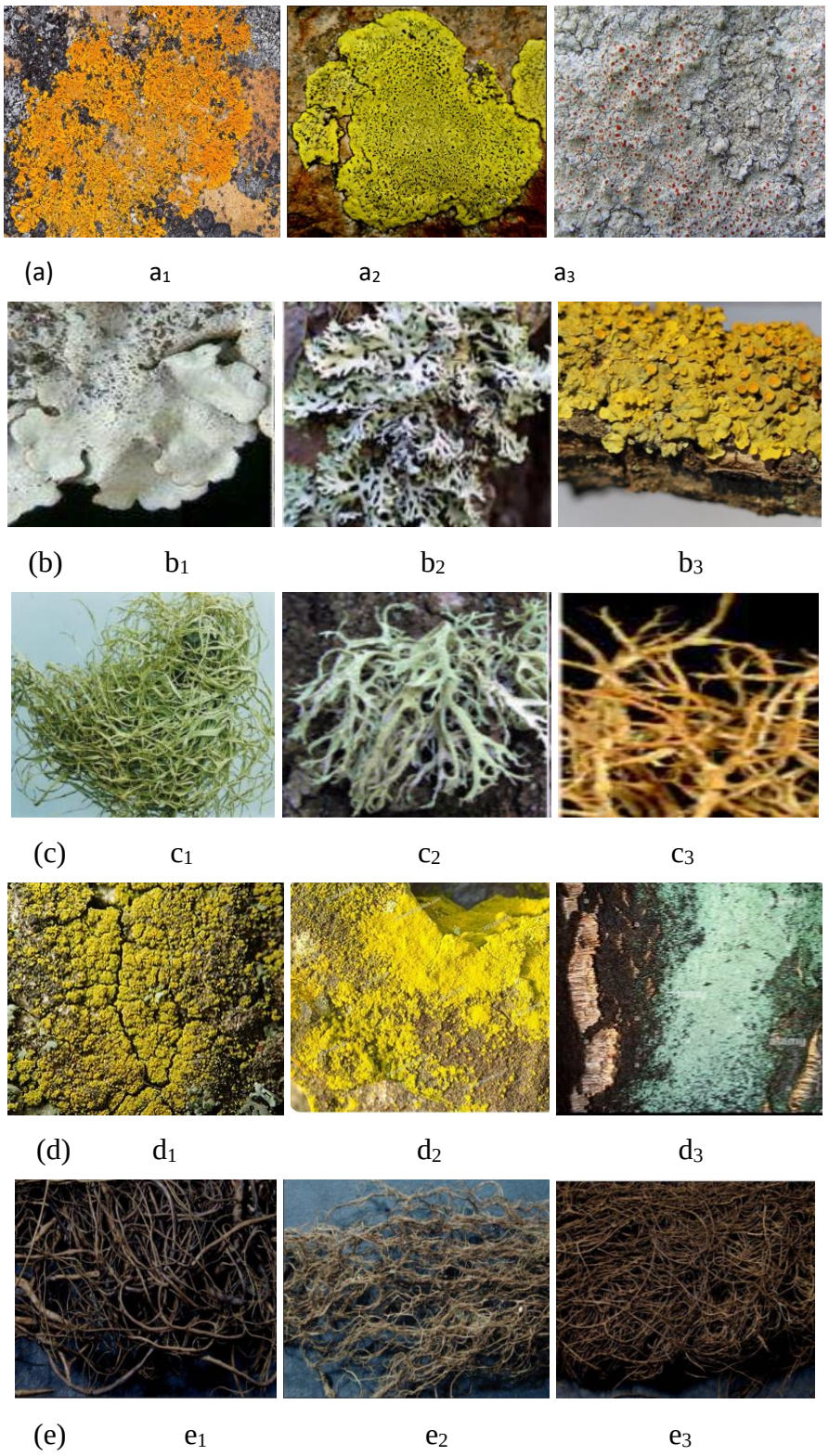

Figure 1. Growth-forms and general appearance of principal type of lichen thalli; (a): Crustose lichens (a_1 : *Lecanora* sp., a_2 : *Rhizocarpon geographicum*, a_3 : *Haematomma puniceum*) (b): Folisose lichens (b_1: *Parmelia*, b_2: *Hypogymnia*, b_3: *Xanthoria*), (c): Fructicose lichens (c_1: *Usnea*, c_2: *Ramalina*, c_3: *Teloschistes*), (d): Leprose lichens (d_1 : *Candelariella efflorescens*, d_2: *Chrysothryx candelaris*, d_3 : *Lepraria incana*), (e): Filamentous lichens (e_1: *Bryoria fremontii*, e_2: *Bryoria fuscescens*, e_3: *Bryoria pseudofuscescens*) (Alison, 2006; Nimis *et al*., 2017).

Figure 2. Growth-forms and general appearance of squamulose and gelatinous lichens; (a): Squamulose lichens (a_1*: Toninia auriculata*, a_2: *Psora decipiens*, a_3: *Squamarina lentigera*), (b): Gelatinous lichens (b_1: *Collema* subflaccidum, b_2: *Collema flaccidum*, b_3: *Collema nigrescens*) (Nimis *et al.*, 2017).

2.3. Lichen Ecology

Lichens can develop on a variety of substrates and in a variety of weather situations: **(1)** Corticolous lichens are those that grow on tree trunk and bark; **(2)** Ramicolous lichens live on twigs; **(3)** Lignicolous lichens live on wood; **(4)** Saxicolous lichens live on rocks and boulders; **(5)** Muscicolous lichens live on moss; **(6)** Terricolous lichens live on soil; **(7)** Foliicolous lichens live on evergreen leaves (epiphyllous). Any lichen that grows on another plant is generally referred to as epiphytic (Singh *et al.*, 2016).

2.4. Lichen Reproduction

Lichens frequently combine sexual and vegetative reproduction. Vegetative means can simply involve thallus fragmentation or specialized organs like soredia or isidia. The fungal partner is often the sole one involved in sexual reproduction, and the variety of fruiting bodies or ascomata (sing. ascoma) is astounding. Not only that, but they also differ considerably among species and higher taxonomic levels like genus and family in terms of internal architecture such as tissue arrangement, spore sacs (known as asci), and the spores themselves (Perlmutter, 2009).

2.4.1. *Vegetative reproduction*

The lichen complex can spread globally, either in the form of thallus fragments, or through the set of special organs, soralia and isidia (Figure 3). A soralia is a crack in the cortex (formed by the fungus) at the level of the medulla of the lichen which resolves into a floury dust of soredia which are glomeruli formed of a few cells of algae surrounded by a bunch of hyphae of very small size and easy dispersal. While the isidia are small buds carried on the surface of the thalli, these isidia, which are heavier than the soralia, cannot be transported as far, they rather ensure colonization of the substrate, they are generally considered as organs of multiplication (Ozenda, 2000).

2.4.2. *Sexual reproduction*

It is carried out by the production of spores of the fungus, which, by germinating then give hyphae which capture the algae. Indeed, two sexually differentiated fungal hyphae merge and give the surface of the thallus structures in the form of buttons (apothecia), or more or less closed cups (perithecia) (Figure 3), in which specific cells (asci) will develop ascospores (generally 8 spores per ascus but the number can vary, and post-miotic mitoses make it possible to obtain in certain cases 32, 64, … ascospores, or much less) (Honegger, 1998).

a b c d

Figure 3. Organs for vegetative and sexual reproduction; a: soredia, b: isidia, c: apothecia, d: perithecia (Nimis *et al*., 2017).

2.5. Composition of Lichen Substances

Primary and secondary lichen compounds can be separated into two categories: Primary lichen compounds play structural and metabolic roles in biological processes. The majority of these are found in other plants. Lichens create a wide variety of peculiar secondary products that are not present in other plants. The functions of secondary lichen compounds are still not fully understood. They most likely function as antibiotics (acids), photosynthesis-related molecules (atranorin), light filters (parietin), or agents that speed up the transfer of carbohydrates from the photobiont to the mycobiont or have roles in degrading the mineral substrates (Podterob, 2008). Lichen metabolites act as a major factor in the metal homeostasis and pollution tolerance of lichen (Bhattacharyya *et al*., 2016).

2.5.1. *Primary metabolites*

Primary metabolites include proteins, amino acids, lipids, carbohydrates, polyols, carotenoids, vitamins, and other organic compounds vital to lichen metabolism and structure (Elix & Stocker-Wörgötter, 2008; Mitrović *et al.*, 2011).

2.5.2. *Secondary metabolites*

A wide variety of chemical groups are represented in lichen secondary metabolites (Nayaka & Haridas, 2020), such as aliphatic acids, sugar alcohols, quinines, chromones, xanthones, dibenzofurans, depsides, depsidones, depsones, terpenoids, steroids, carotenoids, and diphenyl ethers (Yousuf *et al.*, 2014). Secondary metabolites are produced by mycobionts, which then store them in the cortex or the medullary layer (Calcott *et al.*, 2018).

2.6. Uses of Lichens

2.6.1. *Medicinal uses of lichens*

In traditional medicine, several lichens such as *Usnea, Evernia, Cetraria Cladonia Ramalina Lobaria*, *Peltigera* and *Xanthoparmelia* are most frequently used by cultures across the world, particularly in temperate and arctic regions to treat wounds, skin problems, respiratory, and obstetrical difficulties. They have been used for both their carbohydrate and secondary metabolite contents (Crawford, 2015). Likewise, due to their bioactive compounds, lichens are used as antimicrobial and antioxidant agents (Elkhateeb *et al.*, 2021) also as antiviral agent (Bhattacharyya *et al.*, 2016). Currently, lichens are employed to treat a variety of human illnesses because of their anti-cancer, antigenotoxic, anti-inflammatory, analgesic and antipyretic activity (Nugraha *et al.*, 2019; Šeklić *et al.*, 2022). In addition, the lichen's metabolites are being tested for their potential as hepatoprotective, cardiovascular protective, gastrointestinal protective, antidiabetic, and probiotic agents (Nayaka & Haridas, 2020).

2.6.2. *Uses of lichens as food*

Because of its richness in polysaccharides, certain enzymes and some vitamins, some lichens serve as important source of food for animals (*Cladonia rangiferina*, *Cetraria islandica*, species of *Stereocaulon*, *Evernia*, *Parmelia* and *Lecanora*) and humans during famine (*Parmelia* species, *Lecanora esculenta, Cetraria islandica, Lecanora esculenta, Umbilicaria esculenta*) (Elkhateeb *et al.*, 2022).

2.6.3. *Uses of lichens for mineral production*

Lichens living on rocks can pull oxalate and turn it into oxalic acid, particularly calcium oxalate monohydrate and calcium oxalate dihydrate (Marques *et al.*, 2016).

2.6.4. *Uses of lichens as bioindicators of air pollution*

The possibility that lichens are sensitive to air pollution was suggested as early as 1866 from observations made around Paris, France. Since then, several studies have been carried out in large urban areas on several continents, whose gaseous substances most involved in the decline of lichens appear to be sulphur dioxide which mainly affects the algal component by disrupting the process of photosynthesis and various fluorine compounds (Weaver, 1975). Because they are sensitive to changes in temperature, air pollution, and water availability, lichens are excellent markers of planetary change (Bajpai *et al.*, 2018). Their biodiversity is substantially impacted by even minor environmental changes (Eldridge & Delgado-Baquerizo, 2018). According to the researchers, urbanization and environmental pollution appear to have an impact on every functional aspect of lichens (Koch *et al.*, 2019).

They are used in the bio indication of air quality (Kuldeep & Prodyut, 2015; Pescott *et al.*, 2015; Sulaiman *et al.*, 2018; Benítez *et al.*, 2019; Mohamed *et al.*, 2020; Quijano-Abril *et al.*, 2021). Lichens are capable of absorbing large amounts of trace elements from the atmosphere (Caggiano *et al.*, 2015; Darnajoux *et al.*, 2015). Additionally, they serve as biomonitors (Kularatne & de Freitas, 2013; Ite *et al.*, 2014; Conti & Tudino, 2016; Demková *et al.*, 2017; Abas, 2021; Tarawneh *et al.*, 2021) and bioaccumulators of heavy metals (Węgrzyn *et al.*, 2016; Winkler *et al.*, 2019; Rola, 2020; Vannini *et al.*, 2021). They can develop on challenging supports, like coastal rocks (Dévéhat *et al.*, 2014), and are distinct from the majority of other eukaryotic species in terms of their physiology, anatomy, and capacity to tolerate extreme stresses (Expósito *et al.*, 2022). Despite their distribution and diversity are influenced by climate, soil chemistry and geography (Škvorová *et al.*, 2022), lichens can resist various stressful conditions such as extreme drought and temperatures (Beckett *et al.*, 2021), salinity (Chowaniec & Rola, 2022), heat (Kraft *et al.*, 2022), nutrient deficiency (Hauck *et al.*, 2009) heavy metals (Rola, 2020) and fluoride (Roberts & Thompson, 2011). These stresses are the source of ROS and the most important adaptation mechanism used by lichens for tolerance to stressful conditions is the scavenging of these ROS (Kranner *et al.*, 2009). Lichens can also exhibit typical stress-tolerant characteristics, such as reduced growth rates, significant longevity, low nutrient requirements, the presence of specific morphological and physiological adaptation, and changes in ecological behavior for surviving in the most hostile environments in the world (Armstrong, 2017).

3. GENERAL DATA Fluorine

3.1. History and General Properties of Fluorine

After the discovery of sodium and potassium by the English chemist Humphrey Davy (1778-1829), André-Marie Ampère (1775-1836) grasped the idea that chlorine and fluorine were both chemical elements but did not publish his hypotheses. He pointed to the analogies between hydrochloric acid and hydrofluoric acid and concluded that an element first called oxy-fluoric and then fluorine must exist. On 1st November 1810, André-Marie Ampère even suggested the possibility of isolating the element fluorine by electrolysis of anhydrous hydrofluoric acid. In 1813, he announced that a new element had been discovered (Moissan, 1886).

Fluorine, the most electro-negative element of the halogen group, its electronegativity in the Panling scale is 4 against 3.5 for O_2 and 3 for Chlorine. Fluorine is a chemical element with the atomic number 9 and is represented by the symbol F. At ordinary temperature and pressure, fluorine gas is an elemental form of the element fluorine. F_2 is the formula for fluorine gas. Fluorine gas doesn't exist freely in nature due to its high reactivity. Fluorine combines directly with all elements other than oxygen and nitrogen, reacts vigorously with most oxidizable substances and organic compounds, which explains its toxicity (Chappuis, 1991).

3.2. Sources of Fluoride

Parent rock is the main natural source of inorganic fluorides in the soil. Fluoride is naturally released into the environment by mineral weathering, volcanic emissions, and marine aerosols (Fuge, 2018). Different industrial uses exist for inorganic fluorine compounds. Fluoride is a chemical that is used in the manufacturing of aluminum, as a flux in the production of steel and glass fiber, and as a waste product that is released into the air, water, and land. Additionally, it may be discharged into the environment during manufacturing phosphatic fertilizers, bricks, tiles, and ceramics (Bonvicini *et al.*, 2006; Walna *et al.*, 2007).

3.3. Fluorine and Main Derivatives

3.3.1. *Gaseous mineral compounds*

Gaseous forms of fluorine include: **(1)** Fluorine: a yellow-green gas at ordinary temperature, (fluorine 18) prepared in nuclear reactors and used in animal experiments (Chappuis, 1991); **(2)** Hydrogen fluoride: also called hydrofluoric acid, it is the fluorinated compound whose industrial production is the most important, it is a strong corrosive chemical that can cause intense irritation, severe burns and necrosis (eye, lung, digestive tract) (Kim and Su, 1999); **(3)** Silicilated fluorine derivatives: also called silicon tetrafluoride (SiF4): is a very toxic gas, industrial pollutant of many industries (coal combustion, aluminum industry, superphosphate plant, brickworks, tile works, glassworks...), SiF_4 is also observed in terrestrial volcanic gases and is predicted to be the major F-bearing species in low-temperature (Schaefer & Fegley, 2005).

3.3.2. *Fluorides*

The main forms of fluorides are: **(1)** Alkaline fluorides such as calcium fluoride (CaF_2), strontium fluoride (SrF_2), Barium fluoride (BaF), magnesium fluoride (MgF_2), lithium fluoride (LiF) and sodium fluoride (NaF) (Song and Williams, 1993). Except LiF, all alkaline fluorides are soluble in water. The most common alkaline fluoride is NaF responsible for most accidental or voluntary poisoning by the fluoride ion (confusion with edible compounds, ingestion of insecticides, fungicides ...). In addition, NaF has a detrimental effect on immunity and humoral immunity by causing the reduction of the population of T and B lymphocytes (Kuang *et al.*, 2016); **(2)** Main mineral forms: such as fluorspar (CaF_2), Cryolite (3NaF, AlF_3) and Fluor-apatite [$3Ca_3$ (PO_4) $_2$, CaF_2] constituting phosphate rocks and frequently responsible for endemic fluorosis (Choudhary *et al.*, 2019).

3.3.3. *Organic fluorine compounds*

In recent decades, scientists have become interested in organic fluorine compounds. This is owing in significant part to their ubiquitous use in medicines, agrochemicals, and functional materials (Li *et al.*, 2018) like: **(1)** Chlorofluorocarbones (CFCs), commonly referred to as Freon, generally non-toxic. These are gases or more rarely liquids used as refrigerants, solvents and, until recent years, as aerosol propellants (Mishra *et al.*, 2022); **(2)** Fluorocarbones: characterized by a high gas-dissolving capacity (oxygen, carbon dioxide, inert gases), low viscosity, and chemical and biologic inertness. The reason why they are used therapeutically as oxygen transporters instead of hemoglobin (van Hulst *et al.*, 2008); **(3)** Fluorinated anesthetics: widely used as an anesthetic agent, these are enflurane, isoflurane, sevoflurane, methoxyflurane, and fluorinated hydrocarbons such as halothane (Chen *et al.*, 2015).

3.3.4. *Natural organic compounds*

They are rare, including fluorooleic acid and fluoroacetic acid found in some plants. After ingestion, fluoroacetate is rapidly converted to the more toxic fluorocitrate (Talcott, 2018), while sodium fluoroacetate, very toxic, is used as a rat poison (Goncharov *et al.*, 2020).

3.4. Use of Fluorine

Fluorine is a trace element since it represents only 0.0037 % of the human body, to do this, it is beneficial for human health in low concentrations, but is toxic in excess (Fordyce, 2011).

3.4.1. *Caries and periodontal disease*

The beneficial effect of fluoride derivatives in the prevention of tooth decay has been known since 1930, fluoridation of water or salt prevents tooth decay at the communal level as well as on an individual level through use at home and professional application (Pollick, 2018). Low levels of NaF exhibit a pharmacological role in the inflammatory response against the development and progression of periodontal disease (Kim *et al.*, 2019).

3.4.2. *Therapeutic use of fluorine*

Fluorouracil is used to treat actinic and solar keratosis, as well as carcinomas of the head and neck, colon, rectum, breast, stomach, bladder, and pancreas (Vardanyan & Hruby, 2006). Fluorine has a significant impact on the hydrophobic contact between the drug molecule and the receptor because of its ability to make the molecule more lipophilic, for this reason the fluorine atom is used in the production of several therapeutically helpful medications combating numerous life-threatening disorders (Gupta, 2019).

Fluorinated molecules are widely used in bioengineering and nanotechnology due to its properties in forming C-F bonds, which can adapt membrane permeability and improve the pharmacokinetic properties of drugs (Zhang *et al.*, 2022a). The most common example of fluorination is that of fluorination of steroids, this one has been very remarkable especially in the anti-inflammatory field, this is the case of 9α- and 6α- fluorosteroids used in the treatment of rheumatoid arthritis and fluticasone propionate used as an anti-inflammatory in allergic rhinitis and asthma (Filler & Saha, 2009; Yamazaki *et al.*, 2009).

3.4.3. *Prevention of osteoporosis and otosclerosis*

Once bone has been destroyed, fluoride offers the greatest potential as an osteoporosis treatment, essentially common postmenopausal osteoporosis (Pak *et al.*, 2009). In 1961, Rich and Ensinck employed sodium fluoride for the first time to treat osteoporosis (Kleerekoper, 1998). According to low-quality study, patients with otosclerosis may benefit from sodium fluoride to retain hearing and lessen vestibular symptoms (Cruise *et al*., 2010).

3.5. Toxicokinetic of Fluorine

Fluorine absorption can be done by: **(1)** Inhalation exposure: specific for hydrogen fluoride or mixtures of hydrogen fluoride and fluoride, this type of exposure is one of the industrial accidents that causes serious damage (Lee and Jeong, 2021); **(2)** Oral exposure: the gastrointestinal system easily absorbs soluble fluoride compounds such as sodium fluoride, hydrogen fluoride, and fluorosilic acid (Whitford, 1996), about 80 to 90 % of fluoride ingested through this route by passive diffusion (Zohoori & Duckworth, 2017); **(3)** Dermal exposure: the rapid entry of fluoride ions into the epidermis by hydrofluoric acid results in soft tissue necrosis, decalcification, and bone deterioration (Dennerlein *et al.*, 2016). Accidental cutaneous exposure to anhydrous hydrogen fluoride has been associated with systemic fluoride poisoning. After ingestion, fluorides pledge the plasma with maximum absorption within 60 minutes of exposure initiation (Buzalaf & Whitford, 2011). Once in the blood plasma, fluoride is distributed, to soft and mineralized tissues and reversibly incorporated mainly in bone, being released back to plasma during bone remodeling (Ekstrand, 1996; Whitford, 1996).

About 60 % of fluoride that has been ingested by healthy adults is eliminated in urine via the kidneys, compared to about 45 % for children (Villa *et al.*, 2010; Buzalaf & Whitford, 2011). Most of the fluoride not absorbed from the stomach is absorbed from the small intestine and eliminated in feces (Buzalaf & Whitford, 2011). Fluoride is also excreted in sweat (Fawell *et al.*, 2006), and saliva (Ingram *et al.*, 2005).

3.6. Toxic Effects of Fluorine

Due to the excessive intake of fluoride through drinking water, fluoride toxicity adversely affects physiological and biochemical parameters in either agricultural crops, plants, animals, or human consumption (Tak, 2018).

3.6.1. *Toxic effects on the environment*

The use of chlorofluorocarbones (CFCs) as aerosol propellants expected to decrease and even disappear due to the action of freon components on atmospheric ozone (Mishra *et al.*, 2022). Fluorinated gases (F-gases) used as refrigerants, blowing agents and electrical insulators are powerful greenhouse gases and, therefore, their release into the environment creates a significant contribution to global warming (Sheldon & Crimmin, 2022).

3.6.2. *Human toxicity*

Fluoride has recently been grouped with hazardous metals (lead, methylmercury, arsenic) and polychlorinated biphenyls in epidemiological research, because of their adverse effects obtained in animal experiments, in particular on developmental toxicity, and the molecular mechanisms by which it can cause effects. Given the extensive fluoridation of drinking water and the ubiquitous use of fluoride in dental hygiene products like toothpaste, if this evaluation is accurate, it would be quite pertinent (Guth *et al.*, 2020). Acute Toxicity may be caused by accidental ingestion or, for suicidal purposes, of sodium fluoride, fluorosilicate, hydrofluoric acid or fluorosilic acid solutions, the symptoms of which are vomiting, diarrhea, salivation, respiratory arrest, cardiac depression, convulsions, and leading coma (Norman & Arden, 1991).

Dental fluorosis is among the most well-researched long-term effects of fluoride in people (EFSA, 2013). Indeed, excessive incorporation of fluoride into tooth enamel before teeth eruption leads to hypomineralization of developing teeth. This problem is found when fluoride intake exceeds a concentration in drinking water of approximately 1 mg/L of fluoride, under conditions where drinking water is the only relevant source of fluoride. Another example of the long-term effects of fluoride is skeletal fluorosis (osteosclerosis and other bone abnormalities) caused by persistent fluoride toxicity, which results in excruciating pain and weakness, due to exposure to the environment or industry (Choubisa, 2021; Mohideen *et al.*, 2022).

Higher doses of fluoride (> 10 mg/L) can be correlated with debilitating fluorosis and carcinogenic risk. Confined studies suggest that fluoride may speed up the development of cells that would eventually turn cancerous, but this claim is debatable because there is no conclusive evidence linking fluoride to the influence of carcinogenicity (Bajpai, 2013; Ali *et al.*, 2019). It should be mentioned that fluoride ions exhibit a number of genotoxic characteristics and may possess mutagenic effects when in prolonged contact with biological components, but there are only a few studies discussing the ability of fluoride ion to increase the level of genotoxic effects. The danger of genotoxicity associated with human exposure with fluoride compounds is still an open question (Ribeiro *et al.*, 2017; Mikheeva *et al.*, 2020)

3.6.3. *Fluoride phytotoxicity*

One of the most phytotoxic chemical elements for plants, fluoride is present in many environmental matrices and can be harmful to them even in small amounts (Banerjee & Roychoudhury, 2019). Fluoride can cause slower plant growth and browning of their leaves, generally poisoning begins with chloroses, followed by the destruction of tissues, color change and finally the appearance of necrosis. The most remarkable toxic effect of fluoride on plants is leaf damage (Figure 4); indeed, plant leaves are the site of fluorine accumulation which causes stomata closure and inhibition of photosynthesis, hence the appearance of necrosis. Fluorides also induce degradation of photosynthetic pigments, thereby inhibiting photosynthesis, interfere with active metabolism, decrease the rate of cell division and expansion which decreases the germination and development of seed (Ram *et al.*, 2014; Sharma and Kaur, 2018; Choudhary *et al.*, 2019; Pelc *et al.*, 2020; Sahariya *et al.*, 2021), length of root, length of shoot, plant height, number of leaves, size of leaf, number of flowers per plant and fruit-set percentage (Singh *et al.*, 2013; Sodani *et al.*, 2021). Fluoride toxicity also has an adverse effect on development, mineral nutrition, respiration, reproduction, and the activity of cellular enzymes (Sahariya *et al.*, 2021).

Figure 4. Leaf spots due to fluorine toxicity (Chatterjee *et al.*, 2020).

3.6.4. *Toxic effects of fluoride on lichens*

Fluorinated pollution can cause an almost total disappearance of some lichen species. A precise cartographic study makes it possible to establish the existence of a lichen desert in the immediate vicinity of the factories. As one moves away from it, the lichen flora gradually enriches (Perkins, 1992). Numerous studies have shown a relationship between lichen damage and fluorine accumulation. This is because lichens are able to quickly accumulate large amounts of fluoride. This accumulation varies according to the lichen species, the time and the distance from the source of pollution (Deruelle & Lallement, 1983), climatic factors (Conti & Cecchetti, 2001), and fluoride concentration (Chakrabarti *et al.*, 2014).

As a result of the stressful environment, lichens suffered numerous biochemical and physiological alterations. The most prevalent metric used to study the toxicity of air pollutants on lichens is chlorophyll degradation (Šujetovienė, 2015). Fluoride has the greatest impact on photosynthesis and respiration (Sharma & Kaur, 2018). Significant decrease in chlorophyll a, chlorophyll b and total chlorophyll contents in *Xanthoria parietina* under fluoride stress was noted by Benhamada *et al.* (2023b), however significant increase of chlorophyll a/b ratio was obtained correlating with exposure time to NaF (r = 0.818, p < 0.001), indicating that chlorophyll b is more affected than chlorophyll a in *X. parietina*.

In *X. parietina* under fluoride-induced stress, the color change of the pinkish gray thalli suggests that it is accompanied by a destruction of lichenic acids, this is due to the destruction of chlorophylls a and b, and transformation into phaeophytin, before the complete degradation of all pigments including carotene and xanthophyll (Deruelle & Lallement 1983; Zhang *et al.*, 2016; Yang *et al.*, 2019). Fluoride accumulation would also create favorable conditions for exosmosis causing permanent plasmolysis of lichen algal cells. This phenomenon of plasmolysis is explained by the inhibition of the metabolism of the cell wall compounds, there will be a slight dehydration resulting in the folding of the lichen upper cortex, including the necrosis formation (Figure 5). According to Nash III (1971) and Gilbert (1973), this necrosis is due to the destruction of lichenic acids.

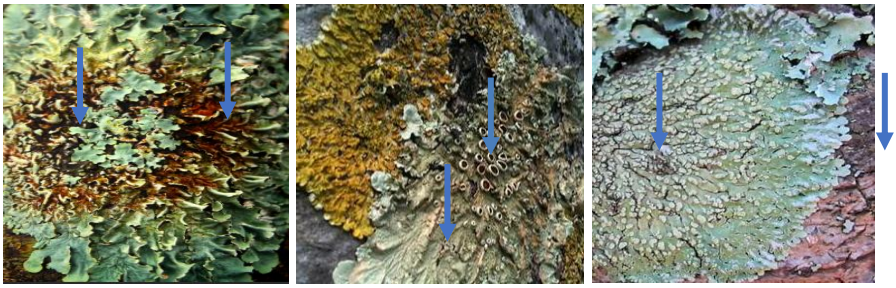

Figure 5. Fluoride injury symptoms (necrosis) in epiphytic lichens (LeBlanc et *al.*, 2011).

Fluoride also decreases germination of spore. Semadi (1989) studies have shown that low concentrations of NaF inhibit spore germination in *Physonia pulverulacea* lichen. Plants boost the activity of antioxidant enzymes such as catalase in order to trap ROS and detoxify their effects (Lei *et al.*, 2022). Several studies have shown that catalase expression increases significantly correlating with increasing concentrations (Mondal, 2017; Elloumi *et al.*, 2017;

Sharma & Kaur, 2019; Benhamada *et al*., 2023b) and with increasing exposure time to sodium fluoride (NaF) (Sharma *et al*., 2019).

Several studies have found that lichens employ glutathione for detoxification; GSH increases in plants under stressful situations and is part of plant adaptation methods to battle and survive stress (Sanità di Toppi *et al*., 2008; Nahar *et al*., 2017; Gong *et al*., 2018). Results obtained by Benhamada *et al.* (2023b) showed that the GSH content increases correlating with increasing concentrations of NaF in *X. parietina*. During lipid peroxidation, a wide range of aldehydes are produced, including MDA. MDA is a promising biomarker and diagnostic for lipid oxidative damage in drought-stressed plants (Amine-Khodja *et al*., 2022; Toto *et al*., 2022). Compared to the control test, results obtained by Benhamada *et al.* (2023a) showed a significant increase in MDA contents ($p < 0.05^{*}$) in exposed *X. parietina* to NaF solutions. Similar results were archived by Dzubaj *et al.* (2008) and Pisani *et al.* (2009) who showed that *X. parietina* reacts against fluorine by increasing MDA content.

Proline is part of a broader adaptive response to adversity (Liang *et al*., 2013; Ghosh *et al*., 2022). Proline accumulation might be thought of as a stress indicator that changes depending on the plant type (Amine-Khodja *et al.*, 2022). According to Benhamada *et al*. (2023a), a significant increase in proline content in *X. parietina* was noted correlating with increasing exposure time to NaF concentrations ($r = 0.783$, $p = 0.00032$). Soluble sugars and polyphenols like proline, are part of plant adaptation strategies to battle and tolerate stressful situations (Gangola & Ramadoss, 2018; Khan *et al.*, 2020; Amine-Khodja *et al.*, 2022). Results obtained by Benhamada *et al.* (2022) showed significant increase in polyphenol contents correlating with increasing concentration of NaF in *X. parietina* thalli. Significant increase in the content of soluble sugars correlating with increasing concentration of NaF ($r = 0.678$, $p = 0.0010$) was also noted in *X. parietina* under NaF stress (Benhamada *et al*., 2023a).

4. GENERAL DATA ON LEAD

4.1. Definition and History

Lead (Pb) from the “Latin plumbum” with atomic number 82 and atomic mass of 208, is a blue-gray non-radioactive metal that turns gray once exposed to air, with a melting point of 327.5 °C and a boiling point at atmospheric pressure of 1740 °C. It is the most universally widespread element of heavy metals and the second most toxic heavy metal after arsenic. Lead and its oxides typically create covalent bonds when reacting with acids and bases (Boldyrev, 2018). It has been commonly used for nearly 6000 years in human activities (Papanikolaou *et al.*, 2005), and since at least 200 B.C. humans have been aware of its harmful effects, indeed Asia Minor saw the earliest known usage of lead between 6000 and 4000 B.C. (Hernberg, 2000). Meanwhile and with the huge development of population and economic growth, lead pollution has increased from an estimated 10 tons to 1 000 000 tons per year, with a global production about ten million tonnes in 2014 (Boldyrev, 2018).

4.2. Origin of Lead

Lead occurs naturally in soil, water, plants and animals (Tiwari *et al.*, 2013). It is found naturally in the earth's crust at concentration of 15–20 mg/kg. In general, lead concentrations are around 5 to 25 mg/kg in soil, 1 to 60 µg in surface water, and around 1µg/m^3 in air (Lu, 1992). Lead can be emitted naturally either by erosion that carries it to the soil, surface water, and sediment, or by terrestrial or underwater volcanic eruptions (Popescu *et al.*, 1998). Industrial lead pollution originates from several industrial processes, especially from mining and smelting, plastics, printing and phosphate industries, manufacturing batteries, metallurgy, and lead refining complexes (Kapusta & Sobczyk, 2015; Kabir *et al.*, 2020) as well as car traffic and leaded aviation gasoline (Lin *et al.*, 2011).

4.3. Lead, Lead Compounds and Uses

(1) Metallic lead (Pb): lead is primarily used to create rechargeable storage batteries. Pb makes up the gray negative electrode in battery (Crompton, 2000); **(2)** Lead oxides: such as α-PbO (litharge) and β-PbO (cutter) used in the industries of glassware, enamels, drying oils of accumulators, α-PbO_2 (scrutinyite) and β-PbO_2 (plattnerite) used as energetic oxidants and make up the red color positive electrode in battery (Crompton, 2000), and Pb_3O_4 (leas tetraoxide or minium) used in the composition of anti-rust paints (Pavlov, 2017); **(3)** Lead sulphides (PbS): formed by chemical reaction of lead acetate with thiourea at room temperature. It provides varnishes of yellow color, which can be colored brown by addition of manganese oxide or green by addition of copper oxide (Bhatt *et al.*, 2011); **(4)** Lead arsenate $PbAH_sO_4$ and lead stearate $Pb(C_{17}H_{35}COO)_2$: used in insecticides and herbicides (Gad and Pham, 2014); **(5)** Lead iodide PbI_2 and lead base acetate $Pb(CH_3\text{-}COO)_2$: used as a mordant in printing and dyeing textiles, as a lead coating for metals, as a dryer in paints, varnishes and pigment inks, and as a dye in hair dyes (Ghazi & Millette, 1964); **(6)** Alkyl derivatives of lead and organic lead compounds: such as tetraethyl lead and tetramethyl lead used as antiknock agents in gasoline (Filella & Bonet, 2017); **(7)** Lead nitrate $Pb(NO_3)_2$: used in the manufacture of matches and explosives, as a heat stabilizer in nylon, and as a coating on paper for photothermography (Nielsen, 2013); **(8)** Insoluble lead compounds: such us lead carbonate $Pb(CO_3)_2$ and lead sulfate ($PbSO_4$) used in paints synthesis (Nielsen, 2013), lead chromate ($PbCrO_4$) used as a yellow pigment in paints, rubber, plastics, and ceramic coatings (Gad and Pham, 2014), lead fluoride (Pb_3F_8) used as a pigment in ancient Rome and in anti-corrosion coatings (Bose *et al.*, 1983) with additional uses in the electronic and optical industries, lead naphthenate used as catalysts and as a varnish drier, lead phosphate and lead stearate used as stabilizers in the plastics industry and lead sulfate with zinc in galvanic batteries (Nielsen, 2013).

4.4. Toxicokinetic of Lead

We distinguish respiratory absorption specific to small Pb inorganic lead particles and aerosols that can be almost completely absorbed, while larger particles can be moved by mucociliary clearance to the oropharynx and swallowed (James *et al.*, 1994); gastrointestinal absorption influenced by age (40–50 % for children compared to 3–10 % for adults), diet, nutrition and physiological characteristics of Pb in the ingested medium (Ziegler *et al.*, 1978) and dermal absorption specific for inorganic forms of lead but less frequent than inhalation and oral exposure.

Ingested lead undergoes the action of hydrochloric acid from gastric juice and that of bile secretions, which by determining partial solubilization and, consequently, increasing toxicity; absorption is maximum in the small intestine and duodenum. Once in the blood, lead mainly attaches to red blood cells before being distributed to the bones (Barry,1981), lead can also be transferred from the mother to the fetus and also from the mother to infants via maternal milk (Papanikolaou *et al.*, 2005). Inorganic forms of lead are not metabolized, so they form complexes with a variety of proteins and non-protein ligands, while organic compounds are actively metabolized in the liver by oxidative dealkylation by cytochrome P-450 enzymes (Philip & Gerson, 1994). About 50 - 60 % of eliminated lead is carried out mainly through the urinary and biliary excretion route (Ziegler *et al.*, 1978), lead elimination can be also done through sweat, saliva, hair, nails, breast milk and seminal fluid (Barbosa *et al.*, 2006).

4.5. Lead Toxicity

4.5.1. *Human toxicity*

Lead poisoning originates from paints, water, food, dust, but in the majority of cases by oral ingestion and absorption through the gut. Acute lead-related toxicity is manifested by headaches, muscle tremors, abdominal cramps, kidney damage, loss of memory with encephalopathy (Papanikolaou *et al.*, 2005). Lead exposure has a negative impact on the hematological, renal, reproductive, neurological, digestive and respiratory systems. Lead also

causes generation of ROS that results in critical damage to various biomolecules such as DNA, enzymes, proteins and membrane-based lipids, while simultaneously altering the antioxidant defense system (Wani *et al.*, 2015). Once in the blood, lead can inhibit certain enzymes responsible for heme synthesis, particularly δ-aminolevulinic acid dehydratase (Sakai, 2000).

4.5.2. *Phytotoxic effects of lead*

In plants, lead is mainly absorbed by the root system and in minor amounts through the leaves. Once inside the plant, lead accumulates in the roots, but part of it is transferred to the aerial parts. At the cellular level, it accumulates in the wall, vacuoles and forms small deposits in the endoplasmic reticulum (Sharma & Dubey, 2005). Figure 6 shows an example of morphological distortion observed in the leaves of *Vicia faba* plants under lead stress.

Lead toxicity causes severe oxidative damage to plants, it limits the synthesis of photosynthetic pigments, proteins, and affects net assimilation rate, sweat rate, and stomatal conductance (Arif *et al.*, 2019). Lead also reduces the rate of seed germination, and plant growth, and causes retardation of carbon metabolism, and blocks potassium in plants which causes membrane damage and stomatal closure (Zulfiqar *et al.*, 2019). Lead stress also inhibits the activities of several enzymes especially that of the Calvin cycle (Sharma & Dubey, 2005), reduces gas exchange attributes, and induces the oxidative stress markers and activities of antioxidant enzymes (Bamagoos *et al.*, 2022).

Figure 6. *Vicia faba* leaves grown on Hoagland nutrient solution under Pb stress, 0.0 mM (left) and 48 mM (right) (Kamel, 2008).

4.5.3. *Toxic effects on lichens*

Foliose lichens are commonly utilized as biomonitors of metal contamination in the environment due to their proclivity to accumulate high quantities of dangerous metals (Subhashini & Suganthi, 2014; Purvis, 2014; Caggiano *et al.*, 2015; Darnajoux *et al.*, 2015). Lichens, according to Abas and Awang (2017), are good biological indicators for biomonitoring. *X. parietina* can accumulate substantial levels of heavy metals when compared to other fruticulous lichens such as *Ramalina farinacea* (Belguidoum *et al.*, 2021). Using a natural sensor for the sampling of biological material, the lichen *X. parietina* was capable of accumulating four heavy metals (Cr, Zn, Cu and Ni) in the Setif region, North-East of Algeria (Douibi *et al.*, 2015). Transplantation of lichen thalli of *Evernia prunastri* and *Ramalina farinacea* from an unpolluted site to a polluted site showed significant accumulation of lead after 3 months of exposure time (Šujetovienė & Česynaitė, 2021). Additionally, Benhamada *et al.* (2023c) verified that lead accumulation in *X. parietina* thalli is correlated with longer exposure times and higher lead nitrate concentrations $Pb(NO_3)_2$. Lead has been demonstrated to infiltrate cells, particularly in fungal cortical compartments, even though lichens still have significant extracellular quantities of the metal, primarily coupled to cell walls and extracellular polymers, once inside the cell, the first important sign of its toxicity is the reduction of photosystem II (PSII) photochemical reactions, and consequently the reduction of integrity and chlorophyll content (Branquinho *et al.*, 1997). Mechanisms of tolerance to lead in lichens are shown in Figure 7.

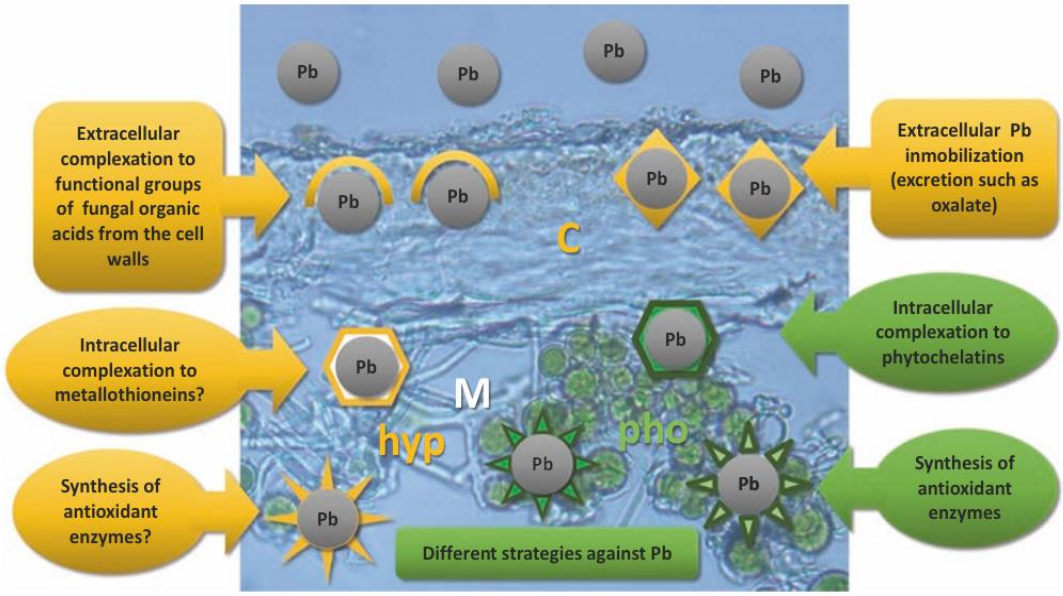

Figure 7. Mechanisms of tolerance to lead in lichens. The mechanisms of tolerance to lead of the mycobiont are marked in yellow and those of the phycobionts in green. When there is more than one phycobiont, these may have different strategies against lead. Different microalgae are represented with different thicknesses and shade of greens. C cortex, M mycobiont, hyp hyphae, pho phycobiont (Expósito *et al.*, 2019).

Unlike fluoride which showed symptoms related to lesions in epiphytic lichens (Figure 5), the accumulation of heavy metals, with the exception of Cd, did not show significant morphological differences between stressed thalli and control (Šujetovienė et *al.*, 2019). In addition to the control test, *X. parietina* thalli were treated with $Pb(NO_3)_2$ concentrations for 24, 48, and 96 hours, and total chlorophyll content was quantified as a direct effect of lead stress, chlorophyll levels fell as lead concentrations in solution increased; the lowest levels of this pigment were found in thalli treated with high quantities of lead, showing that Pb had a detrimental impact on chlorophyll a content (Carreras & Pignata, 2007; Benhamada *et al.*, 2023c). Aboal *et al*. (2008) discovered that a decrease in chlorophyll a/b ratio is an obvious marker of degradation caused by senescence or stress. Additionally, Bajpai *et al*. (2010) discovered that heavy metals (Zn, Cd, and Cu) cause physiological changes and inhibit photosynthesis even at very low concentrations, while lead causes a decrease in total chlorophyll and Ca/b ratio.

Protein content in *X. parietina* decreased significantly with increasing $Pb(NO_3)_2$ concentrations (r = -0.790, p = 0.0002) (Benhamada *et al.*, 2023c) . Esposito *et al.* (2012) and Chetia *et al.* (2021) discovered a decrease in total protein levels to correspond with Pb, Cd, Zn, Cu, Co, Ni, and Cr in lichens growing in different contaminated locations. Catalase levels in plants exposed to lead increase considerably, according to Abu-Muriefah (2015) and Khan *et al.* (2021). Several other research have found that heavy metals promote oxidative stress in biomarkers (Taiwo *et al*., 2014; Batool *et al*., 2018; Ullah *et al*., 2021; Benhamada *et al.*, 2023c).

Lead causes membrane damage, especially through water loss and ion leakage (Expósito *et al.*, 2019), indeed, lipid peroxidation, which is one of the main consequences of prooxidant pollutants examined in thalli lichen, can cause these impairments (Álvarez *et al.*, 2015; Gurbanov & Unal, 2019). Indeed, membrane damage results in the degradation of its phospholipids which accumulate in the form of MDA residues (Zoungranan *et al.*, 2019). Benhamada *et al.* (2023a) found a positive correlation between MDA contents in *X. parietina* and increasing concentrations of $Pb(NO_3)_2$ (0.865, p = 0.000031).

Lead has the ability to stimulate the formation of ROS (Kováčik et *al.*, 2018; Wieners et *al.*, 2018, Benhamada *et al*., 2023c) that react with several vital biomolecules of the species causing serious morphological, metabolic and physiological abnormalities (Zoungranan *et al.*, 2019). To neutralize and trap free radicals produced by lead, lichens accumulate polyphenols, usnic acide, cysteine and tripeptide glutathione, proline, soluble sugars, GSH and catalase (Kováčik *et al.*, 2017; Expósito *et al.*, 2019; Gurbanov & Unal, 2019).

Plants respond to stress by storing proline and soluble sugars in order to protect the structure of their macromolecules (Alhasnawi, 2019). Proline determination in lichens is an investigative method for various stress events. Benhamada e*t al.* (2023a), showed significant increase in proline and soluble sugar contents correlating with exposure time of *X. parietina* thalli to lead nitrate (r = 0.811, p = 0.00013 and r = 0.780, p = 0.00036, respectively).

5. CONCLUSION

Based on this literature search, we were able to conclude that fluoride must be categorized as one of the most toxic air pollutants, even though lead was more toxic than fluoride. This opens the field to further research comparing the toxicity of fluorine to that of heavy metals on various other ecosystems. It will be highly beneficial to conduct more research to identify the cellular, intracellular, and molecular sites of action of lead and fluorine as well as to find out what additional compounds this lichen has produced to counteract stressful environments. Several other studies will be requested to compare the toxicity of heavy metals with that of fluoride and thus to control the use of fluoride, particularly in drinking water disinfection processes.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Ouahiba Benhamada: Investigation, resources, visualization, software, formal analysis, and writing original draft. **Nabila Benhamada:** Language revision. **Essaid Leghouchi:** Supervision and validation.

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Research Article

Protective and therapeutic effects of hesperidin in an experimental colitis model

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Abstract: Ulcerative colitis is a gastrointestinal problem with increasing prevalence. In this study, the effect of hesperidin (commonly found in citrus white peel) on colitis was investigated. For this purpose, 28 Wistar albino female rats, 3–4 months old and weighing between 250–350 g, were used in the study. Rats were randomly divided into four groups: control (n=7), colitis (n=7), protective effect (n=7), and treatment (n=7) groups. Colitis was induced in rats with acetic acid (except control), and hesperidin was then administered at 150 mg/kg for 1 week before and after colitis. At the end of the study, IFN-γ and IL-6 values in blood, TAS, TOS, and OSI in tissue were evaluated. Intestinal tissues were assessed visually by haematoxylin and eosin staining. Our results showed that the levels of IFN-γ and IL-6 were highest in the colitis group and lowest in the treatment group, with statistical significance. The most histopathological damage was seen in the colitis group, while less prevalent in the treatment and control groups. The results of the study show that hesperidin had limited protective and therapeutic effects on experimental colitis mouse models.

1. INTRODUCTION

Inflammatory bowel disease is a chronic problem that begins with intestinal inflammation, progresses with impaired immune response, and significantly affects patients' quality of life (Nazlıkul, 2020). Although the exact cause is unknown, inflammatory bowel disease is reportedly affected by the immune system, genetic factors, and changing intestinal microbiota (Galipeau *et al*., 2020). These diseases affect millions of people worldwide (Tahvillian *et al*., 2020). Attacks and remission are characteristic of ulcerative colitis, while the aetiology is unknown. Aminosalicylates, antibiotics, systemic steroids, local steroids, immunomodulators, methotrexate, cyclosporine, biological agents, and mycophenolate mofetil are used medically for ulcerative colitis. Untreated ulcerative colitis leads to hospitalisation, surgery, and an increased risk of colorectal cancer.

Hesperidin is the most abundant flavonoid glycosides found in citrus peels. Its consumption is beneficial for health and helps prevent heart, brain and eye diseases. Hesperidin has analgesic, anti-inflammatory, anti-hypercholesterolemic, and anti-hypertensive properties (Victor *et al*., 2020). Crespo *et al*., (1999), showed that 10 mg/kg and 25 mg/kg doses of hesperidin reduced

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colon damage in rats. Furthermore, others have reported that administering 10, 40, and 80 mg/kg hesperidin doses had beneficial effects on ulcerative colitis in mice (Xu *et al*., 2009). There are studies on the therapeutic effect of hesperidin on colitis; however, its protective effects are unreported. This study aimed to examine the protective effects of hesperidin in a rat model of ulcerative colitis.

2. MATERIALS and METHODS

The experimental study began after receiving the local ethics committee's approval (Approval number: 64583101/2021/140), and special attention was paid to international ethical rules (2010/63/EU). 28 female Wistar albino rats were used in the study. Rats were kept in standard transparent plastic cages, paying attention to cleanliness and hygiene conditions. They had 24-hour access to drinking water and food and were kept in a temperature-controlled room (22±2°C) under a 12-hour light/dark cycle. Rats were separated into four groups: the control (n=7), colitis (n=7), protective effect (n=7), and treatment (n=7) groups. Hesperidin (Sigma Aldrich, Cat: H5254) was dissolved in 0.5% carboxymethyl cellulose as it does not fully dissolve in water. To the protective group, 150 mg/kg hesperidin was administered via gavage to the protective effect group, 7 days before colitis occurred. For the treatment group, after inducing colitis with acetic acid, hesperidin was administered orally at 150 mg/kg for 7 days, starting from day 0. To induce colitis in rats, 4% acetic acid solution (1 mL, pH 2.3) was slowly applied to the rectum via an intrarectal catheter under anaesthesia (Oruc *et al*., 2008). After colitis, the rats were kept in a head-down position. Once the treatments were completed, the rats were euthanised by the cervical dislocation method under ketamine (50 mg/kg) and xylazine (5 mg/kg) anaesthesia. Blood and intestinal tissues were taken and stored.

2.1. Biochemical Analysis

Blood taken from the rats was centrifuged at 3500 rpm for 10 minutes, and the supernatant for measurements was transferred to tubes. Samples were placed in a -20°C freezer and stored until the day of analysis. Total antioxidant level (TAS) and total oxidant level (TOS) were measured using the Rel Assay Diagnostic test kit according to the manufacturer's instructions. The method specified by Erel (2004) was used in the analyses. The results were calculated as mmolTrolox Equiv/L and μmolH_2O_2 Equiv/L. OSI (Oxidative stress index) was calculated using TAS and TOS values. Serum IFN-γ levels were measured with a kit purchased from Bioassay Technology Laboratory (Cat: E0103Ra). IFN-γ levels are expressed as ng/mL. For serum measurement, IL-6 levels were purchased from Bostonchem (Cat: BLS-1158Ra).

2.2. Histopathological Evaluation

Tissue samples were placed in 10% formaldehyde solution for fixing. 5 μm thick sections were cut and stained with haematoxylin and eosin. The samples were then examined with a trinocular research microscope (Pereira *et al*., 2010), and microphotographs were taken (Olympus BX51, Tucsen 5MP digital camera). When evaluating colitis lesions, mucosal epithelial degeneration and necrosis, inflammation, hyperaemia, oedema, necrosis, and haemorrhage criteria were used and classified according to the severity and extent of the lesion: (-) none, (+) light, (++) medium, (+++) severe, and a semiquantitative scoring was made as (++++) very Severe.

2.3. Statistical Evaluation

Compliance with normal distribution was evaluated with the Shapiro–Wilk test. All results were compared with each other statistically, and the changes were tested to determine for significance. For this purpose, the one-way ANOVA method was applied. Tukey's HSD was used as a post hoc test for within-group significance. A value of $p<0.05$ was considered statistically significant.

3. RESULTS

3.1. Biochemical Test Results

According to biochemical analyses, serum TAS values showed a significant difference in the control, colitis, protective effect, and treatment groups. In the intergroup evaluation, a significant difference was found between the control and colitis group ($p<0.001$). There was a decrease in TAS values between the control and protective effect and treatment groups, which was not significant ($p>0.05$, Table 1). In the TOS evaluation, a significant difference was observed between the groups ($p<0.001$). For intragroup TOS evaluation, a significant difference was determined between the protective effect ($p<0.05$) and treatment ($p<0.001$) groups. The TOS value, which increased in the colitis group, decreased in the treatment and protective effect groups; a significant difference was observed only in the treatment group. As a result of the ANOVA analysis performed with the data obtained in the oxidative stress index (OSI), These values showed a significant difference between the groups ($p<0.001$). When OSI values were examined, a significant difference was seen between the control and colitis groups (Table 1). OSI values were higher in the colitis group compared to the other groups. A significant difference was determined between the colitis group and the protective ($p<0.05$) and treatment ($p<0.05$) groups.

Table 1. TAS (total antioxidant status), TOS (total oxidant status), and OSI (oxidative stress index) values in colon tissue.

	Control	Colitis	Protective effect	Treatment	*p*
TAS (mmol Trolox Eq/L)	1.22±0.16	0.85±0.16	1.05±0.19	1.11±0.19	0.007
TOS (μmol H_2O_2 Eq/L)	9.98±1.49	16.43±2.14	13.57±2.20	14.37±1.82	0.001
OSI (index)	0.83±0.16	1.98±0.47	1.32±0.32	1.34±0.39	0.001

IFN-γ levels were significantly different between the groups ($p<0.001$). When the serum IFN-γ levels of the groups were examined, a significant difference was seen between the control, protective effect ($p<0.001$), and treatment ($p<0.001$) groups. An increase in IFN-γ levels was observed in the colitis, protective effect, and treatment groups compared with control. Serum IL-6 levels showed a statistical difference between groups ($p<0.001$). When the IL-6 levels within the groups were examined, a significant difference was observed between the control and protective effect ($p<0.001$) groups. IL-6 levels were not significantly different between the colitis group and the protective effect and treatment groups ($p>0.05$, Table 2).

Table 2. Blood IFN-γ (İnterferon gama) and IL-6 (Interleukin-6) levels.

	Control	Colitis	Protective effect	Treatment	*p*
IFN-γ (ng/mL)	25.08±2.01	43.87±5.57	36.41±4.84	33.35±4.28	0.001
IL-6 (pg/mL)	3.51±0.86^{c}	5.13±0.64^{a}	4.85±0.58^{b}	4.42±0.48^{b}	0.001

$^{a, b, c}$: different letters in the same column indicate a statistically significant difference.

3.2. Histopathological Findings

In the control group, there was normal colon histology with villi containing widespread goblet cells making papillary extensions towards the lumen between the intestinal mucosa surface epithelium and crypts. There were a small number of lymphoplasmacytic cells and a few eosinophil leukocytes in the lamina propria observed in a few cases (Figure 1). In the colitis group, widespread and advanced necrotic changes were observed, starting from the lamina epithelialis, including the lamina propria and submucosa, affecting the tunica muscularis to varying degrees and sometimes extending to the serosa. In the protective effect group,

widespread necrosis involving all intestinal layers was observed; however, histopathological changes were noted in the submucosal region, characterised by severe hyperaemia in the vessels, widespread oedema, intense inflammatory cell infiltrates, and haemorrhages of varying degrees. Although the tunica muscularis thickened with a homogeneous appearance and suffered necrosis in three cases, the areas above the submucosa were generally unaffected and there were no pathological changes in the epithelial layer or crypts. In the treatment group, while deep necrosis and haemorrhages involving the entire colon mucosa layer were detected in two cases, these changes were severe and affected the whole colon surface. In other instances, pathological changes were generally limited to the submucosa, characterised by hyperaemia, oedema, and severe inflammation, and the epithelial layer was not affected in these cases (Table 3 and Figures 1 - 2).

Table 3. Semiquantitative scoring of histopathological findings.

Groups	Epithelial degeneration	Inflammation	Hyperaemia	Oedema	Necrosis	Haemorrhage
Control	-	-	-	-	-	-
Colitis	+ + +	+ + + +	+ + +	+ + +	+ + +	+ + + +
Protective effect	+	++	+	+ +	+	+ +
Treatment	+ +	+ +	++	+ +	+ +	+ +

(-) normal, (+) mild, (++) moderate, (+++) severe, and (++++) high severe inflammation.

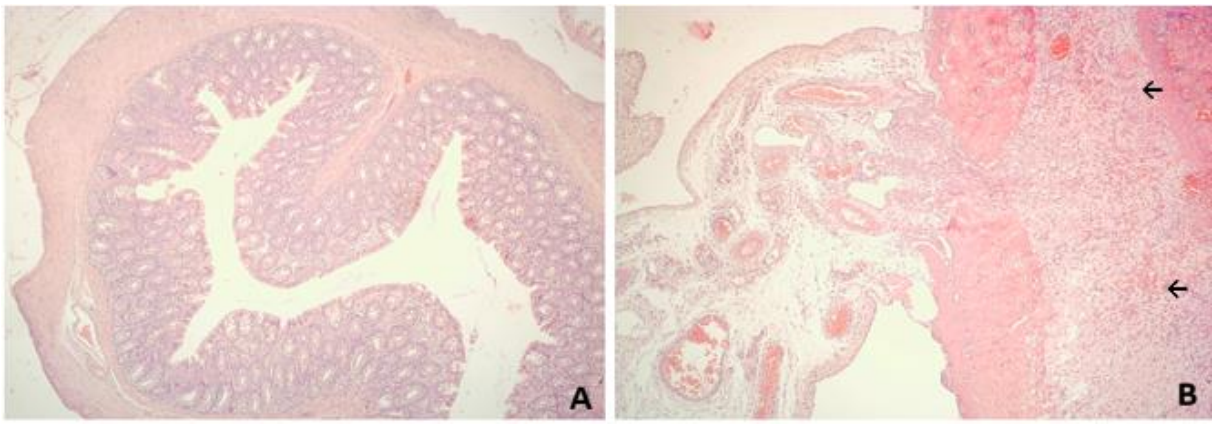

Figure 1. Histopathological images of colon tissue (H&E staining). **(A)** Control group: normal intestinal tissue appearance. **(B)** Colitis group: diffuse and advanced necrotic changes, bleeding, and inflammation in the intestinal tissue.

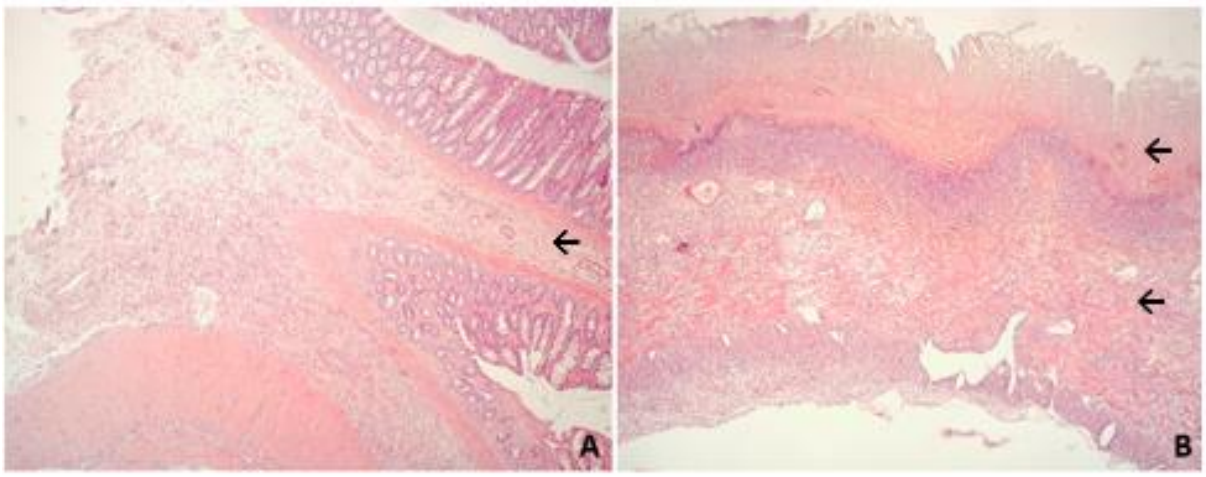

Figure 2. Tissue from hesperidin-treated rats. **(A)** Protective effect group: widespread necrosis involving all intestinal layers, pathological changes were shallow in three cases. **(B)** Treatment group: while deep necrosis and haemorrhages involving the entire colon mucosa layer were detected in two cases, pathological changes are generally limited to the submucosa in other cases characterised by hyperaemia, oedema and severe inflammation, and the epithelial layer was not affected.

4. DISCUSSION

Colitis is a highly prevalent inflammatory bowel disease. Although the exact cause is unknown, it is thought that genetic factors, the immune system, environmental factors, and changing intestinal microbiota might play a role in its aetiology. Immunological mechanisms play an active role in ulcerative colitis inflammation (Lu *et al*., 2022). Stimulation of immune cells results in the release of large amounts of cytokines and inflammatory mediators. Hesperidin is a flavonoid, is abundant in citrus fruits, and has a broad pharmacological effect (Xu *et al*., 2009). Hesperidin has anti-inflammatory, antioxidative, solid, and antimicrobial characteristics (Onal *et al*., 2022). In this study, there was a decrease in TAS values between the protective effect and treatment groups compared with the control group, and no significant difference was seen with these decreases ($p>0.05$). With TOS values, there was a significant difference between the groups ($p<0.001$). The decreases in the protective effect group were more significant than in the treatment group. The OSI values increased in the colitis group and decreased in the treatment and control groups, suggesting that hesperidin has a partial healing and protective effect on colitis by increasing the total antioxidant capacity and reducing the oxidant capacity. A study by Murata *et al*. (1995) showed increased levels of proinflammatory cytokines in ulcerative colitis. In another study by Funakoshi *et al*. (1998), they reported increased expression of IL-1β, IL-6, IL-8, and TNFα mRNA in patients with Crohn's disease and ulcerative colitis compared with the control group. In our study, there was a significant decreasing effect between the colitis group and the protective effect ($p<0.05$) and treatment ($p<0.001$) groups regarding serum IFN-γ levels. This finding shows that IFN-γ (essential in macrophage stimulation) is suppressed and has a reducing role in the severity of inflammation. There was a significant difference in IL-6 levels between the groups ($p<0.001$). In the same parameter, the protective effect and treatment groups showed statistically insignificant changes (Table 2).

In ulcerative colitis, significant pathological changes occur in the colon wall. The leading cause of this tissue damage is usually inflammation. As a result of inflammation, the immune system response is activated, and events such as the proliferation of lymphocytes, accumulation of neutrophils, and increased production of cytokines occur. In this study, normal colon histology was observed in the control group whereas in the colitis group, there was advanced colon epithelial degeneration and necrosis, inflammation, hyperaemia, oedema, necrosis, and haemorrhage (Figure 1). Lower scores were observed in the hesperidin protective effect and therapeutic groups compared with the colitis group. The lesions detected in the hesperidin

treatment group were observed to be more advanced than the protective effect group in severity and prevalence (Figure 2).

5. CONCLUSION

This experimental animal study concluded that hesperidin administered orally at 150 mg/kg/bw could partially reduce oxidative damage and tissue damage caused by colitis by exhibiting antioxidant and anti-inflammatory activity.

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Declaration of Conflicting Interests and Ethics

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Authorship Contribution Statement

Esra Güzel: Experimental study. **Serdar Aktaş:** Experimental study and experimental study. **Serdal Ogut:** Project manager.

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Research Article

Dyeing of banana-silk union fabrics with cochineal using different concentrations of bio-mordant

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Abstract: Banana (*Musa* × *paradisiaca* L.) is a lignocellulosic natural fiber that can be processed to produce eco-friendly and biodegradable hybrid fabrics when combined with other fibers. Natural-dyed banana fiber can be utilized in the production of sustainable materials for wearable products, household linens, technical textiles, and apparel. In this study, banana-silk union fabrics, pre-treated with bio-mordant at concentrations of 1%, 2%, 3%, 4%, and 5%, were dyed separately using cochineal insect extract (*Dactylopius coccus* Costa). To assess the dyeability of the banana-silk union fabric, Anatolian black pine cones (*Pinus nigra* subsp. *pallassiana*) were utilized as a bio-mordant, with varying quantities. The CIEL*ab values of the dyeings were measured and compared. Additionally, the chemical composition of the cochineal extract, pinecone mordant, raw fabric, and selected dyed fabrics was analyzed using Fourier Transform Infrared (FTIR) spectroscopy. The washing, light, and rubbing fastness properties of the dyed banana-silk union fabrics were also evaluated and compared. This study developed a method for weaving union fabric by blending two distinct natural yarns, namely banana and silk, to reduce dependence on a single fiber and promote the utilization of agricultural waste.

1. INTRODUCTION

Natural colorants have been used for centuries to color textiles, food, and other materials (Alegbe & Uthman, 2024). The application and dyeing process of natural dyes is highly efficient, allowing dyeing to be carried out at atmospheric temperature without the need for an external energy source. The entire process is, therefore, economical (Nayab-Ul-Hossain *et al.*, 2023). Compared to synthetic dyes, natural colorants offer several advantages, including their availability, accessibility, and the reduced need for complex chemical extraction processes (Leite *et al.*, 2023). Therefore, the adoption of sustainable natural dyeing techniques in textile production is gaining increasing acceptance. Numerous natural finishing agents and dyes can be utilized to produce UV-protective and antibacterial fabrics. Therefore, natural fibers that have been treated with certain natural finishing agents and dyed with the appropriate natural colorants can provide significant protection against ultraviolet (UV) radiation, microbes, and even mosquito bites (Samanta, 2020). Cochineal insects are one of the best natural sources of red-purple color, and dyestuff is extracted, particularly from the bodies of the female bodies

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from the bodies of female cochineal insects since they grow larger and have purer dye compared to their male counterparts. These insects are typically cultivated on the prickly pear cactus, which serves as their host plant and source of sustenance and they are the source of carminic acid and numerous products, including meals, cosmetics, beverages, medicines, and textiles are colored using carminic acid. (Dapson, 2007). Natural dyes necessitate a mordanting process to ensure the fixation and permanence of color on textile fibers. So, prior to applying natural dyestuffs, it is essential to pretreat the fibers with mordanting agents. This pre-treatment step is crucial as it enhances the bonding between the dye molecules and the fiber, thereby improving colorfastness and overall durability of the dyed textile. (Deveoglu *et al*., 2019). Mordants are categorized into two primary types: metallic mordants and bio-mordants. The use of bio-mordants improves the dyeing process and enhances fastness properties. The textile industry is the largest consumer of water, and comprehensive data on effluent production has also been gathered and extensively documented in the literature. Due to their biodegradability and environmental friendliness, bio-mordants have been proposed by researchers as a safe and effective alternative to metallic salt mordants in textile dyeing. (Hosseinnezhad *et al*., 2021; Rani *et al*., 2020). A range of bio-mordants, such as pomegranate peel, mango peel, tamarind, aloe vera, acacia bark ash, orange peel, amla powder, mustard powder, sodium alginate, lemon peel, and pine cone, have had their dyeing properties extensively studied in the literature. (Shahmoradi Ghaheh *et al*., 2021; Yaqub *et al*., 2020; Yasukawa *et al*., 2017).

The banana (*Musa* × *paradisiaca* L.) is a plant from the Musaceae family and is one of the first domesticated plant species. Historically, the oldest indication of banana stems being utilized as a source of fiber dates to the 13th century. The banana plant is a tall, perennial herb with pseudo-stems formed from leaf sheaths. It can grow to heights of 10–40 feet (3.0–12.2 meters) and is surrounded by 8–12 broad leaves. The leaves may grow up to 2.7 meters long and 2 feet (0.61) broad (Pitimaneeyakul, 2009). Natural fibers, particularly cellulosic-based fibers, have multiple benefits, including low density, high stiffness, and environmental friendliness. Approximately 120–150 million tons of bananas are produced globally each year, yet only 12% of the plant, including the fruit, leaves, and stem, is edible. One hectare of banana cultivation generates approximately 220 tons of waste after the fruit is harvested, accounting for over 60% of the total biomass used in the cultivation process. (Vajpayee *et al*., 2023). More than 1.2 billion tons of stemming bananas are left to rot globally each year. The pseudo stem trunk of a banana plant is discarded as a large agro-waste (Paramasivam *et al*., 2022). Originating in Southeast Asia, the banana is cultivated over an area of 11,154 hectares in Turkey (TÜİK, 2021). The provinces of Antalya and Mersin account for the majority (97.7%) of banana production in Türkiye. The provinces of Antalya and Mersin produce the majority of the bananas (% 97.7) grown in Türkiye (Demirel & Hatirli, 2022; Phromphen, 2023). Banana fiber is predominantly obtained mechanically from the pseudo-stem of the banana plant (Kamel, 2023). As a byproduct, a stem weighing around 37 kg produces roughly 1 kg of high-quality fiber (Shroff *et al*., 2015). Banana fiber consists of the following chemical components: cellulose (50–60%), hemicelluloses (25–30%), lignin (12–18%), pectin (3–5%), fat and wax (3–5%), water-soluble chemicals (2–3%), and ash (1–1.5% of the total fiber (Balakrishnan *et al*., 2019). Banana fibers' low elongation may primarily be caused by their lower microfibrillar angle (11°) and comparatively high crystallinity percentage. Banana stem fibers have tensile properties similar to those of jute and other lignocellulosic fibers (Reddy *et al*., 2015). Textiles, floor mats, and composites are only a few materials made from banana fibers. Lignocellulosic natural fibers, such as those from bananas, can be utilized to produce hybrid or blended fabrics in combination with most other natural fibers (Hassan *et al*., 2022).

The objective of this study was to assess the color properties of cochineal insects (*Dactylopius coccus* Costa) when applied with natural mordants to banana/silk hybrid fabrics. Anatolian black pine (*Pinus nigra* subsp. *pallassiana*) was employed as a bio-mordant to enhance the color characteristics. The color of each fabric was analyzed using CIEL*a*b* values. The fastness characteristics of the dyed samples were evaluated in terms of washing, rubbing, and light

resistance. The chemical compositions of the fibers, bio-mordants, and dyestuffs were analyzed using Fourier Transform Infrared (FTIR) spectroscopy.

2. MATERIAL and METHOD

2.1. Material

Cochineal insects were provided by Natural Dyes Company (Istanbul, Türkiye). Anatolian black pine (*Pinus nigra* subsp. *pallassiana*) wood specimens were obtained from the Marmara region (Istanbul, Türkiye). Banana/silk hybrid fabric was obtained from T.C. Alanya Municipality. The weight of the fabric was 200 g/m^2. The warp density per cm is 18, and the weft density per cm is 20. The warp yarn is Ne 30 (silk) and the weft yarn count is Ne 5 (banana).

2.2. Method

2.2.1. *Extraction*

The insects were ground into powder in a mortar before the extraction. The traditional aqua method process was used with a dye concentration set at 5% of over the weight of the fabric (owf) for all dyeings. The liquor-to-fabric ratio was kept to 1:20, lasting 1 hour at boiling temperature. Finally, the temperature of the solution was decreased to 25°C, and it was filtered. The extraction of natural dye is represented in Figure 1.

Figure 1. Extraction of natural dye

2.2.2. *Fabric pre-treatment*

A bleaching process was used to examine the effect of pretreatment applied to the fabrics, and certain samples were pretreated. The bleaching solution comprised sodium hydroxide (3 g/L), soda ash (2 g/L), and a wetting agent (1 g/L) at 70 °C for 60 minutes. Fabric pre-treatment was performed only on samples coded between BF5 and BF10.

2.2.3. *Preparation in powder form of pine cones*

Pine cones were ground into a powder using a vibrating disc mill at 1,000 rpm for 60 seconds. Pine cone powder was added to the dye solution based on the concentration of the bio-mordant required for each dyeing bath. Graining of pine cones for powder form is presented in Figure 2.

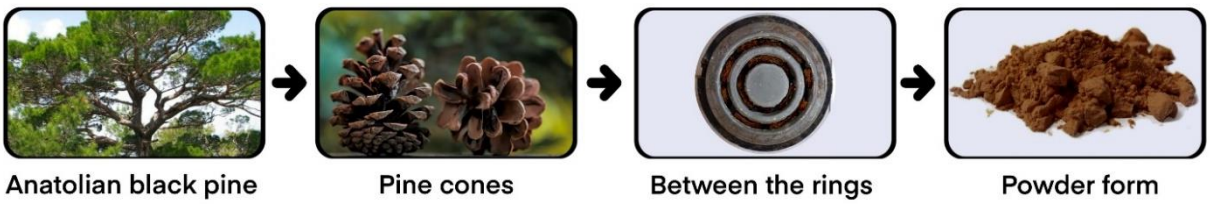
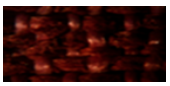
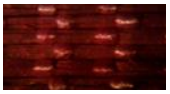
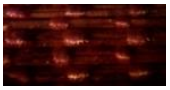


Figure 2. Graining of pine cones for powder form.

2.2.4. *Dyeing procedure*

In this study, banana-silk hybrid fabrics were dyed through a meta-mordant dyeing method with diverse percentages of concentrations. Table 1 summarizes the dyeing procedure.

Table 1. Dyeing procedure.

Dyeing code	Mordant (%)	Pre treatment	Tem. (°C)	Time (min)	Dyeing pH	Shade	
						Microscope	Real
RB	0 RB (RAW)	-	-	-	-		
BF0	0	-	90	120	6-7		
BF1	1	-					
BF2	2	-					
BF3	3	-					
BF4	4	-					
BF5	5	-					
BF6	1	√					
BF7	2	√					
BF8	3	√					
BF9	4	√					
BF10	5	√					

2.2.5. *Fastness properties*

Banana fabrics that had been colored underwent washing, rubbing, and light fastness using ISO standard methods. The colored materials were exposed to xenon arc lamp light for 48 hours (250W). The results of light (ISO 105: B02), rubbing (ISO 105: X12), and washing fastness (ISO 105: C06 -A1S-) tests of dyed fabrics are given in Table 2.

2.2.6. *Color measurement*

The color characteristics were analyzed using a colorimeter. The CIELab values of dyed fabrics are shown in Table 3. The Kubelka-Munk equation for K/S and ΔE*, as shown in Equations 1 and 2.

Equation 1: $$K/S = \frac{(1-R)^2}{2R}$$

Equation 2: $$\Delta E* = \sqrt{(\Delta L*)^2 + (\Delta a*)^2 + (\Delta b*)^2}$$

2.2.7. *FTIR analysis*

FT-IR (Fourier Transform Infrared Spectrophotometer) was used to examine dyed and uncolored fabrics in the range of 4000–650 cm^{-1}. FTIR analyses are presented in Figure 4.

3. RESULTS

Producing woven fabric using two different fibers in two directions, warp and weft, is termed union fabric. That is, these woven fabrics combine different types of yarns to create a new product with specialized properties. In union fabrics, the weft yarns are made of one fiber while the warp yarns are made of another (Kaur & Choudhuri, 2020). Banana/silk union (hybrid) woven fabric was weaved by using banana yarn (Ne 5 in the weft) and silk yarn (Ne 30 in the warp). The coloring compounds of cochineal consist primarily of carminic acid and trace amounts of flavokermesic acid. The coloring component (carminic acid) is water-soluble (Karadag, 2023a). The crude natural dye (*Dactylopius coccus* Costa) was extracted from the cochineal insect using the aqueous method. Different bio-mordant concentrations of pinecone were used on union fabrics that had been naturally dyed to evaluate their dyeability. Pine cones contain lignin, cellulose, and tannin (Lee *et al*., 2018). HPLC analysis revealed that pine cones include gallic acid [$C_6H_2(OH)_3CO_2H$], ellagic acid ($C_{14}H_6O_8$), valeric acid ($C_5H_{10}O_2$), as well as minor colorants (Pars, 2024).

Since ancient times, the traditional aqua method has been one of the simplest and oldest methods to extract natural coloring components. In this method, the raw ingredients are first dried, roughly chopped, and frequently ground into a powder before the color components are extracted in boiling water. Following a set amount of time in the boiling process, the substance is cooled to room temperature and filtered. The cochineal insects were ground in a mortar. According to each dyeing recipe, natural coloring extracts of insects were heated up to 100 °C in separate beakers for one hour. Then, the solution temperature was reduced to 25 °C and filtered with filter paper. The meta-mordant dyeing method was used to color the fabrics. The meta-mordant dyeing process was carried out by simultaneously mordanting and dyeing. This technique involves putting the plant or insect having the dyestuff and the mordant material for dyeing into the dyeing bath, which helps to save both cost and energy. The fabrics were immersed in a dye bath solution comprising both bio-mordant that was put into the dye solution and insect dye (prepared in the aqua method) at a liquor ratio (L/R) of 1:50 (at 90 °C for 120 min). All fabrics were dyed with 5% cochineal (owf) with different concentrations of pinecone (1 %, 2%, 3%, 4%, and 5%) in separate beakers. The dyed materials were then rinsed and dried at room temperature. The dyeing scheme is presented in Figure 3. The dyeing was done per the Natural Organic Dye Standard (NODS). By nature, hazardous substances, heavy metals, carcinogenic dyes, toxic dyes, pesticides, and synthetic dyes are excluded from the NODS. Therefore, brands and the textile industry can accomplish more by creating materials that abide by the NODS while also promoting sustainability and eco-friendly dyeing (Karadag, 2023b). The procedure of dyeing, microscope shades, and real shades are given in Table 1.

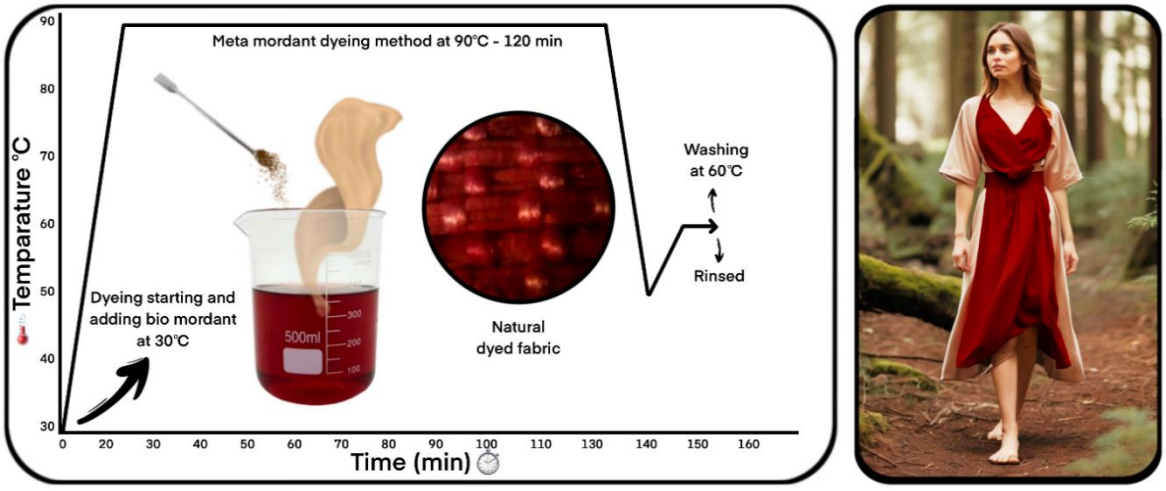


Figure 3. Dyeing scheme.

The lightfastness of all the fabrics was found to be varyingly low, which is typical of natural dyeing in cellulosic-based materials. The results of washing fastness tests on dyed fabrics were good. All of the dyed fabrics exhibit good rubbing fastness ratings of 4-5 in dry conditions, with the lowest ratings being 3-4 in wet conditions. Table 2 provides the results of the light, rubbing, and washing fastness tests for the dyed fabrics.

Table 2. The results of light, rubbing, and washing fastness tests of dyed fabrics.

Code	Light	Rubbing		Washing-Staining					
		Dry	Wet	Acetate	Cotton	Nylon	Polyester	Avyrlic	Wool
BF0	-	4-5	3-4	4-5	4-5	4-5	4-5	4-5	4-5
BF1	2-3	4-5	3-4	4-5	4-5	4-5	4-5	4-5	4-5
BF2	2-3	4-5	3-4	4-5	4-5	4-5	4-5	4-5	4-5
BF3	2-3	4-5	3-4	4-5	4-5	4-5	4-5	4-5	4-5
BF4	2-3	4-5	3-4	4-5	4-5	4-5	4-5	4-5	4-5
BF5	3	5	3-4	4-5	4-5	4-5	4-5	4-5	4-5
BF6	2-3	4-5	3-4	4-5	4-5	4-5	4-5	4-5	4-5
BF7	2-3	5	3-4	4-5	4-5	4-5	4-5	4-5	4-5
BF8	2-3	5	3-4	4-5	4-5	4-5	4-5	4-5	4-5
BF9	3	5	4	4-5	4-5	4-5	4-5	4-5	4-5
BF10	3	5	4	5	5	5	5	5	5

The chemical components in the cochineal, pinecone, raw, and selected dyed fabrics (BF5 and BF10) were detected using FTIR analysis. The bands between 1440 cm^{-1} to 1000 cm^{-1} can be related to carbohydrate functional groups. The aromatic ring at 1513 cm^{-1} and the aromatic methoxyl at 1239 cm^{-1} were bands related to lignin (Pereira *et al.*, 2014). The –OH stretching was identified as the cause of the distinctive broad peaks around 3277 cm^{-1}, which correspond to the stretching vibrations of cellulose, hemicellulose, and absorbed moisture. The intensity of the - OH stretching peaks increased after dyeing. It was also determined that the peaks at 2918 to 2919 cm-1 were caused by the C-H stretching vibration from the -CH_2 group of cellulose and hemicellulose (Oyewo *et al.*, 2023). The molecular vibrations at 1543 and 1242 cm^{-1} may be attributed to the presence of C–C and C–OH groups in the cochineal molecule (Kumar *et al.*, 2016). The peak characteristic of cochineal around 2850 cm^{-1} was attributed to the asymmetric

stretching vibration of the C–H bond in alkanes. These bonds of cochineal were also determined in the selected samples (BF5 and BF10) at 2853 cm^{-1} and the presence of hydroxyl –OH and carbonyl -C=O groups in the structure of pinecones may indicate an improvement in the color properties of dyed fabrics. IR spectra revealed evidence of the interaction between the dye, bio-mordant, and fiber, indicating the formation of a complex. The results of the FTIR analysis are presented in Figure 4.

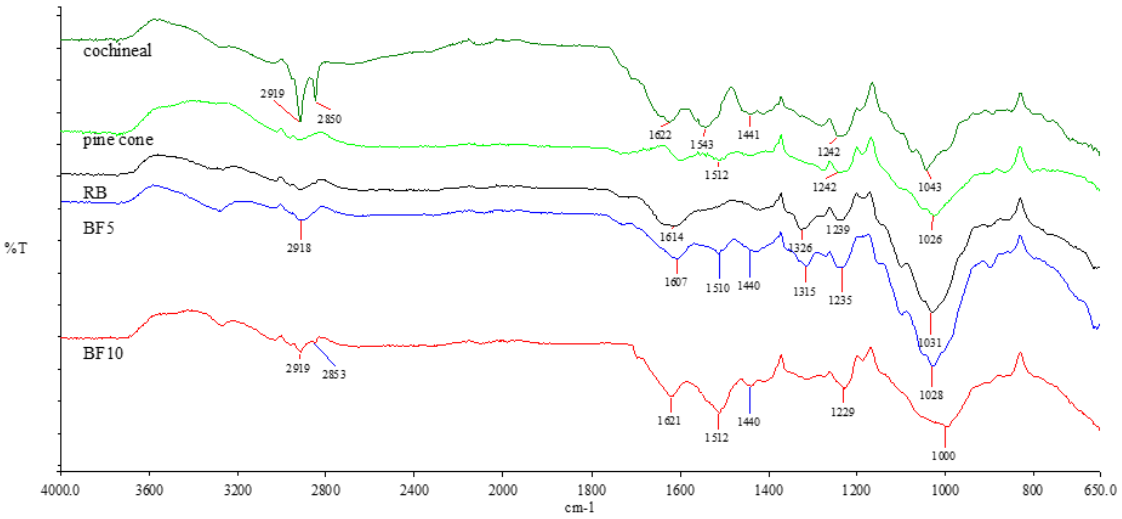


Figure 4. The analysis results carried out by FTIR.

The color of each dyed fabric was investigated based on the CIELAB (L*, a*, and b*) values. Before dyeing to increase hydrophilicity, a fabric pretreatment process was carried out to remove oil, wax, and natural impurities from the banana. For the pretreatment process, only fabrics coded between BF5 and BF10 were bleached with sodium hydroxide (NaOH) at a quantity of 3 g/L with 2 g/L soda ash and 1 g/L wetting agent running at 70 °C for 60 min at a 1:50 liquor ratio. Thus, the effect of the pre-treatment applied to the material on the fabric was examined. The CIEL*a*b* values of dyed fabrics are given in Table 3. The measurements were taken at 22°C (65 ±2% relative humidity), D65 daylight, with a 10° standard observer, and the raw fabric was used as the standard. Three values (L*, a*, and b*) are used to represent color in the CIE-LAB color space. From black to white, the perceptual lightness is expressed by L* (brightness) [between 0-100]. Red, green, blue, and yellow are the four hues that a* and b* refer to. With a value range of a* representing the green-red opponent colors and b* representing the blue-yellow opponent colors. The BF10-coded fabric had the highest color yield of all the colored fabrics (K/S = 10.31) at the wavelength of maximum absorption. As shown in Table 3, the sequence of color intensity (K/S value) of different dyed fabrics is BF10 > BF9 > BF8 > BF7 > BF6 for pretreated fabrics. This indicates that the mordant with 5% pinecone produced the highest color yield. The color difference (ΔE*) values of the dyed fabrics were found in the range of 4.19 to 10.31 when the sample "RB" was used as a standard (the highest ΔE* value was found to be 10.39 for the sample BF10 and the lowest color difference was 16.16 for the sample BF0). Banana fiber contains cellulose, which is the highest among the fibers obtained from other parts of the banana plant, hemicellulose, lignin, wax, ash, and moisture (Karuppuchamy *et al*., 2024). Thus, fabric pre-treatment was found to enhance color strength, and before the dying and mordanting processes, cellulosic fabrics may undergo pre-treatment.

The novel method of bio-mordant utilized in this study is gaining recognition for its ability to produce new shades with exceptional fastness qualities. Varied colors were produced by using different ratios of bio-mordants. The color strength was affected by the mordant concentration (ranging from 1% to 5%).

Table 3. The CIELab values of dyed fabrics.

Code	*L**	*a**	*b**	*C**	*h°*	K/S	ΔE*
RB	74.07	5.06	15.92	16.71	72.39	-	-
BF0	61.68	11.34	7.66	13.69	34.05	4.19	16.16
BF1	59.42	14.06	5.32	15.03	20.73	4.20	20.19
BF2	57.54	13.02	6.15	14.40	25.28	5.00	20.79
BF3	57.85	13.37	6.14	14.71	24.65	5.79	20.69
BF4	49.58	13.51	6.82	15.13	26.80	7.07	27.46
BF5	48.06	13.70	5.84	14.90	23.08	7.45	29.20
BF6	50.35	15.41	4.51	16.05	16.31	6.50	28.28
BF7	48.06	13.70	5.84	14.90	23.08	6.63	30.79
BF8	47.01	14.95	3.32	15.31	12.51	7.03	31.44
BF9	41.93	14.65	4.10	15.21	15.66	9.45	35.56
BF10	37.03	14.56	3.86	15.06	14.85	10.31	39.10

Annually, enormous amounts of pinecones, a common agricultural waste, are produced and utilized in a variety of industries. Numerous research studies on banana fiber have spurred investigations into its novel uses in the textile sector, including value addition and enhancement of fabric qualities. Furthermore, Environmental concerns, which are driving the demand for natural materials as alternatives to synthetic reinforcements in composite products, suggest that the use of banana fiber as reinforcement in polymers is likely to increase in future technical textile applications (Makinde-Isola *et al*., 2024). The study developed a method of weaving the union fabric by combining two various quality natural yarns like banana and silk to reduce the dependence on a single fiber and to promote the use of agricultural waste.

4. CONCLUSION

Banana/silk union fabrics bio-mordanted at rates of 1%, 2%, 3%, 4%, and 5% were separately dyed with cochineal (*Dactylopius coccus*). Various ratios of Anatolian black pine (*Pinus nigra* subsp. *pallassiana*) have been used effectively as bio-mordants. Banana fibers can be dyed with natural colors to a significant extent, which will help mitigate pollution and address ecological imbalances, contributing positively to environmental conservation. The principal objective of this current study was to evaluate the effects of bio-mordants including natural dye extract on banana fiber in terms of color measurement and color fastness. The hydrophilicity of banana-silk hybrid fabrics was improved by diminishing lignin, fats, and wax contents with fabric pre-treatment. It was determined that the color strength was influenced by the pretreatment and bio-mordant concentration used. Bio-mordants enhance the bonding of natural dyes to the fiber and may be used as biodegradable versus metallic mordants. The coloristic properties of the dyed fabrics were enhanced due to the high concentration of bio-mordant, which utilized pinecones to form complexes with carminic acid and the fiber. A lingo-cellulosic natural fiber, bananas can be treated to combine with other fibers to develop a hybrid fabric that is both biodegradable and ecologically safe. Banana fiber that has been naturally dyed may be utilized to create eco-friendly materials for clothing, technical textiles, wearable technology, and home linens.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

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Research Article

Comparison of high antioxidant ZnONPs produced from different fungi as alternative biomaterials

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Abstract: In this study, zinc oxide nanoparticles (ZnONPs), a promising alternative biomaterial, were synthesized using a non-toxic, cost-effective green synthesis approach using various fungal species (*Penicillium citrinum, Fusarium solani, Aspergillus flavus* and *Aspergillus niger)*. The effect of different fungal species on the structural, optical, morphological and antimicrobial properties of ZnO nanoparticles (ZnONPs) was compared. ZnO nanoparticles (ZnONPs) crystallized in a hexagonal wurtzite structure with grain sizes ranging from 45 to 61 nm. Fungal species had a significant effect on the surface plasmon resonance (SPR) peak observed at 302 nm. ZnONPs were obtained in different morphologies such as nanodiscs, nanospheres, nanorchins and nanonuts, and it was determined that fungal species had a significant effect on these structures. The antibacterial activity of ZnONPs against *Candida albicans*, *Streptococcus mutans*, *Pseudomonas aeruginosa*, *Eosinophilic pneumonia* and *Staphylococcus aureus* was investigated. The effect of these nanoparticle shapes on antibacterial activity was evaluated. ZnONPs were found to have a significant antimicrobial effect especially on *Candida albicans* and *Streptococcus mutans*. ZnONPs produced only with *Aspergillus niger* fungus were found to have a strong antimicrobial effect especially on *Staphylococcus aureus*. Based on these results, the biosynthesis of ZnO nanoparticles (ZnONPs) using *Penicillium citrinum*, *Fusarium solani*, *Aspergillus flavus* and *Aspergillus niger* fungal species is proposed for the production of ZnONPs as a biomaterial with remarkable antibacterial properties and various morphologies.

1. INTRODUCTION

In recent years, numerous studies have been conducted on the production and characterization of nanomaterials to discover intriguing aspects of nanotechnology. In particular, the distinctive physical characteristics of metallic nanoparticles (MNPs) or metal oxide nanoparticles (MONPs) have garnered attention. In response to environmental concerns associated with traditional chemical methods for producing these nanoparticles, biogenic synthesis has emerged as a viable alternative. Green synthesis is a clean, non-toxic, and cost-effective method that utilizes plants and microorganisms to synthesize nanoparticles (NPs) (Abdelhakim *et al*., 2020).

Due to their good conductivity, chemical stability, catalytic properties, photonic and optical properties, ZnO is among the most extensively researched metal oxide nanoparticles (MONPs)

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(Ahmad *et al*., 2020). A review of the literature reveals that ZnO nanoparticles (ZnONPs) are predominantly synthesized using plant-based methods in the green synthesis process (Alavi & Nokhodchi *et al.,* 2021; Bhardwaj *et al.,* 2017; Bhuyan *et al.,* 2015; Brady *et al.,* 2023; Chauhan *et al.,* 2015; Chaurasia *et al.,* 2010; Cooper *et al.,* 1955). However, in the green synthesis approach, various microorganisms, including fungi, bacteria, and algae, can also be utilized alongside plants for the production of ZnO nanoparticles (ZnONPs). Among these, fungi play a particularly significant role by utilizing microbial cells, enzymes, proteins, and other biomolecules to drive the synthesis of ZnO nanoparticles. In this process, ZnONPs can be obtained in the desired size and shape by optimizing the reaction conditions during microbial synthesis**.** The production method used determines the morphology and size of nanoparticles. ZnONPs can exhibit a diverse range of morphologies, including nanoflowers, nanowires, nanorods, nanopellets, nanosprings, nanoplates, nanorings, nanocombs, nanohelix, nanosheets, nanospheres, and nanourchins, which differ in size and shape from other nano-metal oxides (Rajiv *et al.,* 20132). In contrast to expensive and environmentally harmful chemical or physical methods, fungal-mediated synthesis offers cleaner, environmentally friendly, non-toxic, and biocompatible alternative. In the recent years, various fungal species have been used to produce chemical-free and non-toxic ZnO nanoparticles, particularly in biomaterial applications. Fungi can produce metal oxide nanoparticles with minimal energy, thus making them increasingly become prominent in the production of ZnO nanoparticles in recent years. Fungi, offer significant advantages over other microorganisms due to their high metal tolerance, metal absorption capabilities, wide pH-temperature range, rapid accumulation, and cost-effectiveness. ZnONPs synthesized via fungi have stable, biocompatible, and antibacterial properties. ZnONPs exhibit broad-spectrum antibacterial and antifungal activities. They are particularly promising for combating fungal infections, including those caused by multi-drug-resistant fungal strains. These nanoparticles can serve as an alternative approach to deal with the growing threat of drug-resistant fungal infections (Pesika *et al.,* 2003). Therefore, they are widely utilized in various industries including, biomedicine, environmental cleaning, food preservation, agriculture, textile, and pharmaceutical distribution. Especially, the biological applications of nanoparticles are influenced by several factors, as size, shape, surface properties, chemical composition, solubility, and dispersion (Patterson *et al.,* 1939).

Although, in recent years, there have been some studies that explored the production of zinc oxide nanoparticles (ZnONPs) using fungi, these studies remain relatively limited (Dhillon *et al.,* 2012; Venkatesh *et al.,* 2013; Vettumperumal *et al.,* 2016; Wang *et al.,* 2017; Yavuz & Yılmaz, 2021). Abdelhakim *et al.* synthesized ZnONPs using *Alternaria tenuissima* fungus and investigated their light scattering, zeta potentials, antimicrobial, anticancer, antioxidant, and photocatalytic activities (Guilger-Casagrande, M., & de Lima, 2019). Chauhan et al. conducted a study on the synthesis of ZnONPs using *Pichia fermentans* JA2 fungus and their structural, surface, optical, and antibacterial effects (Gupta *et al.,* 2024). Vlad et al. investigated the behaviors of a group of ZnO molecules. In this study, polyurethanes were examined, and a widespread presence of *Aspergillus brasiliensis* was observed. The biological activity was evaluated, and numerous films were created to defend against the attack of Sabouraud-Agar fungi in a nutrient medium. The fungus growth was monitored visually. The study revealed that polyurethane membranes, which have been modified with zinc nanoparticles, possess unique antibacterial and antifungal characteristics, making them essential in medical and biological applications (Günay *et al.,* 2021).

Rajan *et al.* utilized *Aspergillus fumigatus* JCF for the synthesis of zinc oxide (ZnO), resulting in the formation of white agglomerates composed of zinc oxide nanoparticles. The antimicrobial efficacy of these nanoparticles against Gram-positive and Gram-negative bacteria including *Klebsiella pneumoniae*, *Escherichia coli*, and *Pseudomonas aeruginos*a was investigated. The findings of the study demonstrated the efficacy of zinc oxide in effectively combating these harmful microorganisms (Jain *et al.,* 2023). Mohamed et al. synthesized ZnONPs using two recently isolated strains of fungi, *Fusarium keratoplasticum* (A1-3) and

Aspergillus niger (G3-1). Variations in the size, shape, and structure of the biosynthetic ZnONPs were assessed. This study examines the impact of microbial biostimulation using composite ZnONPs and investigates the structural properties of green composite ZnONPs. The properties, antimicrobial activities, and cytotoxic properties of various biosynthesized NPs were analyzed (Jain *et al.*, 2014). Ahmad *et al.* synthesized ZnO from three types of fungi: *D. seriata*, *B. dothidea* and *A. mali*. ZnONPs functioned as inhibitors in addressing fungal diseases in apple orchards. The results suggest that zinc oxide nanoparticles can significantly contribute to combatting fungal pests and improve the protection of plants (Kalpana *et al.*, 2018). Garrett *et al.* produced ceramic ZnO nanoparticles using three different filamentous fungi: *Aspergillus* sp., *Penicillium* sp., and *Paecilomyces variotti*). They used different zinc sources during the production process. Particle sizes ranged from 1 nm to 1000 nm. They synthesized zinc oxide nanoparticles (ZnO) exhibiting conical, cubic, and spherical morphologies (Kalpana *et al.*, 2022). However, there still remain numerous unexplored aspects regarding the green synthesis of ZnONPs using fungi.

In studies of this nature, the antifungal efficacy is influenced by the size and morphology of the antifungal agent. The morphology of ZnO nanoparticles can be regulated by selecting appropriate synthesis methods. The existing literature indicates a substantial number of studies that focused on the production of ZnONPs utilizing plant extracts; however, research involving fungi for ZnONP synthesis remains relatively limited. Various fungal species, including *Psathyrella candolleana*, *Pichia kudriavzevii*, *Aspergillus aereus*, *Fusarium solani*, and *Alternaria alternate*, have been investigated for their potential in the production of zinc oxide nanoparticles (ZnONPs) (Rajiv *et al.*, 2013; Sarkar *et al.*, 2014; Senthilkumar & Sivakumar, 2014; Singh & Singh, 2019; Soosen *et al.*, 2009; Tauc & Menth, 1972; Urbach, 1953; Vlad *et al.*, 2012).

The aim of our study is to determine the effect of fungi on the structural, morphological, elemental, antibacterial and optical properties of ZnO nanoparticles, with particular focus on the role of fungal species and metal precursors in the production of ZnO NPs. ZnO nanoparticles are produced by fungi through the interaction between fungal exudates and metal ions of ZnO. Fungi reduce metal ions to NPs by producing extracellular reducing enzymes that interact with metal ions. Moreover, during this synthesis process, nanoparticles are coated with biomolecules produced by the fungus, increasing the stability and biological activity of the NP (Yoldas & Partlow, 1985; Zeghoud *et al.*, 2022). Metal nanoparticles can be produced from several fungal species such as *Fusarium* sp., *Aspergillus* sp., *Verticillium* sp. and *Penicillium* sp. (Vettumperumal *et al.*, 2016; Wang *et al.*, 2017; Yavuz & Yılmaz, 2017; Yoldas & Partlow, 1985; Zeghoud e*t al.*, 2022). However, the factors that affect this process, such as physical, chemical, and biochemical parameters, remain inadequately understood.

In this study, we used the fungal species Penicillium citrinum, Fusarium solani, Aspergillus flavus and Aspergillus Niger, which have previously been reported to have high reductase activity and high yields in AgNP synthesis. The study also compared the effects of these fungal species on the structural, morphological, elemental, antimicrobial and optical properties of ZnONPs.

2. MATERIAL and METHODS

2.1. Obtaining Fungal Cultures

In this study, the biosynthesis method successfully yielded ZnONPs without the need for hazardous chemical agents. ZnONPs were produced via the biosynthesis method using four different fungal isolates *Penicillium citrinum*, *Fusarium solani*, *Aspergillus flavus*, and *Aspergillus niger*. Each fungal strain was inoculated into 250 ml erlenmeyer flasks containing 160 ml of fungal broth medium and agitated at 25 °C, 125 rpm. Subsequently, the flasks were placed in a water bath for incubation over a period of 7 days. After a 7-day incubation period, the fungal strains were filtered through filter paper and subsequently washed three times with

sterile distilled water to eliminate any residual media components. This process resulted in the collection of the fungal biomass. Each of these fungal masses was placed in 250 ml Erlenmeyer flasks containing 160 ml sterile distilled water. The flasks were then incubated in a shaking water bath at a temperature of 125 rpm for 48 hours. Then, the cell-free filtrates were filtered through filter paper, and the remaining filtrate was used to synthesize ZnONPs. Experimental studies regarding the production of fungal cultures are presented in Figure 1, and the biomass of the fungi used for establishing the fungal cultures can be found in Table 1.

Table 1. Fungal biomass used in obtaining fungal culture.

Fungal Culture	Code	Fungal Biomass (gr)
Penicillium citrinum	*(P.C)*	8.05
Fusarium solani	*(F.S)*	3.70
Aspergillus flavus	*(A.F)*	5.09
Aspergillus niger	*(A.N)*	5.52

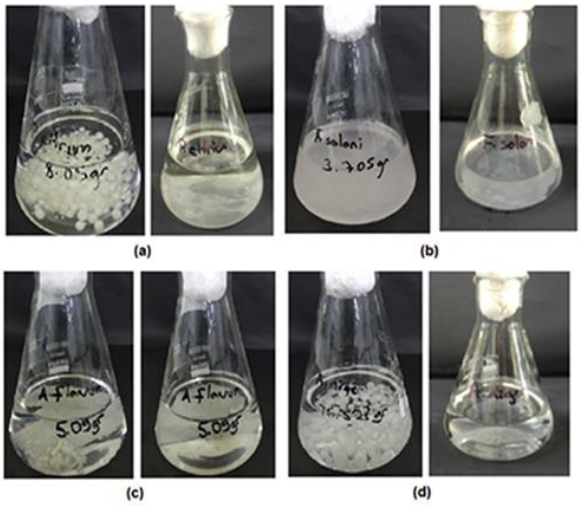

Figure 1. Fungal culture production stages (a) *P. citrinum* (b) *F. solani* (c) *A. flavus* (d) *A. niger*.

2.2. Biosynthesis of ZnONPs Using Cell-Free Filtrate

For four distinct fungal isolates, namely *P. citrinum*, *F. solani*, *A. flavus* and *A. niger*, 2 mM (6.92 g) zinc acetate [$Zn(CH_3CO_2)_2$] was added individually to 160 ml of cell-free filtrate and left for 48 hours at 125 rpm and 25 °C. Subsequently, these solutions were incubated in a water bath with vigorous shaking, yielding four different ZnONPs using cell-free filtrates obtained from the four respective fungal isolates. Figure 2 displays the images of the cell-free filtrates employed as the initial solution for the ZnONPs.

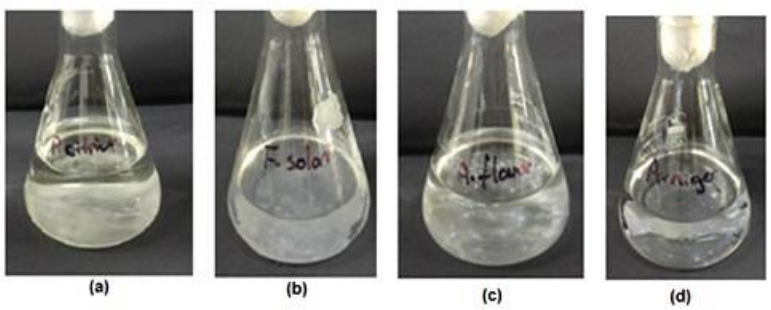

Figure 2. Cell-free filtrates obtained from fungal cultures (a) *P. citrinum* (b) *F. solani* (c) *A. flav*us (d) *A. niger*.

2.3. Obtaining ZnONPs from ZnO Solutions Prepared in Different Fungal Isolates

ZnO nanoparticles (ZnONPs) were synthesized using ZnO solutions derived from different fungal isolates. The solutions of ZnO prepared from the fungal isolates *P. citrinum*, *F. solani*, *A. flavus*, *A. niger* were combined and stirred at 85 °C for 5 hours to gelate the solutions. The method involved heating the gelled ZnO solutions at 400 °C for 10 minutes, yielding slightly yellow- and off-white powder samples of ZnONPs.

2.4. Characterization of ZnONPs

The physical and antibacterial properties of the green synthesized ZnONPs were assessed through range of characterization techniques. The UV-Vis spectra were scanned at wavelengths of 200-800 nm using the SHIMADZU UV-1800 instrument and the data was collected through using UV-Probe software program. The absorbance spectra of ZnONPs were analyzed using the obtained spectra, and the nanoparticles' band gaps, radii, Urbach energies and steepness parameters were determined. The X-ray diffraction (XRD) (Rigaku Ultima-IV) and field emission scanning electron microscopy (FESEM) (Carl Zeiss-Sigma 300 VP) were applied to investigate the structural, morphological and elemental properties of ZnONPs. Additionally, the antibacterial activity of ZnONPs was assessed using the agar diffusion method.

3. RESULTS

3.1. Structural Properties of ZnONPs

Figure 3 illustrates the XRD patterns of ZnONPs generated through green synthesis techniques employing a range of fungal isolates. The patterns were utilized to determine the structural characteristics of the ZnO nanoparticles (ZnONPs), including their phases, Miller indices, grain sizes, and dislocation. As per Figure 3, all films exhibited a hexagonal wurtzite ZnO structure (JCPDS: 04-005-4711), and none of the fungal isolates led to any structural alterations in the nanoparticles. The synthesis of ZnO nanoparticles was successfully achieved using four different selected isolates. The crystallization levels and half-peak widths of all ZnONPs are nearly identical, except for those produced with the *A. flavus* fungal isolate. The crystallization level of ZnONPs produced solely by the *A. flavus* isolate was found to be lower than that of other ZnONPs, and the peak widths were larger. Some structural parameters of ZnONPs, including diffraction angle (2θ), interplanar distance (d), Miller indices (hkl), full width at half maximum (FWHM), grain size (G) and dislocation density (δ) were determined. The grain size and dislocation density of the ZnONPs were calculated using the Scherrer equation (Kumar *et al.*, 2022).

$$D = \frac{0.9\,\lambda}{\beta \cos\theta} \tag{1}$$

$$\delta = \frac{1}{D^2} \tag{2}$$

where λ is wavelength, β is full width at half maximum, θ is Bragg angle and D is grain size. These values are given in Tables 2.

Bragg reflections found in the structures of ZnONPs; produced in *P. citrinum* isolate, correspond to values of 31.7124°, 34.385°, and 36.205°. It was observed that there was dominant growth in the (100), (002), and (101) planes. The grain sizes for these three predominant orientations were calculated, and they were found to be 51.5 nm, 58.1 nm, and 51.6 nm. Additionally, it was observed that the dislocation density in the structured nanoparticles had similar values to the grain size. Bragg reflections can be observed in ZnONPs produced by the *F. solani* isolate at values of 31.725°, 34.384°, and 36.209°, indicating that the (100), (002), and (101) planes exhibited dominant growth. The grain sizes of these three dominant orientations were calculated to be 60.0 nm, 62.4 nm, and 60.6 nm. Bragg reflections were observed in the structures of ZnONPs produced in *A. niger* isolates. The values for these

reflections were 31.705°, 34.336° and 36.2191°. It was noted that there was dominant growth in the (100), (002) and (101) planes. The grain sizes for these three dominant orientations were calculated to be 60.0 nm, 61.6 nm and 61.0 nm, respectively. Bragg reflections in the structures of ZnONPs produced in *A. flavus* isolates correspond to values of 31.782°, 34.417°, and 36.250°. It was observed that dominant growth occurred in the (100), (002), and (101) planes. The grain sizes for these three dominant orientations were calculated to be 45.8 nm, 46.1 nm, and 44.1 nm, respectively.

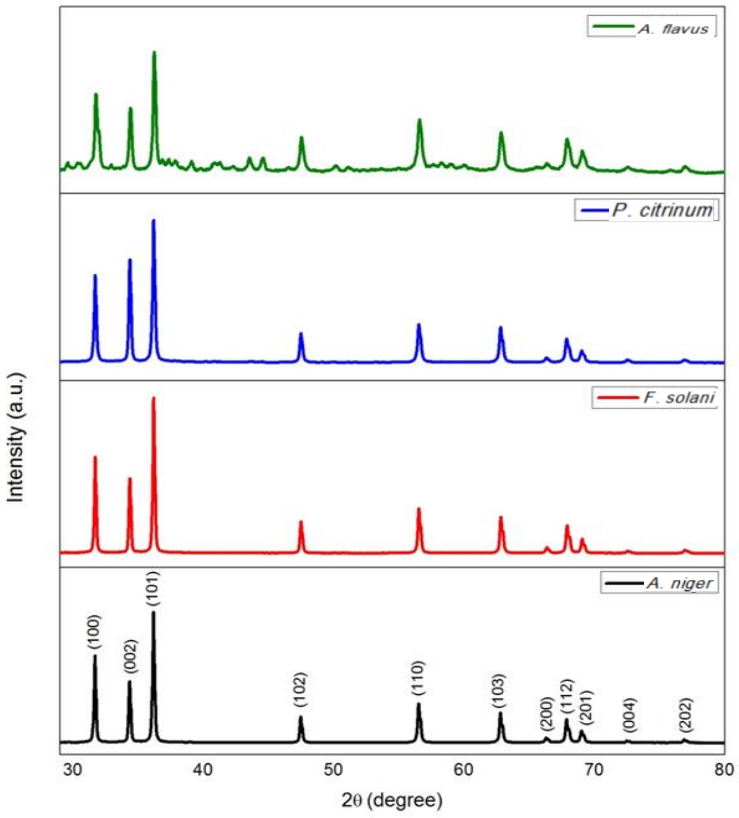

Figure 3. The XRD pattern of ZnONPs.

When examining all films, it was discovered that the grain sizes in the dominant orientations were consistent across each film. Notably, the utilization of the *Aspergillus flavus* isolate in the production of ZnO nanoparticles resulted in the smallest grain size when employing these methods. Furthermore, various unique peaks were identified in these ZnONPs at 10°-25° diffraction angles, which were not present in the other films, indicating possible impurities. Similar structures have also been observed in various studies in the literature on ZnO nanoparticles that were prepared using different methods (Charmaine *et al.*, 2022). The ZnONPs produced by the *P. citrinum* isolate exhibited a dislocation density of 3.77, 2.96, and 3.76 line/nm^2. The (100), (002), and (101) planes experience dominant growth. The dislocation density of zinc oxide nanoparticles (ZnONPs) in *F. solani* isolates was determined to be 2.78, 2.57, and 2.72 line/nm^2 for growth in (100), (002), and (101) planes, respectively. Technical term abbreviations were defined at their first occurrence in the text. Similarly, the dislocation density of ZnONPs produced in *A. niger* isolates was found to be 2.78, 2.64, and 2.69 line/nm^2 for dominant growth in (100), (002), and (101) planes. The dislocation density of zinc oxide nanoparticles (ZnONPs) produced by *A. flavus* isolates corresponds to values of 4.71, 5.14, and 9.13 lines/nm^2. Dominant growth was observed in the (100), (002), and (101) planes. All nanoparticles were found to grow in the smallest grain size (002) orientation. Notably, only the grain size and dislocation density values differ in the ZnONPs produced by the *A. flavus* isolate. It was observed that the stress level in ZnONPs was the highest despite its smallest grain size compared to other nanoparticles.

Table 2. Some of the structural parameters of ZnONPs.

	2θ (°)	d (Å)	Miller indices (hkl)	FWHM (°)	D (nm)	$\delta \times 10^4$ (Line/nm^2)
ZnONPs-*P.citrinum*	31.724	2.818	(100)	0.167	51.5	3.77
	34.385	2.605	(002)	0.149	58.1	2.96
	36.205	2.479	(101)	0.169	51.6	3.76
	47.480	1.913	(102)	0.174	52.1	3.68
	56.512	1.627	(110)	0.198	47.6	4.41
	62.780	1.478	(103)	0.194	50.2	3.97
	66.274	1.409	(200)	0.213	46.6	4.60
	67.851	1.380	(112)	0.218	45.7	4.79
	68.958	1.360	(201)	0.231	43.6	5.26
	72.481	1.302	(004)	0.198	51.9	3.71
	76.844	1.239	(202)	0.276	38.4	6.78
ZnONPs-*F.solani*	31.725	2.818	(100)	0.143	60.0	2.78
	34.384	2.606	(002)	0.139	62.4	2.57
	36.209	2.478	(101)	0.144	60.6	2.72
	47.497	1.912	(102)	0.145	62.4	2.57
	56.543	1.626	(110)	0.159	59.0	2.87
	62.831	1.477	(103)	0.163	59.6	2.82
	66.351	1.407	(200)	0.170	58.3	2.94
	67.925	1.388	(112)	0.178	56.0	3.19
	69.065	1.358	(201)	0.182	55.3	3.27
	72.521	1.302	(004)	0.241	42.8	5.46
	76.954	1.238	(202)	0.252	42.1	5.64
ZnONPs-*A. niger*	31.705	2.819	(100)	0.143	60.0	2.78
	34.336	2.607	(002)	0.140	61.6	2.64
	36.191	2.479	(101)	0.141	61.0	2.69
	47.481	1.913	(102)	0.145	64.1	2.27
	56.526	1.626	(110)	0.156	60.1	2.77
	62.798	1.478	(103)	0.153	63.3	2.50
	66.306	1.408	(200)	0.164	60.5	2.73
	67.882	1.379	(112)	0.169	59.2	2.85
	69.014	1.359	(201)	0.174	57.9	2.98
	72.507	1.302	(004)	0.163	63.1	2.51
	76.889	1.238	(202)	0.182	58.3	2.94
ZnONPs-*A. flavus*	31.782	2.841	(100)	0.189	45.8	4.71
	34.417	2.603	(002)	0.188	46.1	5.14
	36.250	2.476	(101)	0.198	44.1	9.13
	47.508	1.912	(102)	0.274	33.1	8.35
	56.550	1.626	(110)	0.273	34.6	7.42
	62.829	1.477	(103)	0.265	36.7	5.81
	66.367	1.407	(200)	0.240	41.5	7.63
	67.911	1.379	(112)	0.277	36.2	8.81
	69.070	1.358	(201)	0.299	33.7	2.99
	72.500	1.302	(004)	0.560	18.3	1.38
	76.903	1.238	(202)	0.390	26.9	4.71

Additionally, these nanoparticles were found to exhibit the smallest grain size growth (101). The average grain size values of the major orientations of ZnONPs, generated through fungal isolates obtained from *F. solani*, *A. niger*, and *A. flavus*, were established as 53.7 nm, 61 nm, 61.9 nm, and 45.3 nm, respectively. The ZnONPs generated by the *P. citrinum*, *F. niger* isolates, which share similar structural characteristics; the smallest mean grain size belongs to the ZnONPs produced by *P. citrinum*. The mean grain sizes of the ZnONPs generated by the *F.*

solani and *A. niger* isolates are comparable. Nonetheless, the mean grain size of the ZnONPs generated by the *A. Flavus* isolate was the tiniest. The dislocation density values in the dominant orientations of the ZnONPs produced by fungal isolates from *A. flavus* were calculated to be 3.49 lines/nm², 2.69 lines/nm², 2.70 lines/nm², and 6.32 lines/nm², respectively. Among the ZnONPs produced in *P. citrinum*, *F. solani*, and *A. niger* isolates with, similar structural properties, and the ZnONPs produced in *F. solani* and *A. niger* isolates exhibited the lowest average dislocation density values. The average dislocation density values for ZnONPs produced by *F. solani* and *A. niger* isolates were found to be similar. Nevertheless, the ZnONPs produced by the *A. flavus* isolate exhibited the highest average dislocation density.

3.2. Optical Properties of ZnONPs

UV-Vis spectroscopy was utilized to establish the response of ZnONPs to visible light alongside various optical parameters, including band gap, porosity, Urbach energy and refractive index. Absorbance and transmittance spectra for all materials were recorded in the range of 200-900 nm. The absorbance spectra for ZnONPs are exhibited in Figure 4. In general, the absorbance properties for ZnONPs made utilizing different fungal cultures exhibit notable similarities.

In particular, ZnONPs generated in *P. citrinum* and *A. niger* gave rise to analogous absorbance spectra. The band edges of all ZnONPs were observed to be smooth, with a sharp increase at approximately 340 nm. ZnONPs produced in *F. solani* showed an increase in absorbance values in the visible region. Conversely, the absorbance values of other materials in this region were notably low. For all NPs, absorbance values showed a sharp increase upon transmission of wavelengths below approximately 350 nm. At around 300 nm, the absorption values of all nanoparticles reached their peak. It was discovered that ZnONPs that were synthesized using *F. solani* and *A. flavus* had the highest absorption values in this region. The inflection points in the absorbance spectra were initially identified, and are depicted in Figure 5. These peaks, which represent the highest points on the absorbance spectrum may be correlated with surface plasmon resonance (SPR), which is a unique feature of metal nanoparticles (Moormann & Bachand, 2021).

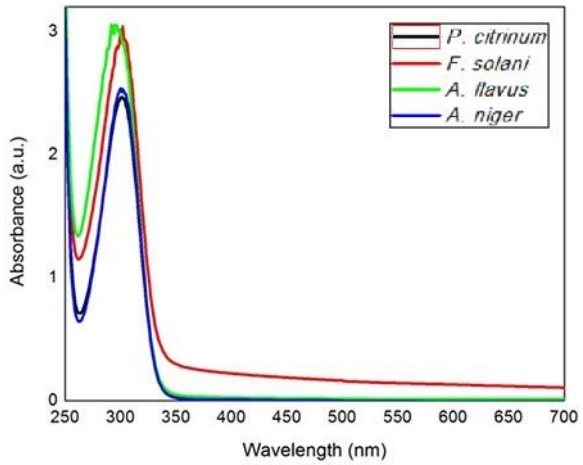

Figure 4. Absorbance spectra of biosynthesized ZnONPs.

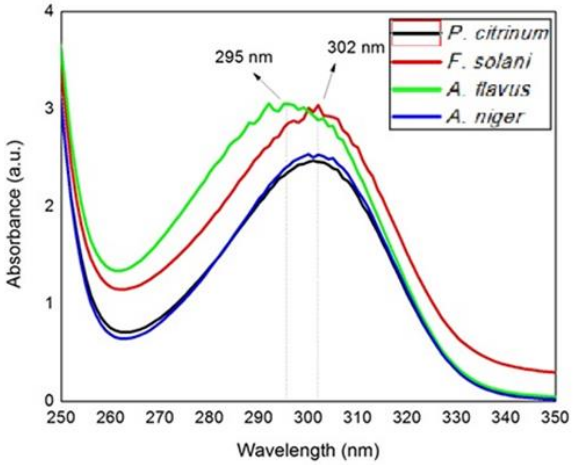

Figure 5. Inflection points in the absorption spectra of ZnONPs.

A shift in the short wavelengths was observed for only one fungal species. This alteration in the absorbance edge may have stemmed from the quantum size effect (Pariona *et al.*, 2020). Particle size was calculated using the effective mass model to characterize the nanoparticle size (r, radius) as it is dependent upon the peak absorbance wavelength (l_p) (Nehru *et al.*, 2023):

$$r\ (nm) = \frac{\left[\frac{1020.72}{\lambda_p} - 26.23012\right]^{1/2} - (0.3049)}{\frac{2483.2}{\lambda_p} - 6.3829} \quad (3)$$

ZnONPs exhibit a peak absorbance of 295 nm and 302 nm, as shown in Figure 8. During the development of equation (3), it was established that m_e = 0.26 m_o, m_h = 0.59 m_o, where m_o represents the free electron mass, ε equals 8.5, and the bulk E_g is 3.3 eV (Mahamuni *et al.*, 2023). Table 3 indicates the sizes of the ZnONPs. A red-shift in the absorbance peak wavelength occurs as the fungal solution varies, attributed to the reduction in quantum confinement resulting from an increase in rising particle size. It was discovered that the absorbance peak of ZnONPs created in the solution of *A. flavus* had shifted to a longer, redder wavelength. Additionally, the particle size was observed to be larger in size. Figure 6 illustrates the transmittance spectra of ZnONPs. The visible region demonstrates high transmittance for all ZnONPs, whereas only those produced in the *F. solani* medium exhibit low transmittance values. This indicates that ZnONPs generated in *F. solani* medium are applicable for absorbing properties, and that those derived in *P. citrinum, A. flavus*, and *A. niger* environments are fit for high transmittance applications.

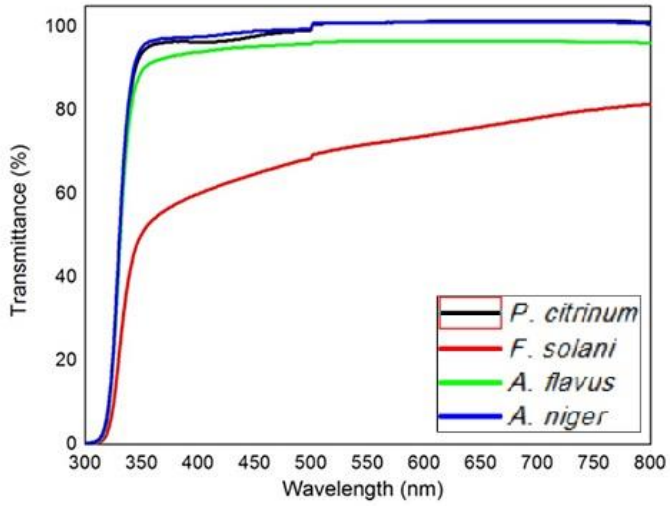

Figure 6. Transmittance spectra of ZnONPs.

Band gap values are crucial optical parameters for technological applications in nanomaterials. Figure 7 presents the αhv^2-hv graphs for ZnONPs and Table 3 displays the band gap (E_g) values derived from these graphs. Based on the results of the analysis of the graphs, it was found that the band gap values of ZnONPs were relatively consistent, measuring at 3.83 eV. The band gap values of ZnO nanoparticles (ZnONPs) produced exclusively in *F. solani* medium are lower than those of other materials. It is evident that the fungal biosynthesis approach has an impact on the band gap values of ZnONPs. The production method yields ZnONPs with enhanced values and a broader band gap. The optical band gap values were calculated using the following equation (Malaikozhundan *et al.*, 2017):

$$(\alpha hv) = A(hv - E_g)^n \quad (4)$$

where A is a constant determined by the refractive index of the material, E_g represents the optical band gap, and hv is the photon energy. The absorption spectra of ZnONPs were used to calculate the average particle size.

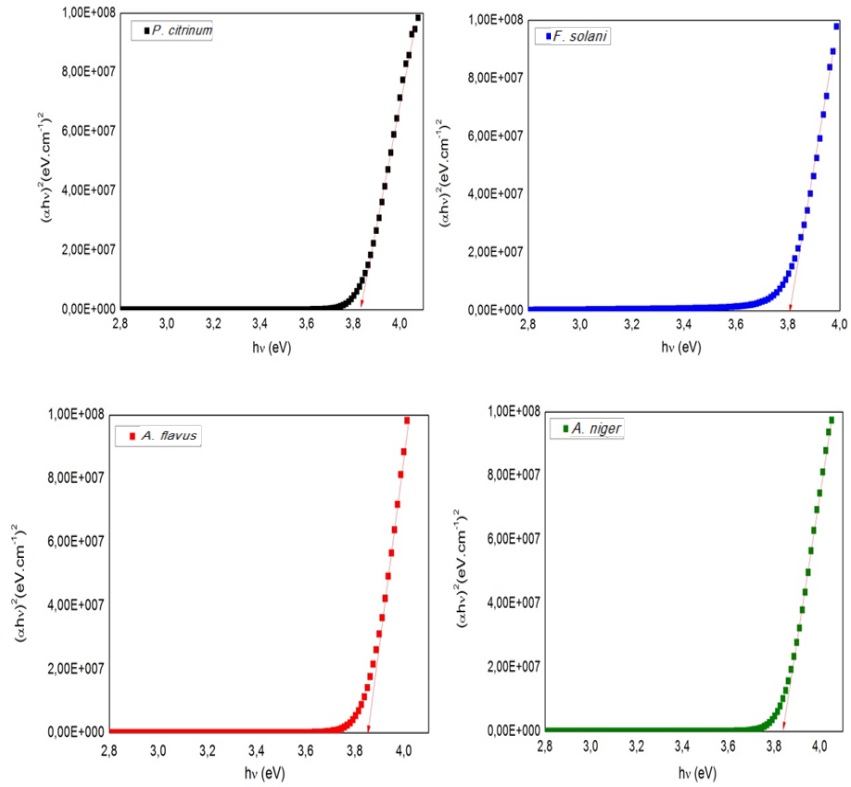

Figure 7. $\alpha h\nu^2$-$h\nu$ graphics of ZnONPs.

Optical transitions between a material's valence and conduction bands, defect states, conduction mechanisms, and disproportionate charge-induced defects depend on the material's bandgap. The identification of these changes and defects in the structure is achieved through detecting the Urbach tails that are formed in the band structure. The energy associated with these Imperfect Urbach tails in the band structure is known as the Urbach energy. Therefore, it is a significant parameter in identifying alterations in the band structure of materials. The Urbach energy was determined by computing the inverse of the gradient of lnα-hν plots, using the following equation (Meruvu *et al.*, 2011):

$$\alpha = \alpha_0 exp^{h\nu - E/E_u} \quad (5)$$

where α_0 is a constant and E_u represents the Urbach energy, which is the width of the tails of localized states in the material's band gap. *lnα-hν* graphs are presented in Figure 8. Another significant parameter that illustrates changes in the band structure is the steepness factor. This parameter is a perpendicularity parameter that indicates the material's band broadening. Additionally, this parameter determines the electron-phonon interaction's potency. Both of these critical parameters can be calculated using the following equations (Moghaddam et al., 2015; Moghaddam *et al.*, 2017):

$$\sigma = \frac{k_B T}{E_u} \quad (6)$$

where σ is the steepness parameter, k_B is the Boltzmann constant, and T is the absolute temperature. The electron-phonon interaction strength ($E_{e\text{-}p}$) depends on the steepness parameter and can be determined using the formula 2/3s. Also, the refractive index of ZnO-NP was calculated through the use of the Ravindra relation (Mohamed *et al.*, 2019). This relationship is linked to the material's band gap of the material, and the equation is provided below:

$$n = 4.084 - 0.62E_g \quad (7)$$

where E_g represents the optical band gap energy calculated from the Tauc plot, and n signifies the refractive index. Furthermore, Figure 9 presents the variations in the optical bandgap-

Urbach energy and optical bandgap values. The porosity values of ZnONPs have been assessed using Equation (8), a quantitative analysis of porosity based on refractive index (Molina *et al.*, 2020):

$$Porosity\ (\%) = \left[1 - \frac{n^2-1}{n_d^2-1}\right] \times 100 \quad (8)$$

where n is the refractive index of ZnONPs and n_d is the refractive index value of pore-free ZnO according to literature. Table 3 presents the calculated values of refractive index and porosity. The results demonstrate that ZnONPs exhibit refractive index values ranging from 1.70 to 1.72 and porosity values of 34.5 % to 35.9 %.

Table 3. The some optical parameters of ZnONPs.

ZnONPs	Peak absorbance peak	Particle R (nm)	E_g (eV)	E_u (meV)	Steepness parameter ($\sigma \times 10^{24}$)	Refractive index (n)	Porosity (%)
P. citrinum	302	1.672	3.83	572	6.58	1.7094	35.9
F. solani	302	1.672	3.81	541	6.96	1.7218	34.5
A. flavus	295	1.581	3.84	566	6.65	1.7032	36.6
A. niger	302	1.672	3.83	571	6.60	1.7094	35.9

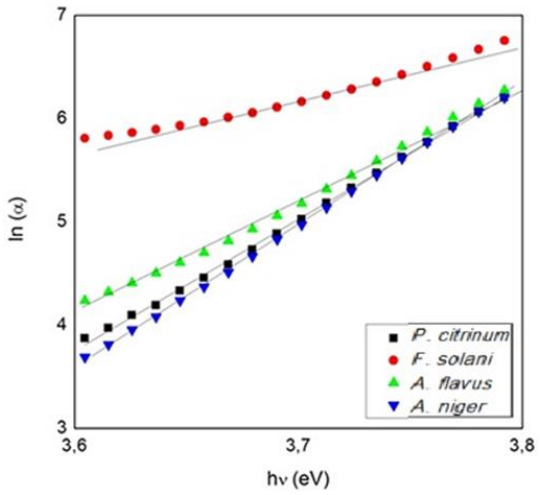

Figure 8. *lnα-hν* graphs of ZnONPs.

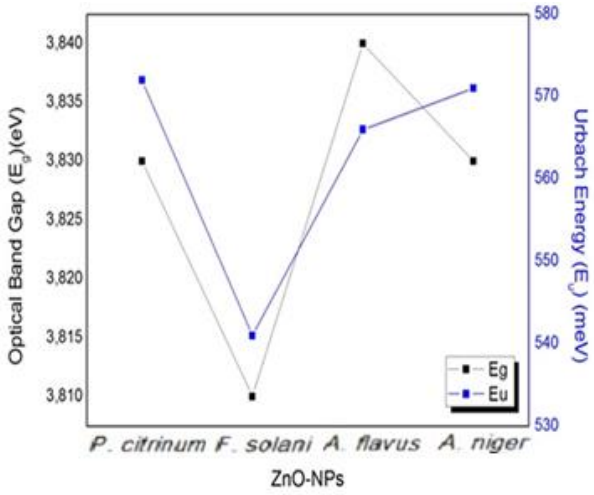

Figure 9. Variation of optical band gap with Urbach energy of ZnONPs.

3.3. Morphological Properties of ZnONPs

Morphological properties of ZnONPs produced in fungal isolates of *P. citrinum*, *A. niger*, *F. solani*, and *A. flavus* were investigated using scanning electron microscopy at 500 x and 3 kx magnifications. FESEM images and the EDX images and elemental analysis findings of ZnONPs are shown in Figure 10 and 11 for *P. citrinum*, *A. niger*, *F. solani*, and *A. flavus* isolates, respectively. When the morphological structures of ZnONPs were examined, it was observed that they all exhibited tightly packed formations, each developing its own distinct morphology. At high magnification (300 kx), the distinct morphologies of all nanoparticles become evident. Nonetheless, upon closer inspection, it was deduced that the morphologies of ZnONPs generated in *P. citrinum* and *A. niger* isolates were corresponding to each other. In Figure 10(a) and 10(c), ZnONPs display a morphology resembling that of cauliflower. Figure 10 reveals that ZnO nanoparticles synthesized by the *P. citrinum* isolate formed larger aggregates, while those produced by the *A. niger* isolate formed smaller clusters. It was observed that both isolates were capable of producing ZnONPs with a cone-shaped morphology.

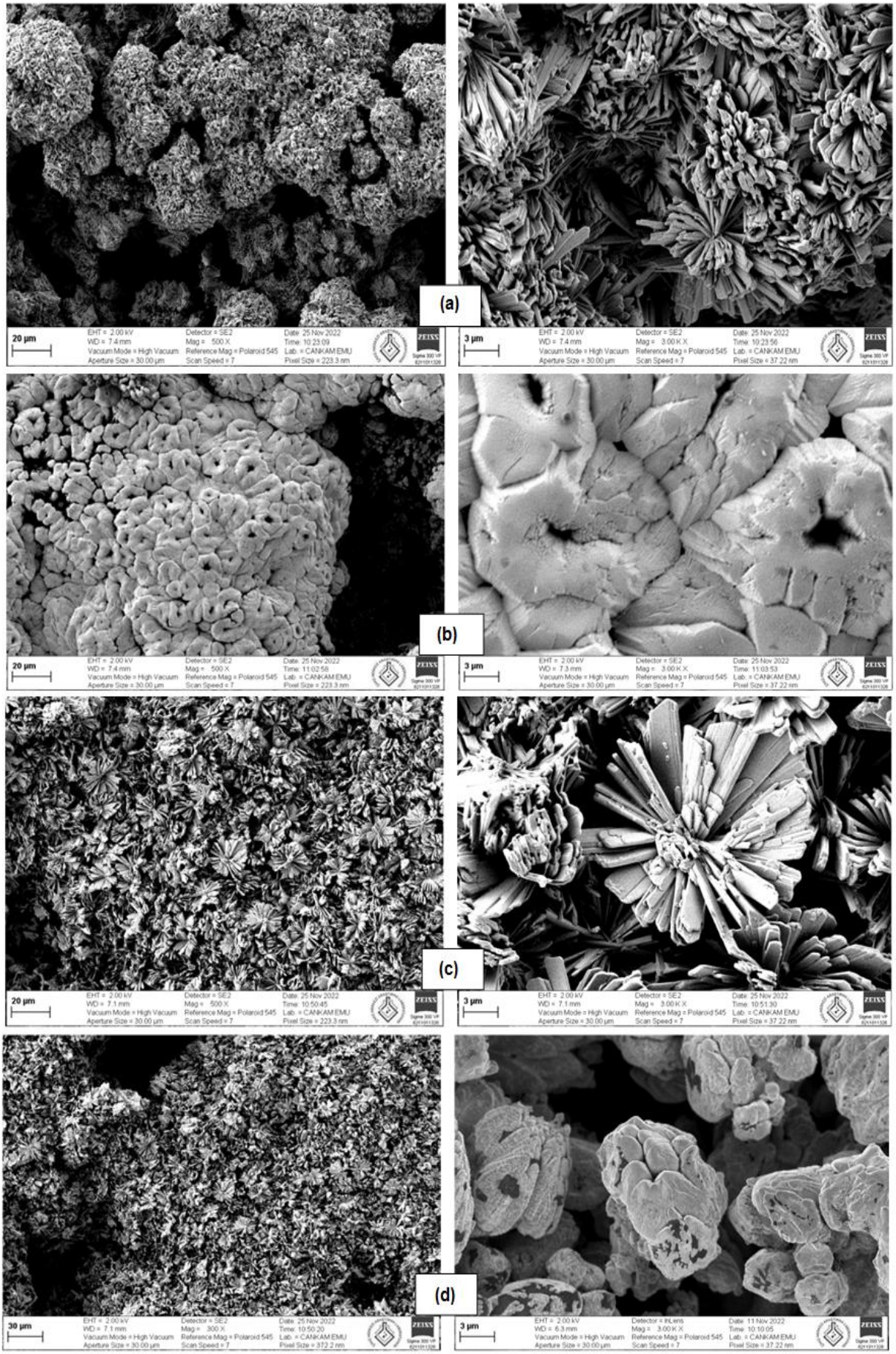

Figure 10. FESEM images of ZnONPs.

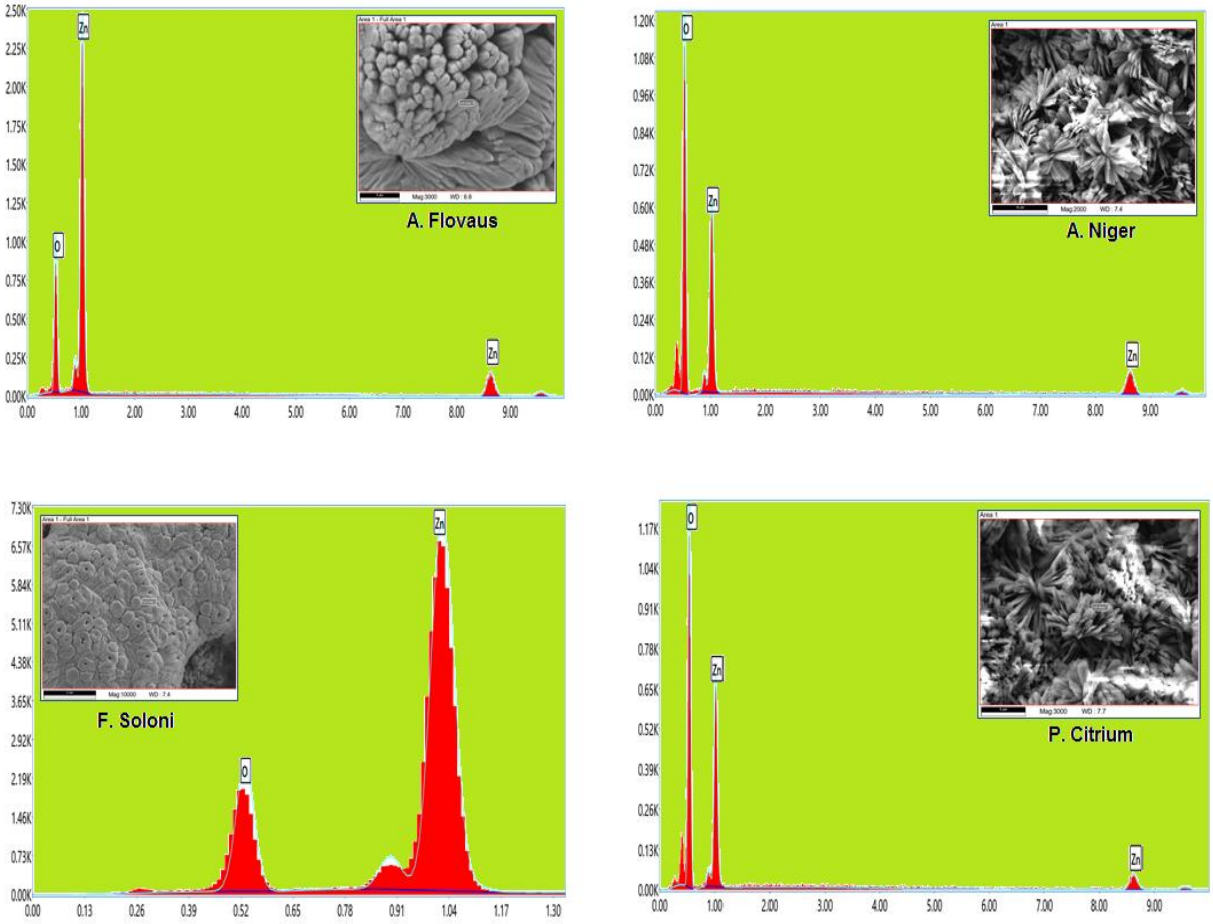

Figure 11. EDX results of ZnONPs.

In addition, the production of nanoparticles in *A. niger* isolation permits for cone-shaped expansion in larger dimensions (Figure 10(c)). Figures 10(b) and 10(d) exhibit the morphologies of ZnONPs created in the *F. solani* and *A. flavus* isolates, respectively. It has been observed that the production of ZnONPs in these isolates resulted in nanoparticles with dissimilar morphologies compared to other isolates. In Figure10(d), ZnONPs created from the *F. solani* isolate displayed greater porosity than nanoparticles produced from other isolates. However, these nanoparticles exhibit a densely packed structure with a void in the center. FESEM images of ZnONPs produced in *A. flavus* isolates are presented in Figure 10(d). It was observed that the ZnONPs had distinct morphologies. A compact, uniform, and foliaceous growth was witnessed for ZnONPs produced in this isolate.

Elemental analysis of ZnONPs synthesized by various fungal isolates was conducted using a field-emission scanning electron microscope (FESEM) device. Figure 11 and Table 4 present the EDX images and elemental analysis findings for ZnONPs obtained from *P. citrinum*, *A. niger*, *F. solani*, and *A. flavus* isolates, respectively. The results indicate the presence of zinc and oxygen atoms in the structure of ZnONPs, which is consistent with the XRD results. When examining the atomic percentages of ZnONPs, it becomes apparent that the nanoparticles with the highest stoichiometric value are those produced in *A. flavus* isolate. Therefore, the ZnONPs produced in these isolates are of lower quality. The atomic percentages of O and Zn atoms in these ZnONPs are very similar. Conversely, the atomic percentages of O and Zn in ZnONPs produced in isolates of *P. citrinum*, *A. niger*, and *F. solani* are significantly different leading to lower stoichiometric values. When examining the atomic ratios in the structure of ZnONPs, it was observed that there was an excess of O and a deficiency of Zn in the nanoparticles produced by *P. citrinum* and *A. niger* isolates. In contrast, the nanoparticles synthesized from the *F. solani* isolate exhibited an excess of zinc and a deficiency of oxygen in their structure. This modified

ratio of Zn and O in the structure is the primary reason for the alteration in the morphology of ZnONPs. The cone-shaped morphological structure of Zn-rich ZnONPs is found to be impaired. This indicates that various fungal isolates are effective in producing ZnONPs with varying atomic percentages.

Table 4. EDX data of ZnONPs.

ZnONPs	Atomic %		Weight %	
	Zn	O	Zn	O
P. citrinum	18.13	81.87	47.51	52.49
F. solani	76.96	23.04	93.17	6.83
A. flavus	40.22	59.78	73.32	26.68
A. niger	23.26	76.74	55.33	44.67

4.4. Antibacterial Properties of ZnONPs

The antibacterial properties of ZnONPs synthesized by various fungal isolates using the biosynthesis method were evaluated through the agar diffusion technique. Using the agar well diffusion method, this study examines the antibacterial impact of ZnONPs on five types of bacteria, namely *Candida albicans*, *Streptococcus mutans* ATCC 10449 s, *Pseudomonas aeruginosa* ATCC 27853, *Eosinophilic pneumonia* NRLLB4420, and *Staphylococcus aureus* ATCC 25923. The minimum inhibitor concentrations were determined by spreading the selected microorganisms onto nutrient agar medium, introducing wells into the medium, and planting sterile vials. The minimum inhibitor concentrations were determined by spreading the selected microorganisms onto nutrient agar medium, introducing wells into the medium, and planting sterile vials. 100 µL of the pertinent biosynthesis product was added to the wells and incubated at 37 °C for 24 hours. Subsequently, the culture dishes were inspected after which the resulting area dimensions were determined. The zone of inhibition (ZOI) was evaluated using the following formula (Moormann & Bachand, 2021):

$$W = \frac{(T-D)}{2} \tag{9}$$

in which *W* represents the width of ZOI (mm), D indicates the diameter of the test specimen (mm), and T refers to the total diameter of the said specimen. These computed figures and the zone of inhibition are illustrated in Table 5 and Figure 13, correspondingly.

Figure 12(a) presents the examination of the antimicrobial properties of biosynthesized ZnO-NP on various fungal isolates, including *P. citrinum*, *F. solani*, *A. flavus* and *A. niger*. It was found that ZnONPs exhibited a significant antibacterial effect against *Candida albicans* microorganisms. Moreover, their resistance to specified microorganism was observed. Notably, the antibacterial effect varied among different fungal isolates against *Candida albicans* microorganisms. The ZOI widths of ZnONPs for this microorganism ranged from 4.2 to 4.5 cm. The *A. flavus* fungal isolate produced ZnONPs with the highest antibacterial activity against the *Candida albicans* microorganism. The smaller particle size of this particular ZnONPs, along with its surface morphology, supports its superior antibacterial activity compared to other ZnONPs. This is because the small size and protruding surface morphology of the particles enable them to penetrate into the cell more effectively, thereby increasing antibacterial resistance (Qianwei *et al.*, 2022; Rajan *et al.*, 2016). Figure 12(b) demonstrates the evaluation of antimicrobial activity of biosynthesized ZnONPs against *Eosinophilic pneumonia* microorganisms using fungal isolates of *P. citrinum*, *F. solani*, *A. flavus* and *A. niger*. The findings suggest that ZnO-NP had limited antibacterial effects against Eosinophilic pneumonia microorganisms and their high resistance against this substance was clear. While the ZnONPs synthesized from the *F. solani* and *A. flavus* isolates exhibited different antibacterial effects on the microorganisms responsible *for Eosinophilic pneumonia*, the other ZnONPs showed consistent antibacterial effects. The ZOI widths of the ZnONPs, which were produced these

isolates against, this microorganism varied from 0.5 to 0.7 cm. *F. Solani* fungal isolate displayed the most potent antibacterial activity towards microorganisms that cause eosinophilic pneumonia. Figure 12(c) displays the evaluation of the antimicrobial impact of biosynthesized ZnONPs on Pseudomonas aeruginosa microorganisms through fungal isolates of *P. citrinum*, *F. solani*, *A. flavus* and *A. niger*. It was found that ZnONPs demonstrated limited antibacterial efficacy against *Pseudomonas aeruginosa* microorganisms with ZOI widths of 0.5 to 0.6 cm.

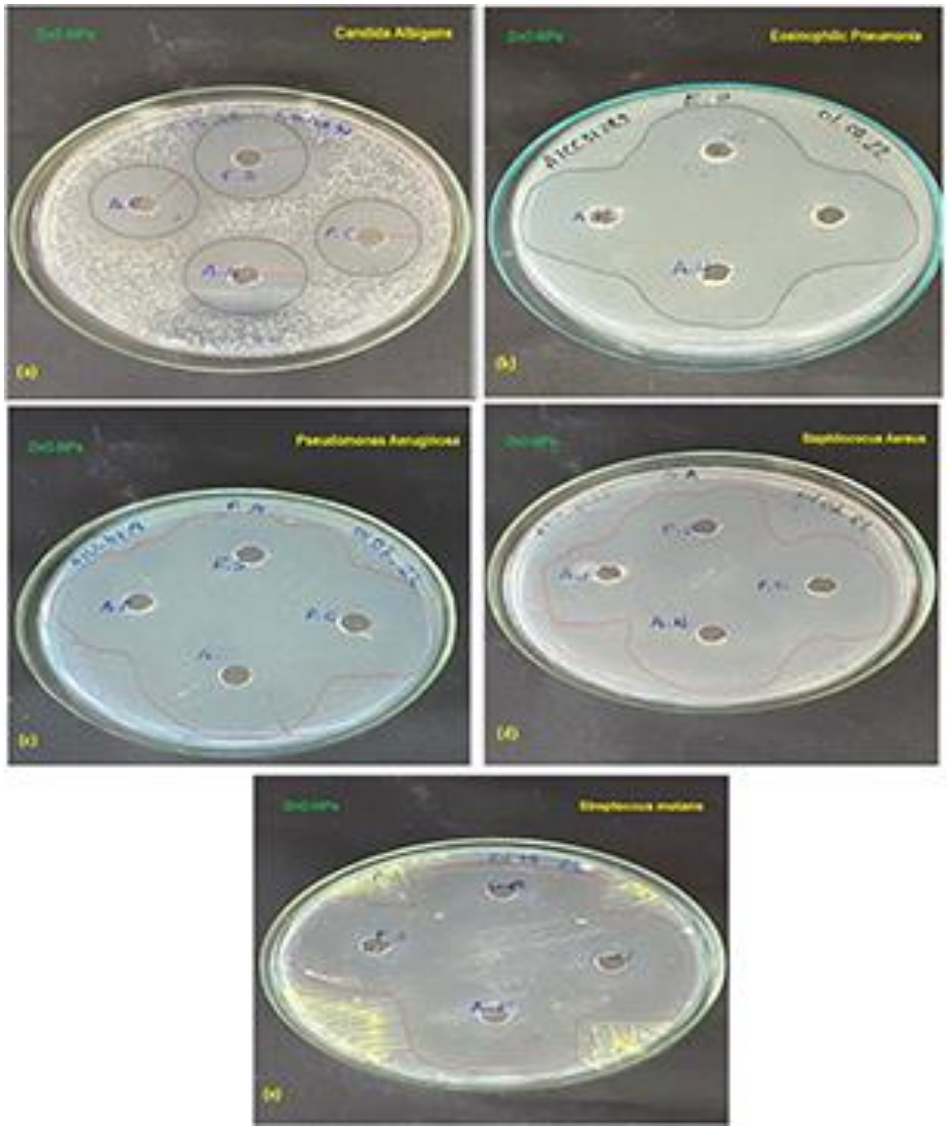

Figure 12. Antibacterial activity tests of ZnONPs (a) *Candida albicans* (b) *Eosinophilic pneumonia* (c) *Pseudomonas aeruginosa* (d) *Staphylococcus aureus* and (e) *Streptococcus mutans* microorganisms.

It was observed that the antibacterial effect of various fungal isolates on the microorganism *Pseudomonas aeruginosa* remained consistently similar. While ZnONPs produced by the *F. solani* fungal isolate exhibited differing antibacterial activity on *Pseudomonas aeruginosa* microorganisms, the remaining three isolates demonstrated identical antibacterial effects. Figure 12(d) displays the results of the testing undertaken to determine the antimicrobial effect of ZnONPs synthesized biologically on the microorganism *Staphylococcus aureus* through the utilization of fungal isolates such as *P. citrinum*, *F. solani*, *A. flavus*, and *A. niger*. Analysis revealed that ZnONPs provided a weaker antibacterial effect on the microorganism *Staphylococcus aureus* compared to other variations of ZnONPs. The ZOI widths of ZnONPs for this microorganism ranged from 0.1 to 0.9 cm. It was noted that the antibacterial impact of various fungal isolates on *Staphylococcus aureus* microorganisms was equal. However, the *A. niger* fungal isolate demonstrated the highest antibacterial activity on *Staphylococcus aureus* microorganisms, as it produced ZnONPs. Figure 12(e) displays the assessment of the

antibacterial properties of biosynthesized ZnO-NPs on *Streptococcus mutans* microorganisms using fungal isolates of *P. citrinum*, *F. solani*, *A. flavus*, and *A. niger*. It was found that ZnONPs exhibited a significant antibacterial effect on Streptococcus mutans. The ZOI widths of ZnONPs for this microorganism ranged from 2.1 to 2.4 cm. It was observed that the antibacterial effect of various fungal isolates on the microorganism *Streptococcus mutans* was comparable. The ZnONPs produced in both *P. citrinum* and *F. solani* fungal isolates exhibited uniform antibacterial activity on *Streptococcus mutans* microorganisms, with *A. flavus* isolates presenting with the slowest antibacterial activity.

As a result of the antimicrobial effectiveness test, it became apparent that the biosynthetic products harvested from the fungal mass had an antimicrobial effect on all the microorganisms tested. Figure 13 displays the variation in the inhibition zone of ZnONPs created from various fungal isolates on different bacteria. Similarly, Figure 14 illustrates the juxtaposition of the inhibition diameters of ZnONPs on the identical bacteria. All ZnONPs exhibited significant antibacterial activity against *Candida albicans* and *Streptococcus mutans*. However, while all ZnONPs had low antibacterial activity against Staphylococcus bacteria, those produced in the *A. niger* isolate demonstrated high resistance. The antibacterial effects of ZnONPs were similar in other bacterial species.

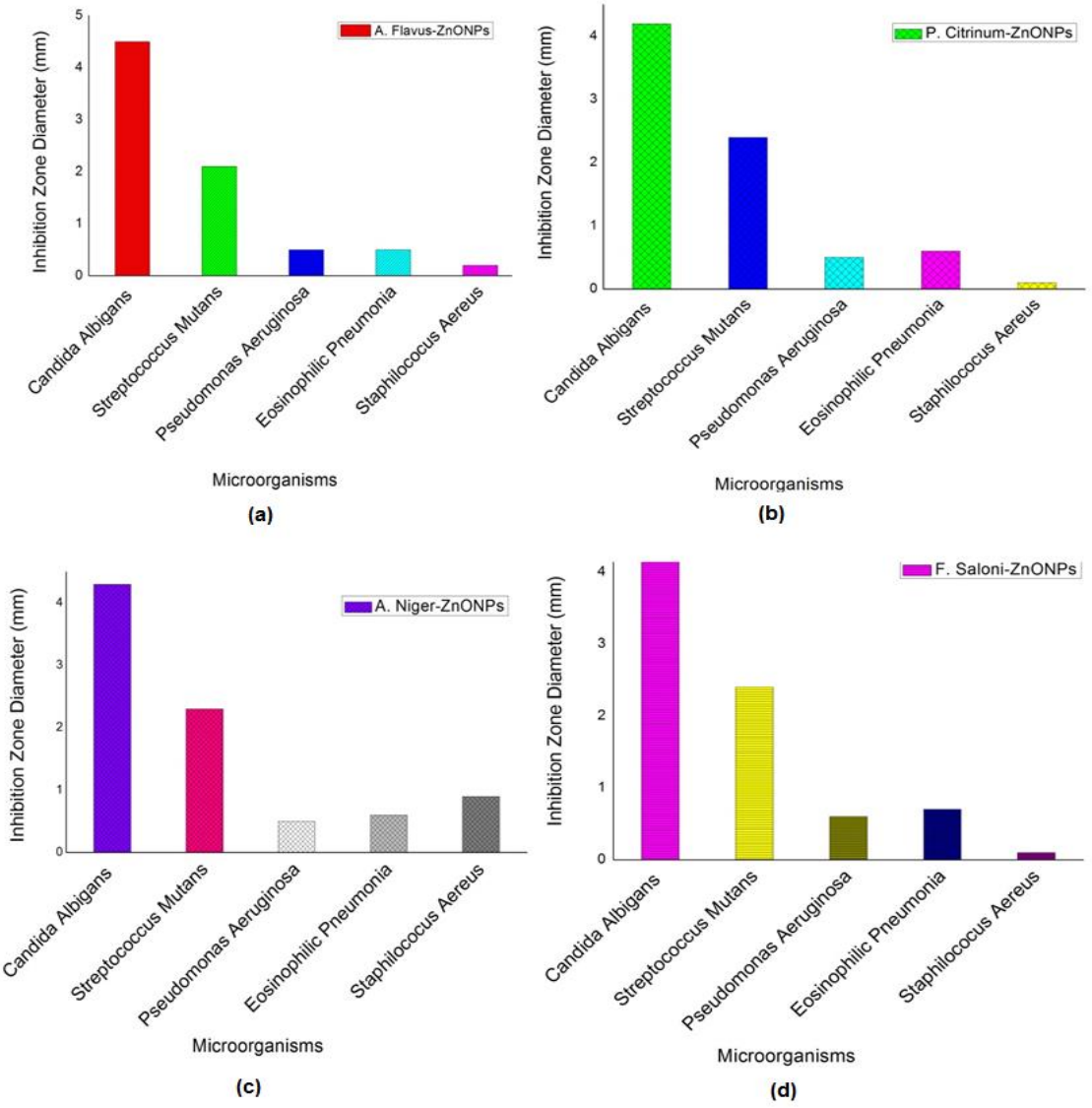

Figure 13. The inhibition zone variation of ZnONPs (a) *A. flavus*, (b) *P. citrinum*, (c*) A. niger* and (d) *F. solani.*

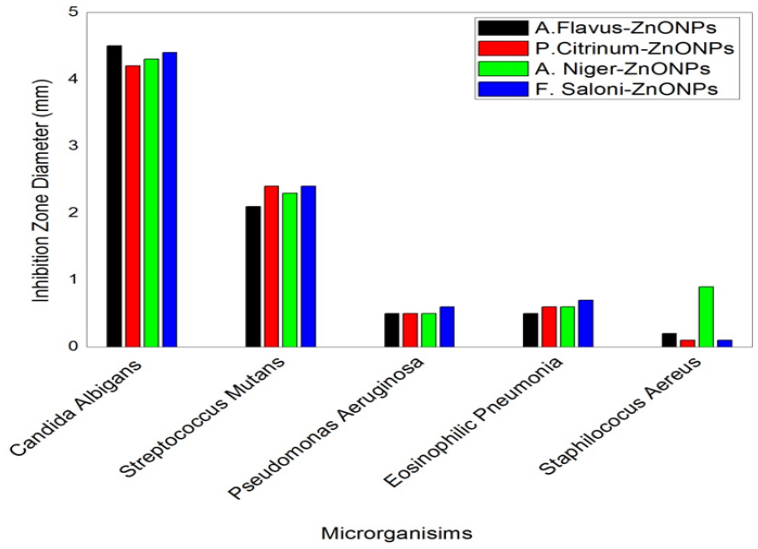

Figure 14. The comparison of the inhibition diameters of ZnONPs on the same bacteria.

Table 5. The zone of inhibition (ZOI) values of ZnONPs.

Bacteria	ZnONPs			
	P. citrinum	*F. solani*	*A. flavus*	*A. niger*
Candida albicans	4.2 cm	4.4 cm	4.5 cm	4.3 cm
Streptococcus mutans ATCC 10449 s	2.4 cm	2.4 cm	2.1 cm	2.3 cm
Pseudomonas aeruginosa ATCC 27853	0.5 cm	0.6 cm	0.5 cm	0.5 cm
Eosinophilic pneumonia NRLLB4420	0.6 cm	0.7 cm	0.5 cm	0.6 cm
Staphylococcus aureus ATCC 25923	0.1 cm	0.1 cm	0.2 cm	0.9 mm

4. DISCUSSION and CONCLUSION

In this study, zinc oxide nanoparticles (ZnONPs) were produced successfully using various fungal cultures. The chosen fungal species for ZnONPs production were observed to have substantial impacts on the physical and antibacterial characteristics of the nanoparticles. As a result of the structural analysis of the nanoparticles, it was found that all fungal cultures were appropriate for the production of ZnONPs. The ZnONPs, formed under hexagonal zinc oxide structure and dominant orientations, were identical in all cases. However, in ZnONPs produced solely in *A. flavus* mushroom culture, different phases formed within the structure, and the crystallization level was lower. The average grain size of the ZnONPs is between 53-61 nm. It was determined that ZnONPs synthesized through *P. citrinum* culture had the smallest grain size and the most favorable structure. ZnONPs synthesized from *P. citrinum*, *A. flavus*, and *A. niger* cultures were highly transparent (at a rate of 90%). The least transparent ZnONPs were synthesized from *F. solani* culture nanoparticles. Particle radii and bandgap values were measured to be in the range of 1-2 nm at 3.83 eV. It was determined that the bandgap, refractive index, and porosity values of the fungal cultures used did not affect the particle size but did affect other aspects of the nanoparticles. ZnONPs displayed high levels of resistance against *Candida albicans* and *Streptococcus mutans* bacteria. Furthermore, in contrast to other types of nanoparticles, ZnONPs produced from the *A. niger* fungal culture exhibited greater resistance against *Staphylococcus aureus* bacteria. Furthermore, the fungal diversity induced significant alterations in the morphological architecture of ZnONPs. The nanoparticles synthesized from *P. citrinum* and *A. niger* fungal cultures resembled each other with their nano-flake structure, while ZnONPs generated from *A. flavus* and *F. solani* fungal cultures adopted distinct morphological compositions.

Consequently, ZnONPs produced with different fungi can be utilized as an alternative biomaterial in applications such as an alternative filling and bracket material, particularly in dental applications due to their in vitro antimicrobial properties against *Candida albicans* and *Streptococcus mutans* bacteria.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Olcay Gençyılmaz: Conceptualization, Methodology, Validation, Data curation, Writing – review & editing, Writing – original draft, Supervision. **Mohanad Fawzi Mutar Mutar:** Methodology and Writing – original draft.

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Research Article

Chemical profile by GC-MS and protective effect of Algerian cloves (*Syzygium aromaticum*) against *Lactobacillus* spp. and *Streptococcus* spp. isolated from dental caries

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Abstract: The oral cavity is home to a large and diversified microbial flora, which plays an important role in the genesis of multiple diseases, including tooth decay. Indeed, tooth decay is the most common ailment in the world, with almost everyone having experienced it at least once in their lifetime. Examining plants used in traditional medicine is one of the research approaches used to discover novel, potent antibacterial chemicals with a broad spectrum of action, as present antibacterials have significant drawbacks. This study aims to examine the chemical composition of Algerian clove *Syzygium aromaticum* using GC-MS and to evaluate the antibacterial activity of the methanol extract against bacteria isolated from dental caries caused by *Streptococcus* spp. and *Lactobacillus* spp. The results show a strong extraction yield of 29.7%, with high amounts of polyphenols and flavonoids calculated at 178.82 mg GAEQ/g and 24.13 mg QEQ/g. The principal chemical elements of *S. aromaticum* peel methanol extract were identified as eugenol (61.23%) and eugenol acetate (26.45%) based on mass spectrum data and retention times. The methanol extract has a significant antibacterial effect against tested strains, with MICs ranging from 111.37 to 445.5 mg/mL. Higher concentrations of polyphenols resulted in a significant increase in inhibition zone diameter against S1 ($r^2 = 0.94$, $p < 0.001$), L3 ($r^2 = 0.94$, $p < 0.001$), L5 ($r^2 = 0.93$, $p < 0.001$), and L9 ($r^2 = 0.96$, $p < 0.001$).

1. INTRODUCTION

Dental caries, one of the most common chronic illnesses of the oral cavity worldwide, persists in the modern period despite access to the most advanced sciences and technologies in dental treatment. Bacterial fermentation of dietary carbohydrates, specifically sucrose, breaks down tooth-hard acellular tissue (Kabra *et al.*, 2012; Riaž *et al.*, 2023). One of the most pressing

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issues of our time is the ineffectiveness of antibiotic treatments. Given the numerous barriers to the use of known antibacterials, it is critical to search for novel, effective antibacterial compounds with a broad spectrum of action.

One of the study methodologies is to investigate plants used in traditional medicine. Because medicinal plants contain a high concentration of antimicrobial compounds, they have been shown to be beneficial in treating a number of ailments, including bacterial disorders (Featherstone, 2000). Several studies have tested plant extracts for antibacterial action against pathogenic bacteria (Dogruoz *et al.*, 2008; Amadi *et al.*, 2016; Mostafa *et al.*, 2018; Srikacha & Ratananikom, 2020). Medicinal plants are widely used in the treatment of dental caries and dental care-associated infections because they contain phytochemicals such as flavonoids, polyphenols, terpenes, and alkaloids (de Oliveira Carvalho *et al.*, 2020; Sharaf *et al.*, 2021; Foda *et al.*, 2022; El-Sherbiny & Mahmoud, 2022). Clove (*Syzygium aromaticum* (L.) Merr. & L.M.Perry) is one of the most expensive spices and has been used for centuries as a food preservative and for a number of medicinal purposes. Clove was originally grown in Indonesia, but it is now grown all over the world, mainly in Brazil's Bahia state. This plant has a high potential for use in food, cosmetics, medicine, and agriculture. It is a rich source of bioactive chemicals such as hydroxycinnamic acids, hydroxybenzoic acids, phenolic compounds, and flavonoids. Cloves' principal phenolic components include eugenol, eugenyl acetate, caryophyllene, and gallic acid, which account for their high antioxidant capabilities (Cortés-Rojas *et al.*, 2014; Gengatharan & Abd Rahim, 2023).

Clove plants are widely used as traditional remedies due to their anti-helminthic, anti-inflammatory, anti-spasmodic, anti-pyretic, anti-allergic, antifungal, anti-carcinogenic, anti-allergic, antiviral, antioxidant, anti-mutagenic, anti-arthritis, and anti-parasitic properties. Cloves are being employed for their antibacterial characteristics (Saikumari *et al.*, 2016; Ajobiewe et *al.*, 2022; Yakubu Bello *et al.*, 2022). This study aims to contribute to a better understanding of this plant and to promote its traditional use for therapeutic purposes. We investigated the chemical composition by GS-MS and in vitro the antibacterial activity of the methanol extract of *S. aromaticum* growing in northern Algeria. The agar-well diffusion method was used against *Lactobacillus* spp. and *Streptococcus* spp. isolated from dental caries.

2. MATERIAL and METHODS

2.1. Plant Material

The species used in this study is *S. aromaticum* (Table 1); however, it has also been known as *Eugenia caryophyllata*, *Eugenia caryophyllus*, and *Eugenia aromatica* (Penot, 2016; Kaur & Chandrul, 2017).

Table 1. Scientific classification of clove (Kaur & Chandrul, 2017).

Classification unit	Classification
Kingdom	Plantae
(unranked)	Angiosperms
(unranked)	Eudicots
(unranked)	Rosids
Order	Myrtales
Family	Myrtaceae
Genus	*Syzygium* Gaertn.
Species	*S. aromaticum* (L.) Merr. & L.M.Perry

The flower buds were employed; they are dark brown "nail" in color, 12 to 17 mm long, with a lower calyx (hypanthus) up to 4 mm thick, surmounted by four leathery and divergent lobes made up of the four fleshy sepals spreading in a cross. The four paler, non-spreading petals are yellow-brown and create a headpiece that conceals numerous bent stamens and a short upright style on a nectar-bearing disc at the base (Figure 1a). The bilocular inferior ovary's receptacle

tube is angular, wrinkled, and carries a large number of seeds. These buds have a distinct aroma and flavor that is fragrant, scorching, and pungent (Wichtl & Anton, 1999).

2.2. Methanol Extract Preparation

2.2.1. *Sampling and grinding*

The clove buds were procured at a local store in Jijel, Northeast Algeria, in April 2022. The samples were dried at room temperature for three days, then in an oven at 40°C for five days. Subsequently, the plants were ground with a coffee grinder until a fine dry powder was obtained (Figure 1b).

2.2.2. *Phenolic compounds extraction*

According to Owen *et al.* (1999), methanol is the ideal solvent for extracting phenolic chemicals because it boosts extraction efficacy and is easier to remove. It also works well when coupled with water (80%) (Qasim *et al.*, 2016; Nakilcioglu & Otles, 2021). The complete extract was made by macerating 30 g of clove powder with 300 mL of 80% methanol in a flask on a magnetic stirrer for 48 hours in the dark at room temperature. After maceration, the clove solution was filtered via filter paper and evaporated at 40°C in a Heidolph-type steam rota until the solvent was entirely evaporated (Figure 1c). The extract was collected and kept in the freezer at -20 °C until use.

Figure 1. *S. aromaticum* in forms: clove buds (a), crushed cloves (b), and clove methanol extract (c).

2.2.2.1. Extraction yield determination. The extraction yield is calculated as the weight of the clove methanol extract (Figure 1c) divided by the weight of the powdered plant (Figure 1b). This yield was computed as a percentage using Eq. (1):

$$Y(\%) = W_d/W_p \times 100 \quad (1)$$

where, Y is the extraction yield as a percentage, W_d is the weight of dry extract in grams, and W_p is the weight of the powdered plant in grams.

2.2.2.2. Phenolic compounds determination. The total polyphenols were determined using the Folin-Ciocalteu (FC) method (Slinkard & Singleton (1977), with gallic acid as a standard: 1 mL of clove extract was mixed with 1/10 mL of FC reagent and 2% Na_2CO_3. The mixture was stirred and incubated in the dark at room temperature for 2 hours. The absorbance was measured at 760 nm using a spectrophotometer. The results were expressed in mg gallic acid equivalent per gram dry weight (mg GAEQ/g) using the equation ($y = 6.574$ x, $r^2 = 0.99$) derived from the calibration curve established with gallic acid.

2.2.2.3. Flavonoids compounds determination. Determination was based on the principle of direct determination by aluminum trichloride using the method of Meda et al. (2005). Flavonoids have a free hydroxyl group in positron that, when combined with aluminum trichloride, forms a yellowish complex through ion chelation. The yellow color produced is proportional to the amount of flavonoids in the extract (Basli *et al.*, 2012). Then, 2 mL of crude methanol extract was combined with 2 mL of aluminum chloride methanol solution (2% $AlCl_3$). After 15 minutes, a spectrophotometer was used to read the wavelength at 415 nm against a blank. A calibration curve was generated in parallel under the same operating conditions, with

Quercetin serving as a positive control. The results are expressed in milligrams of Quercetin equivalent per gram dry weight (mg QEQ/g) using the equation obtained from the standard curve (y = 31.68 x, r^2 = 0.99).

2.3. GC-MS analysis of *S. aromaticum* methanol extract

The phytochemical compounds in the previously prepared crude methanolic extract of *S. aromaticum* were analyzed using the Shimadzu GC-MS QP2010 El 70 ev quadrupole model. A mass-selective detector was designed with a 200°C ion source and a 250°C interface. MS analysis was performed using an OV 1701 capillary column with a film thickness of 0.25 µm and a length of 25 meters. Helium was used as a carrier gas in a split-less injection (20:0 split ratio) mode at 250 °C, with a volume of 1µL and a flow rate of 1.00 mL/min. The injection was performed at a constant linear speed of 40.6 cm/sec, with a purge flow of 1.2 mL/min and a total flow of 22.2 mL/min. The temperature was initially set at 90.0 °C and gradually increased to 250 °C at a rate of 10 °C per minute. The sample's total run time was set at 52 minutes. The mass spectrum range was set from 40.00 to 350.00 m/z.

2.4. Phytochemical Compounds Identification

To identify the compounds, their names, structures, and molecular weights were determined by comparing their spectra to those found in the National Institute of Standards and Technology database library (Nist05.LIB). Components were identified using GC retention time (RT), and MS fragment interpretation was performed by comparing the results to the Nisto5.LIB database.

2.5. Evaluation of the Antibacterial Activity of Methanol Extract

2.5.1. *Bacteriological samples*

A sterile excavator was used to collect ten samples from dental surgeons in Jijel wilaya from carious lesions and dentin softenings of patients with tooth decay. These samples were placed in sterile tubes with nutrient broth, transported to the laboratory, and incubated at 37°C for 24 hours.

2.5.2. *Isolation and identification of bacteria*

After incubation, the samples were isolated on Columbia and MRS agar plates to detect *Streptococcus* spp. and *Lactobacillus* spp., which are the most involved in the formation of dental caries. Sterile swabs were soaked in the nutrient bowls for each sample and deposited on the surface of the agar, which had previously been poured into a Petri dish and cooled before being inoculated using the streak technique. The dishes were then incubated at 37°C for 24 h for *Streptococcus* spp. and 48 h for *Lactobacillus* spp. Following incubation, the bacteria were identified in three stages:

• *macroscopic examination* consists of studying colony morphology (shape, appearance, outline, surface, color) from cultures obtained on agar of Colombia and MRS media;

• *microscopic examination* is performed on a bacterial smear, prepared from the suspect colonies in pure cultures, then fixed and stained by the Gram method to determine their morphology and Gram type (positive or negative Gram);

• *biochemical identification of bacteria* was performed using API 20 campy, catalase, and oxidase assay (Delarra, 2007).

2.5.3. *Transplanting bacterial strains*

The various bacterial strains were transplanted onto appropriate agar media using the streak method 24-48 h before antibacterial activity testing and then incubated in an oven at 37°C for 24-48 h to obtain a fresh culture and isolated colonies (La *et al.*, 2008).

2.5.4. *Inoculum preparation*

Colonies well isolated from fresh cultures were transferred to tubes containing sterile physiological water to produce bacterial suspensions with turbidity close to 0.5 McFarland (10^6 CFU/mL) (Kablan *et al.*, 2008; Kuete *et al.*, 2010; Souad *et al.*, 2010).

2.5.5. *Aromatogram*

The antibacterial activity of the methanolic extract was assessed using the Mueller-Hinton agar diffusion method (Souad *et al.*, 2010). The culture medium is poured into 90-mm Petri dishes. After solidification, each dish is inoculated with a sterile swab soaked in bacterial inoculum. After inoculation, sterile 5 mm diameter Whatman No. 1 paper discs were placed on the seeded agar with sterile forceps and filled with 10 µL of different concentrations of clove methanol extract. The plates were kept in the fridge at 5 °C for 2 hours to allow the plant extract to diffuse and then incubated at 37 °C for 24 hours. The absence of microbial growth around the discs creates a translucent halo (inhibition zone). The inhibition zone diameter is measured and expressed in mm. The negative control contained methanol, whereas the positive contained penicillin.

2.5.6. *Determination of minimum inhibitory concentration (MIC)*

The disk diffusion method, as described by Aneja and Joshi (2010) and Mostafa *et al.* (2018), was used to determine the MIC values of *S. aromaticum* extract concentrations in Mueller-Hinton agar, which was then inoculated with pathogenic strain bacterial suspensions. Because of its coagulated appearance, the methanol extract of *S. aromaticum* was first diluted by a decimal dilution (1/10) to a concentration of 891 mg/mL. Different concentrations of clove methanol extract were prepared separately in a geometric progression of 1/2 ratio, with concentrations ranging from 891, 445.5, 222.75, 111.37, and 55.68 mg/mL. Filter paper discs (8 mm in diameter) were moistened with 10 µL of various clove methanol extract concentrations and placed on top of Mueller-Hilton agar plates. All Petri dishes containing the seeded medium were refrigerated at 5 °C for 2 h to allow clove extract diffusion before being incubated at 37 °C for 24 h. The MIC was determined to be the extract's lowest concentration, indicating a distinct zone of inhibition (Nkere & Iroegbu, 2005; Aneja *et al.*, 2009).

2.6. Statistical Analysis

Three repetitions were performed for each concentration to calculate the standard deviation (SD). The ORIGIN 6.0 system was used to conduct the statistical analysis, which involved testing univariate variance (one-way ANOVA). The results were expressed as mean ± SD. A distinction was considered insignificant when $p > 0.05$ (NS), significant when $0.01 < p < 0.05$, very significant when $0.001 < p < 0.01$, and highly significant when $p < 0.001$. The correlation matrices between polyphenols and antibacterial activity were examined using STATISTICA Version 10.

3. RESULTS

3.1. Extraction Yield and Contents of Polyphenols and Flavonoids

The methanol extract of *S. aromaticum* produced a high yield of 29.7%. 8.91 g of dry residue was recovered from 30 g of powdered plant, containing 178.82 mg GAEQ/g polyphenols and 24.13 mg QEQ/g flavonoids.

3.1. Macroscopic and Microscopic Identification of Isolated Strains

Figure 2 and Figure 3 show the macroscopic and microscopic appearance of the isolated strains, respectively. Figure 2 depicts the colonies of isolated bacteria from the Columbia medium as a cream-colored mat. On MRS medium, growth results in the formation of isolated cream-white colonies with a uniform outline.

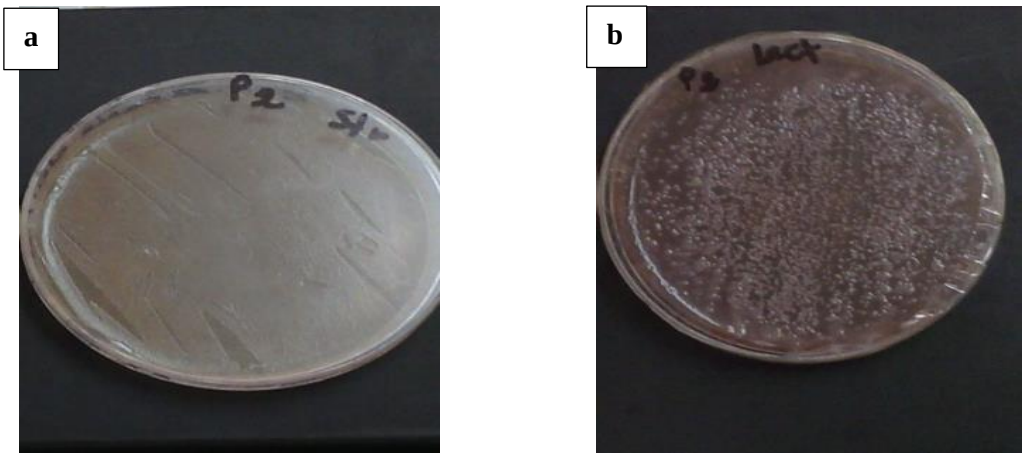

Figure 2. Macroscopic appearance of isolated bacterial colonies on Columbia Agar (a) and MRS Agar (b)

Gram staining revealed two types of cell arrangements: isolated and chain-shaped rounded cocci (Streptococci) from Columbia Agar (a) and isolated bacilli from MRS Agar (b).

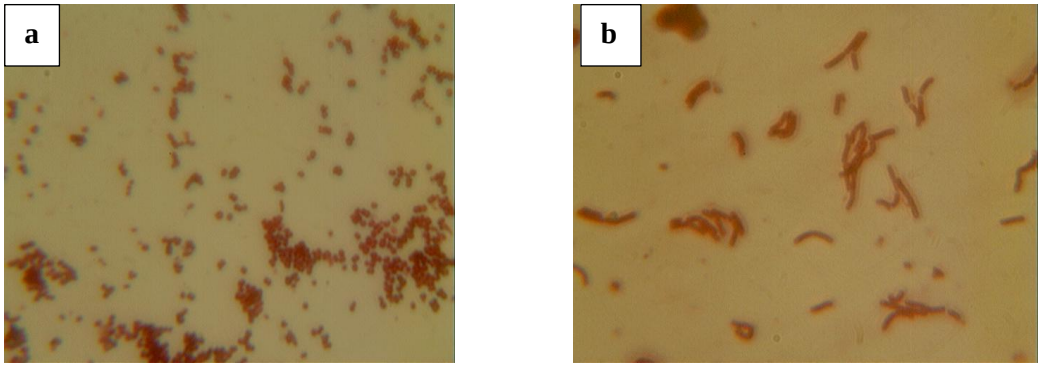

Figure 3. Microscopic appearance of isolated bacterial colonies on Columbia Agar (a) and MRS Agar (b) after Gram coloration

Streptococcus spp. were isolated from all ten samples, but *Lactobacillus* spp. could only be isolated from samples 1, 2, 3, 5, 9, and 10. Streptococci were found to be the most common etiological agents in the formation of dental caries, as opposed to lactobacilli. Table 2 lists the biochemical characteristics of the isolated bacteria. The findings clearly indicate that the samples belong to the genera *Streptococcus* spp. and *Lactobacillus* spp.

Table 2. Biochemical characteristics of isolated bacteria.

Biochemical test	ONPG	LDC	ODC	ADH	Citrate	H_2S	Urease	TDA	Indole	VP	Gelatinase	Glucose	Mannose	Inositol	Sorbitol	Raffinose	Saccharose	Melibiose	Amylase	Arabinose	Catalase	Oxidase
S	+	+	+	+	+	-	+	+	-	+	+	-	+	-	-	+	+	+	+	+	-	-
B	+	+	+	+	+	-	+	+	-	-	-	+	+	-	+	+	-	-	+	+	-	-

S. Streptococci, B. Bacilli, +. Positive reaction, -. Negative reaction

3.3. Estimation of Chemical Constituents of *S. aromaticum* by GC-MS

A GC-MS analysis of the chemical composition of *S. aromaticum* methanol extract revealed 31 peaks (Figure 4). Table 3 lists *S. aromaticum*'s phytochemical components, including their names and chemical formulas, concentrations (%), retention time (RT), and biological activities.

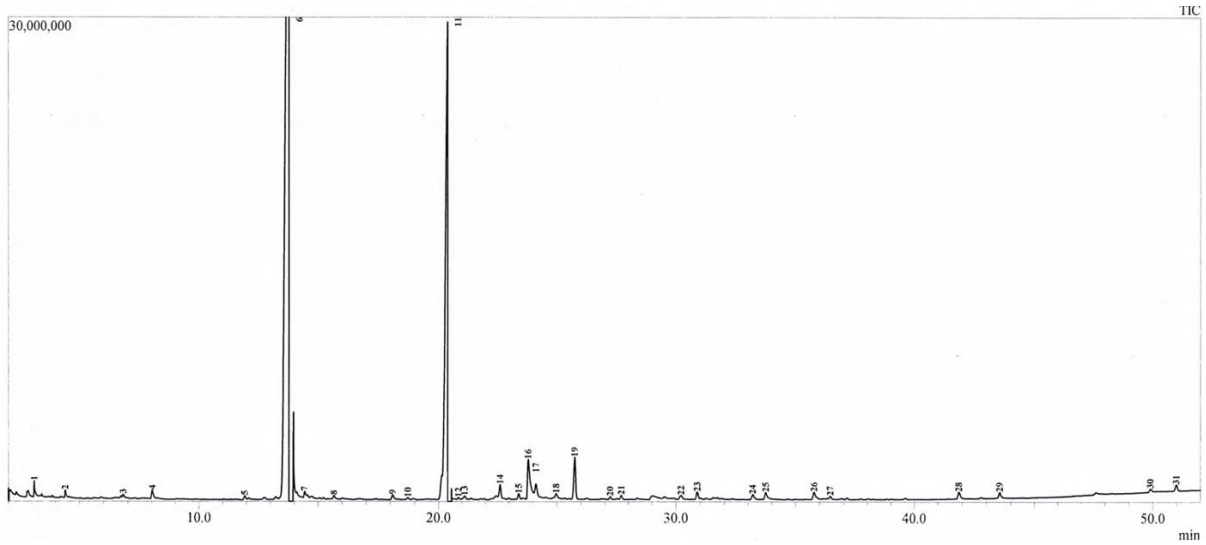

Figure 4. GC-MS chromatogram of methanol extract of *S. aromaticum*, where the x-axis represents retention time (mn) and the y-axis represents abundance.

By comparing mass spectral data and retention times, the main chemical constituent of *S. aromaticum* peel methanol extract was identified as eugenol (61.23%), which was detected at 13.177 mn. The other major constituent found in the methanol extract was eugenol acetate (26.45%) at 20.371 mn, followed by Pyrogallol (2.72%) and 2',3',4' Trimethoxyacetophenone (1.99%), while the other components had low values ranging from 0.09% to 0.74%.

The 20 chemical components of *S. aromaticum* are exhibited in biological activities including Pyrogallol, 2',3',4' Trimethoxyacetophenone, Benzyl Benzoate as antibacterials, 4H-Pyran-one, 2,3-dihydroxy-3,5-dihtdroxy-6-methyl-, Pentadecanoic acid, 14-methyl, methyl ester, 9-Octadecanoic acid (Z)-, methyl ester as antioxidants, caryophyllene, 2-Furancarboxaldehyde, 5-(hydroxymethyl)-, Caryophyllene oxide, estragole as anti-inflammatory, vanillin as anti-carcinogenic, and antioxidant, androsterone as neurosteroid and anticonvulsant, 4H-1-Benzopyran-4-one, 5-hydroxy-7-methoxy-2-methyl as antioxidant and anti-inflammatory, naphthazarin as antibacterial and antifungal, Di-n-octyl phthalate as antimicrobial and insecticidal (Table 3). The major chemical constituents of eugenol and eugenol acetate exhibit a variety of biological activities, including antibacterial, antiviral, and antifungal properties. Eugenol has anticancer, anti-inflammatory, and antioxidant properties.

Table 3. Lists of chemical components of *S. aromaticum* detected by GC-MS.

PN	Name of compounds	Molecular formula	RT	%	Biological activities	References
1	D-Alaninol	C_3H_9O	3.092	0.40	Undefined	
2	dl-Glyceraldehyde dimer	$C_6H_{12}O_6$	4.416	0.22	Undefined	
3	Cyclopentane, 1-acetyl-1,2-epoxy-	$C_7H_{10}O_2$	6.817	0.13	Undefined	
4	4H-Pyran-one, 2,3-dihydroxy-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	8.018	0.40	Antioxidant	Chen *et al.* (2021)
5	Caryophyllene	$C_{15}H_{24}$	11.936	0.15	Anti-inflammatory	Gyrdymova & Rubtsova (2021)
6	Eugenol	$C_{10}H_{12}O_2$	13.771	61.23	Antibacterial, antiviral, antifungal, anticancer, anti-inflammatory and antioxidant	Ulanowska & Olas (2021); Cheikhyoussef *et al.* (2022)
7	2-Furancarboxaldehyde, 5-(hydroxymethyl)-	$C_6H_6O_3$	14.436	0.16	Anti-inflammatory	Brustugun *et al.* (2005); Lu *et al.*

						(2005); Xu *et al.* (2007)
8	2(3H)-Furanone, dihydro-4-hydroxy-	$C_4H_6O_3$	15.666	0.18	Undefined	
9	Vanillin	$C_8H_8O_3$	18.096	0.25	Anti-carcinogenic, antioxidant	Arya *et al.* (2021)
10	Butanoic acid, 3-oxo-, 1-methylpropyl ester	$C_8H_{14}O_3$	18.726	0.09	Undefined	
11	Eugenol acetate	$C_{12}H_{14}O_3$	20.371	26.45	Antibacterial, antiviral, antifungal	Hemeda *et al.* (2022)
12	α-Cedrene	$C_{15}H_{24}$	20.843	0.17	Undefined	
13	Lingustral	$C_9H_{14}O$	21.111	0.25	Undefined	
14	Caryophyllene oxide	$C_{15}H_{24}O$	22.626	0.64	Anti-inflammatory	Gyrdymova & Rubtsova (2021)
15	Androsterone	$C_{19}H_{30}O_2$	23.417	0.24	Neurosteroid Anticonvulsant	Reddy & Rogawski (2012); Zolkowska *et al.* (2014)
16	Pyrogallol	$C_6H_6O_3$	23.803	2.72	Antibacterial	Tinh *et al.* (2016); Oliveira *et al.* (2022)
17	10-12-Pentacosadiynoic acid	$C_{25}H_{42}O_2$	24.130	0.74	Undefined	
18	Acetophenone, 4'-hydroxy-	$C_8H_8O_2$	24.968	0.21	Antifungal	Mohammadi Ziarani *et al.* (2020)
19	2',3',4' Trimethoxyacetophenone	$C_{11}H_{14}O_4$	25.739	1.99	Antibacterial	Freitas *et al.* (2020)
20	2H-1-Benzopyran, 6,7-dimethoxy-2,2-dimethyl-	$C_{13}H_{16}O_3$	27.220	0.13	Undefined	
21	Benzyl Benzoate	$C_{14}H_{12}O_2$	27.692	0.19	Antibacterial	Diastuti *et al.* (2019)
22	Pentadecanoic acid, 14-methyl, methyl ester	$C_{19}H_{34}O_2$	30.217	0.23	Antioxidant	Vijisaral Elezabeth & Arumugam (2024)
23	Palustrol	$C_{15}H_{26}O$	30.888	0.32	Undefined	
24	Clovane diol	$C_{15}H_{26}O_2$	33.196	0.31	Undefined	
25	4H-1-Benzopyran-4-one, 5-hydroxy-7-methoxy-2-methyl	$C_{17}H_{14}O_5$	35.743	0.43	Antioxidant, anti-inflammatory	Gupta *et al.* (2023)
26	9-Octadecanoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	35.777	0.43	Antioxidant	Mazumder *et al.* (2020)
27	Naphthazarin	$C_{10}H_6O_4$	36.455	0.12	Antibacterial, antifungal	Ryu *et al.* (1993) Duvauchelle *et al.* (2021)
28	3-[(Benzo[1,3]dioxol-5-ylmethylene)-amino]-2-methyl-5,6,7,8-tetrahydro-3H-benzo[4,5]thieno[2,3-d]pyrimidin-4-one	$C_{19}H_{17}N_3O_3S$	41.873	0.41	Undefined	
29	Pyrrole-3-carboxylic acid, 5-(3-hydroxypropyl)-2-methyl-5-(1,2,4(4H)-triazol-3-yl)-,ethyl ester	$C_{16}H_{20}N_6O_4$	43.551	0.31	Undefined	
30	Di-n-octyl phtalate	$C_{24}H_{38}O_4$	49.911	0.15	Antimicrobial, insecticidal	Huang *et al.* (2021)
31	Estragole	$C_{10}H_{12}O$	50.984	0.35	Anti-inflammatory	Rodrigues *et al.* (2016)

3.4. Antibacterial Activity of *S. aromaticum* Methanol Extract

The results in Table 4 and Table 5 show the antibacterial activity of *S. aromaticum* methanol extract against *Streptococcus* spp. and *Lactobacillus* spp. isolated from various samples in the form of bacterial growth inhibition halos around the discs, respectively. Table 6 shows the antibacterial activity results for the negative control (filled with methanol) and positive control (penicillin).

Table 4. Antibacterial activity of clove methanol extract against *Streptococcus* spp. $p = 0.011$.

	Inhibition zone diameters (mm)				
	Clove methanol extract concentrations (mg/mL)				
Isolates	891	445.5	222.75	111.37	55.68
S1	23.33±1.52	12±2.64	0	0	0
S2	17.67±0.57	12±1	10±0	9±0	0
S3	9±0	8.5±2.12	0	0	0
S4	22.67±3.05	9.33±0.57	8.67±0.57	8.33±0.57	0
S5	17.33±0.57	9±1	7.5±0.70	7±0	0
S6	23.33±1.15	12.67±0.57	10.33±0.57	9±0	0
S7	11.33±1.52	9.5±0.70	9±1	8.33±1.54	0
S8	15±0	13.69±0.79	0	0	0
S9	15.33±0.57	11.33±1.15	9 .67±0.57	9.67±2.51	0
S10	10±1.73	9.33±0.57	9±0	9.33±1.15	0

S: Streptococcus

The data in the table are represented as the mean ± SD.

Table 5. Antibacterial activity of clove methanol extract against *Lactobacillus* spp. $p = 0.00368$.

	Inhibition zone diameters (mm)				
Isolates	Clove methanol extract concentrations (mg/mL)				
	891	445.5	222.75	111.37	55.68
L1	36.33±1.50	16.5±2.12	10.67±1.15	10±0	0
L2	24.67±2.08	12.5±0.70	12±0	11±0	0
L3	35±0	11.5±2.12	9±0	0	0
L5	40±0	28±2	19±1.41	9±0	0
L9	40±0	18.33±0.57	13	8.50	0
L10	19.67±4.72	9.67±0.57	9.67±0.57	9±0	0

L: Lactobacillus

The data in the table are represented as the mean ± SD.

According to Table 4, *Streptococcus* spp. exhibit remarkable sensitivity to methanol extract, with an increase in the diameters of the inhibition zones observed at various extract concentrations. Except for S1, S3, and S8, all other *Streptococcus* spp. strains were slightly more sensitive, with a zone of inhibition at 111.37 mg/mL. Table 5 shows that Lactobacilli strains exhibit hypersensitivity, as evidenced by the large diameters of the inhibition zones when compared to Streptococci strains. These findings indicate that Lactobacilli are more sensitive to *S. aromaticum*'s methanol extract than Streptococci.

Clove's antibacterial properties demonstrated effective inhibition of test bacterial strains at an extract concentration of 891 mg/mL. The maximum zone of inhibition was against *Lactobacillus* spp. isolates L5 and L9 (40 mm), followed by L1 (36.33 mm) and L3 (35 mm), 2 (24.67 mm), S1 and S6 (23.33 mm), and S4 (22.67 mm). Most of the other extract concentrations tested against the other isolates resulted in inhibition zone diameters ranging from 8.33 mm to 19.67 mm. Except for L3, the inhibitory effect of *S. aromaticum* extract on all tested *Lactobacillus* spp. began at 111.37 mg/mL, with inhibition zones ranging from 8.5 to 11 mm.

Table 6. Antibacterial activity of Penicillin and Methanol (80%) against *Streptococcus* spp. and *Lactobacillus* spp.

Streptococcus spp.	Inhibition zone diameter (mm)		*Lactobacillus* spp.	Inhibition zone diameter (mm)	
	Penicillin	Methanol (80%)		Penicillin	Methanol (80%)
S1	18	0			
S2	17	0			
S3	20	0	L1	22.5	0
S4	19.5	0	L2	23	0
S5	18.5	0	L3	23	0
S6	22	0	L5	24	0
S7	21	0	L9	24.5	0
S8	20	0	L10	22	0
S9	19.5	0			
S10	21.5	0			

Table 7 summarizes the MIC values for *S. aromaticum* methanol extract against *Streptococcus* and *Lactobacillus* spp. It can be noted that 60% of the tested *Streptococcus* spp. had the same MIC of 111.37 mg/mL (S2, S4, S6, S7, S9, and S10). However, except for L3, methanol extract has the same MIC of 111.37 against all tested *Lactobacillus* spp.

Table 7. MIC of *S. aromaticum* methanol extract against *Streptococcus* spp. and *Lactobacillus* spp.

Streptococcus spp.	Minimum inhibitory concentration of *S. aromaticum* extract (mg/mL)	*Lactobacillus* spp.	Minimum inhibitory concentration of *S. aromaticum* extract (mg/mL)
S1	445.5	L1	111.37
S2	111.37	L2	111.37
S3	445.5	L3	222.75
S4	111.37	L5	111.37
S5	445.5	L9	111.37
S6	111.37	L10	111.37
S7	111.37		
S8	445.5		
S9	111.37		
S10	111.37		

3.5. Correlation analysis

Figure 5 and Figure 6 show correlation matrices between polyphenols in mg gallic acid equivalent per g dry weight, corresponding in dilutions $(GAEQ/g)_{cd}$ of the methanolic extract and inhibition zone diameters against *Streptococcus* spp. and *Lactobacillus* spp. (Table 8). The findings reveal significant positive correlations between polyphenols and inhibition zone diameters for all tested strains.

Table 8. Corresponding polyphenols in methanol extract dilutions.

Dilution ratio	Concentrations (mg/mL)	Polyphenols $(GAEQ/g)_{cd}$
1/10	891	159.5
1/20	445.5	79.75
1/40	222.75	39.87
1/80	111.37	19.93
1/160	55.68	9.96

$(GAEQ/g)_{cd}$: mg gallic acid equivalent per g dry weight corresponding in dilutions

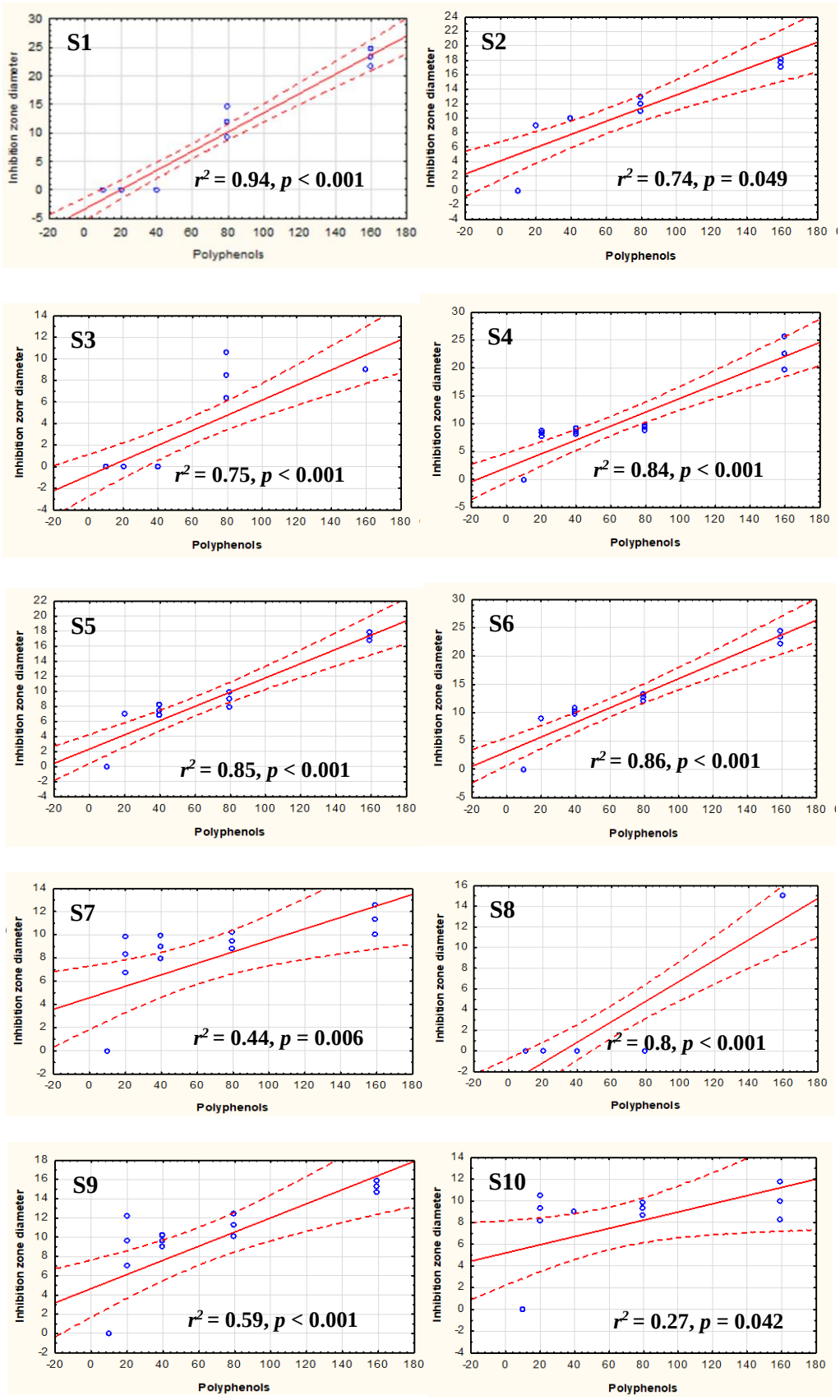

Figure 5. Correlation matrices between polyphenols and inhibition zone diameters against *Streptococcus* spp.

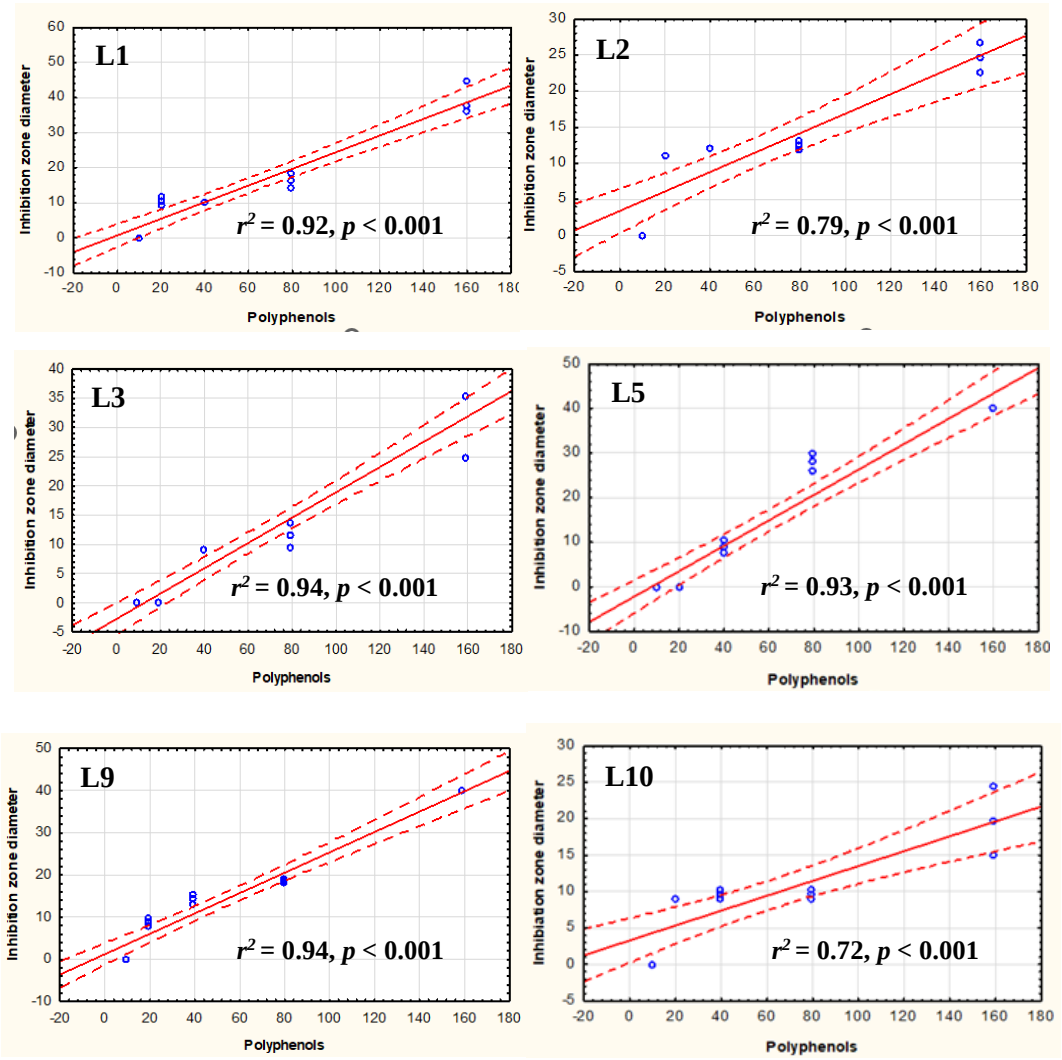

Figure 6. Correlation matrices between polyphenols and inhibition zone diameters against *Lactobacillus* spp.

4. DISCUSSION and CONCLUSION

According to a review of the studies, methanol is typically identified as the most effective solvent for phenolic compound extraction due to its polar property and ability to solubilize and recover optimal amounts of plant active components (Sultana *et al.*, 2014; Qasim *et al.*, 2016; Shafira *et al.*, 2020; Nakilcioglu & Otles, 2021; Benhamada *et al.*, 2022). In the case of our experiment, we were able to obtain a high extraction yield estimated at 27 %, which differs from the results found by Sabiu-Haxhijaha *et al.* (2021), who obtained yields of 5.67% and 1.07% using hydrodistillation and ultrasound extraction methods, respectively. Khan *et al.* (2022) indicated that the maximum yield of clove extract was 28.2%.

Using GC-MS for quantitative phytochemical screening, the *S. aromaticum* methanol extract was discovered to contain 31 compounds, with the largest peak area at 13.77 RT and 20.37 RT, indicating the presence of eugenol (61.23%) and eugenol acetate (26.45%), respectively, among its principal chemical constituents. Bhuiyan *et al.* (2010) identified 31 components in *Syzigium caryophyllatum* bud oil, the most important of which were eugenol (49.7%), caryophyllene (18.9%), benzene,1-ethyl-3-nitro (11.1%), and benzoic acid,3-(1-methylethyl) (8.9%). Eugenol was the most abundant oil constituent in *S. aromaticum* buds (72.08-82.36%), with eugenyl acetate essential oil (8.6 - 21.3%) coming in second (Kaur *et al.*, 2017).

Our findings are consistent with those of Ratri *et al.* (2020), who discovered that eugenol (85.01%) and eugenyl acetate (13.06%) are the main components detected by GC-MS in Island clove oil. Nonetheless, the petroleum ether extract of *S. aromaticum* contained the primary

chemical constituents eugenol (21.72%), phenol, 2 methoxy-4-(2-propenyl)-acetate (16.75%), espintanol (10.41%), and caryophyllene oxide (9.55%) (Alghazzaly *et al.*, 2022). Several other studies have also reported that eugenol is the primary component of cloves (Cortés-Rojas *et al.*, 2014; Cheikhyoussef *et al.*, 2022: Jadhav *et al.*, 2022; Khan *et al.*, 2022; Kiralan & Ketenoglu, 2022; Frohlich *et al.*, 2023; Gengatharan & Abd Rahim, 2023).

Bacteria are one of the leading causes of tooth decay. In this study, microbial analysis of tooth decay revealed the presence of two Gram-positive bacterial genera: *Lactobacillus* spp. and *Streptococcus* spp. Munson *et al.* (2004) found that Gram-positive bacteria dominate the oral bacterial flora, and Prajapati and Raol (2013) isolated *Streptococcus* spp. and *Lactobacillus spp.* from dental caries, confirming these findings. Our results are also consistent with those of Aneja and Joshi (2009) and Prajapati and Raol (2014), who indicated that *Streptococcus* spp. and *Lactobacillus* spp. are the primary, culturable agents responsible for caries lesions.

Another study conducted by Almaamori (2023) revealed that bacteria associated with tooth decay include *Staphylococcus* spp., *Streptococcus pyogenes*, *Bacillus cereus*, *Escherichia coli*, and *Proteus* spp. Mallya and Mallya (2020) reported the same conclusions. In fact, *Streptococcus mutans*, *Lactobacillus*, and *Actinomycetes* were the bacteria most frequently associated with tooth decay, despite the fact that *Lactobacillus* spp. is not the caries initiator but does play a role in the development and progression of dentin caries. *Streptococcus* spp. was isolated from 10 samples, leading us to conclude that *Streptococcus* spp. isolates are the most common etiological bacteria of dental caries. Cai and Kim (2023) and Riaz *et al.* (2023) found that *Streptococcus mutans* is the most commonly associated with dental caries.

To assess the efficacy of medicinal plant extracts, which can rival that of antibacterials, we evaluated the antibacterial activity of the methanol extract of *S. aromaticum* in this work. The findings show that methanol clove extract has significant activity against the tested strains, resulting in varying inhibition zone diameters depending on the concentration used ($p = 0.011$ and $p = 0.00368$ against *Streptococcus* spp. and *Lactobacillus* spp., respectively). Our findings are consistent with those of Alghazzaly *et al.* (2022), who found that *S. aromaticum* extract has antibacterial activity against strains of the viridans group Streptococci, indicating a potential natural treatment alternative.

Our results also indicate that methanol extract from cloves has significant antimicrobial activity in the bacteria tested. Hugar *et al.* (2017) reported that clove oil was highly effective against *E. faecalis* and could thus be used as a disinfectant. Yakubu Bello *et al.* (2022) concluded that clove oil could be used as a natural preservative because it inhibits the growth of Bacillus cereus. Ajobiewe *et al.* (2022) mentioned the same result, indicating that clove has antibacterial activity against antibiotic-resistant *Escherichia coli*. The observed variation in inhibition zone diameters can be attributed to either differences in bioactive molecule composition in the extract or their mechanism of action on Gram-positive bacteria.

Numerous studies have demonstrated the antibacterial effect of natural active ingredients. *S. aromaticum*'s antibacterial properties are thought to be due to its high polyphenol and flavonoid content (Shrivastava *et al.*, 2014; Oshomoh *et al.*, 2015). Increasing polyphenol concentrations resulted in a significant increase in inhibition zone diameter against tested strains, especially against S1 ($r^2 = 0.94$, $p < 0.001$), L3 ($r^2 = 0.94$, $p < 0.001$), L5 ($r^2 = 0.93$, $p < 0.001$), and L9 ($r^2 = 0.96$, $p < 0.001$). According to Alghazzaly *et al.* (2022), *S. aromaticum*'s antibacterial activity is due to polyphenolic constituents such as eugenol, phenol, 2-methoxy-4-(2-propenyl)-acetate, espintanol and Caryophyllene oxide.

Saini et *al.* (2019) and Bai *et al.* (2023) reported that eugenol is the main component of clove essential oil, and eugenol clearly has antibacterial effects on *S. aureus* and *E. coli* related to cell wall and membrane damage, inhibition of biofilm formation, oxidative stress-mediated apoptosis, and disruption of DNA synthesis. Joseph and Sugatha (2011) discovered that clove has antibacterial properties against a variety of foodborne pathogens, including *Staphylococcus aureus*, *Staphylococcus epidermis*, *Bacillus cereus*, *Bacillus subtilis*, and *Bacillus sp*.

According to the findings of Shehadi *et al.* (2014), these substances have been shown in vitro to have effective antimicrobial activity against a wide range of microorganisms. Their mode of action is most likely due to their ability to form a complex with soluble extracellular proteins from the bacterial wall, which destroys the cell membrane. In the same context, essential oils, flavonoids, and polyphenols may cause potassium ion leakage at the membrane level, resulting in irreversible membrane damage. This potassium permeability is a precursor to death (El-Haci *et al.*, 2012).

The MIC results of methanolic extracts indicated that *S. aromaticum* could be used to control and prevent pathogenic bacteria. These findings are consistent with those of Mostafa *et al. (*2018), who discovered that the most potent plant extracts with bacteriostatic and bactericidal properties against highly susceptible strains of foodborne pathogenic bacteria (*S. aureus* and *P. aeruginosa*) were ethanolic extracts of *S. aromaticum*. Furthermore, it has been demonstrated that flavonoids' toxicity to microorganisms occurs either through the deprivation of metal ions such as iron or through non-specific interactions such as the formation of hydrogen bonds with proteins in microorganism cell walls (adhesins) or enzymes (Basli *et al.*, 2012).

In conclusion, the obtained results highlight the antibacterial properties of the methanol extract of Algerian clove due to its high bioactive component content, which showed significant growth inhibitions for all *Lactobacillus* spp. and *Streptococcus* spp. isolated from dental caries. These findings led us to propose using essential oils, such as cloves, as a natural antibiotic for the treatment of dental caries, as well as in the preparation of toothpaste and mouthwashes. Future research should focus on extracting, separating and purifying the bioactive components of *S. aromaticum*, as well as studying their mechanisms of action on antibiotic resistance in vivo.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Ouahiba Benhamada: Investigation, Resources, Visualization, Software, Formal Analysis, Methodology and writing original draft. **Nabila Benhamada:** Analysis, Interpretation and Language revision. **Lilia Boussouf:** Interpretation and Language revision. **Essaid Leghouchi:** Supervision and Validation

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