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Having well known board members distinguished scientists from different disciplines with huge experiences on MAPs all over the world, CUPMAP will be indexed in many databases after first issue. The goal of the journal is to be indexed in Thomson Routers in a short time.

CUPMAP is inviting papers for Volume 6 Issue 1, which is scheduled to be published on June, 2023. Last date of submission: June 15, 2023. However, an early submission will get preference in case of review and publication process. Please submit your manuscripts according to instructions for authors by the Journal online submission system.

Sincerely,
Prof. Dr. Nazım ŞEKEROĞLU
Editor-in-Chief
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Reviewers are selected according to their background and experience in some aspect of the subject. The most desirable reviewers identify the strengths and weaknesses of the submitted paper, and analyze it from different viewpoints. The peer reviewers are asked to read and analyze the assigned manuscript and provide a written opinion of its quality, novelty, relevance and suitability for publication in the "Current Perspectives on Medicinal and Aromatic Plants (CUPMAP)" Journal. Peer reviewers also make suggestions to assist the authors in improving the article. Reviewers must not only analyze and comment on the paper, but also provide opinions about general concerns such as clarity and quality of the writing, validity of scientific approach, and whether the article provides new information.

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Produce as careful and objective a review as possible Respect the editor's deadline. Consider with an open mind innovations or approaches different from those of one's own.

Provide a balanced critique targeted not only to identify the strengths and weaknesses of the paper, but also to provide useful feedback to the authors to improve their manuscript, without being overly critical of minor points.

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Direct comments about ethical concerns confidentially to the editors.

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Organized Critique

Assessment of Strengths and Weaknesses: the following should be evaluated: Literature review is up-to-date; Methods align with study purpose or research questions; Methods described in sufficient and appropriate detail; Research design or study approach is adequate; Approach to data analysis is appropriate; Thoughtful consideration given to the study limitations; Manuscript provides new information that is likely to be of interest to our readers.

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Commonly Overlooked Areas: Reviewers should carefully note: title, abstract, tables and figures, references.

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"Current Perspectives on Medicinal and Aromatic Plants (CUPMAP)" is an international journal, which publishes at the highest scientific level on original research articles dealing with Medicinal and Aromatic Plants in the fields of science and technology such as Biology, Molecular Biology and Genetics, Chemistry, Agriculture, Biochemistry, Botany, Ethnobotany, Environmental Science, Forestry, Horticulture, Health Care & Public Health, Nutrition and Food Science, Pharmaceutical Sciences, and so on. Originality, high scientific quality, and citation potential are the most important criteria for a manuscript to be accepted for publication. Manuscripts submitted for evaluation should not have been previously presented or already published in an electronic or printed medium. The journal should be informed of manuscripts that have been submitted to another journal for evaluation and rejected for publication. The submission of previous reviewer reports will expedite the evaluation process. Manuscripts that have been presented in a meeting should be submitted with detailed information on the organization, including the name, date, and location of the organization. All authors submitting their works to "Current Perspectives on Medicinal and Aromatic Plants (CUPMAP)" for publication as original articles attest that the submitted works represent their authors' contributions and have not been copied or plagiarized in whole or in part from other works. It is necessary to agree upon standards of expected ethical behavior for all parties involved in the act of publishing: the author, the journal editor, the peer reviewer and the publisher. "Current Perspectives on Medicinal and Aromatic Plants (CUPMAP)" ethic statements are based on COPE's Best Practice Guidelines for Journal Editors.

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- Manuscripts can only be submitted through the CUPMAP **online** manuscript submission and evaluation system https://dergipark.org.tr/tr/pub/cupmap
- The journal management requires that all authors register to **ORCID** at http://orcid.org/register and get a free ORCID ID.
- All submissions are screened by **iThenticate** similarity detection software and our maximum allowed score is **24%** for the document in which the References section truncated.

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Volume: 5, Issue: 2, December 2022

EXECUTIVE EDITORIAL BOARD _____ ii

JOURNAL INFORMATION _____ v

AIM AND SCOPE _____ vii

OPEN ACCESS STATEMENT _____ viii

COPYRIGHT POLICY _____ viii

PUBLICATION CHARGES _____ ix

PEER REVIEW PROCESS _____ ix

ETHIC RULES AND PLAGIARISM _____ xii

CUPMAP INSTRUCTIONS FOR THE AUTHORS _____ xvi

CUPMAP STRUCTURE OF THE MANUSCRIPT _____ xvii

Current Perspectives on Medicinal and Aromatic Plants
Curr. Pers. MAPs

An International Peer Reviewed Journal
ISSN: 2619-9645 | e-ISSN: 2667-5722

Qualitative and Quantitative Phytochemical Screening and Free Radical Scavenging Activity of Different Parts of Rubus ellipticus Sm.
Ananda LAMICHANE, Susmita KHATRI, Mamata DHUNGANA, Bijaya TRIPATHI, Namrata BHATTRAI, Rishiram BARAL , Nirmala JAMARKATTEL 106

Contribution to the Microscopic Study of Three Plant Species (Parsley, Spanish Scolyme and White Marrube) Commonly Used in Traditional Algerian Medicine
Sara HASSAÏNE, Nassima ELYEBDI, Hasnia MEDJAHED, Khawla YAZID 118

The Antioxidant Capacities of Leaf Extracts from *Salvia viridis* L.
Kemal KARAMAYA, Belgin COŞGE ŞENKAL 127

Anti-Acetylcholinesterase and Synergistic Antifungal Activities of Selected Salvia Species: Correlation with Metabolic Profiles
Gamze BENLI YARDIMCI, Nurnehir BALTACI BOZKURT, Cigdem KAHRAMAN, Ekrem Murat GONULALAN 136

Effect of chronic administration of aqueous extract of Neem (*Azadirachta indica*) leaves on Paracetamol-induced hepatotoxicity in Wistar albino rats
Pranob Jyoti BHARALI, Sushanta Kumar BORDOLOI, Swarnamoni DAS, Kingshuk LAHON.....146

Screening of Biological Potential of Pleurostylia opposita (Wall.) Alston. - A Rare Medicinal Plant of Eastern Ghat
Mahendra CHIKKAMADAIAH, Savitha Ramesh SOWBHAGYA, Ravindra Kembalu NARAYAN.....162

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Research article
Curr. Pers. MAPs, (2022); 5(2): 106-117

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Qualitative and Quantitative Phytochemical Screening and Free Radical Scavenging Activity of Different Parts of *Rubus ellipticus* Sm.

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Abstract

Free radicals are molecules with reactive unpaired electrons which are produced during cell metabolism and create the oxidative stress inside tissue resulting in tissue damage. The aim of the present study was qualitative and quantitative phytochemical screening, determination of total phenolic and total flavonoid content, and free radical scavenging activity of different parts of *Rubus ellipticus* Sm. plant. Root, stem, and leaves of *Rubus ellipticus* Sm. were collected from Annapurna Rural Municipality, Kaski, Western Nepal. Ethyl acetate and ethanol extracts of the plant parts were obtained by subsequent maceration process. The phytochemical screening of most of the extract showed the presence of phenols, carbohydrates, flavonoids, and glycosides. The ethanolic extract of stem showed the higher phenolic content with the value of 343.75 ± 2.21 µg GAE/mg. Ethanolic extract of stem had the highest amount of flavonoid content (1563.17 ± 10.79 µg QE/mg of extract), whereas all the ethyl acetate extracts of root, leaves and stem showed comparable flavonoid content. Ethanolic extracts of leaves showed potent DPPH free radical scavenging activity with IC50 value of 5.03µg/ml while ethyl acetate extract of stem showed the maximum free radical scavenging properties. The result depicted that the ethanolic extract of *Rubus ellipticus* Sm. showed the potent antioxidant activity by scavenging free radicals.

Keywords: Antioxidant, Ethnomedicine, Free Radical Scavenging, Phytochemicals, *Rubus ellipticus* Sm.

1. Introduction

Free radicals are molecules with one or more unpaired electrons (superoxide, hydroxyl, peroxyl) that are produced during cell metabolism are very reactive (Halliwell. 1995). Among different free radicals generated inside the cell during metabolic process, Reactive Oxygen Species (ROS) are most profound oxygen free radicals (Deweirdt et al., 2017; Valko et al., 2007). Because free radicals have a strong oxidizing functional group (Baliyan et al., 2022), they can create the oxidative stress inside tissue resulting in tissue damage (Erdemoglu et al., 2006). ROS are mainly generated into the mitochondria, endoplasmic reticulum, cytosol and plasma membrane of the cell (Polidori & Mecocci. 2022; Sies & Cadenas.

1985). Meanwhile there are some exogenous factors inducing ROS production such as; exposure to radiations, xenobiotic, tobacco etc. (Brown & Borutaite. 2012; Deweirdt et al., 2017). These ROS can cause oxidative damage of lipids, proteins, RNA, DNA, and many small molecules in cells (Halliwell & Gutteridge. 2015). Although the human body produces natural antioxidants to combat those free radicals, but oxidative stress and aging upsurge the production of free radicals in a way necessitating the use of exogenous antioxidants (Indradi et al., 2017).

Antioxidants are the compounds the protect the cells from ROS by donating electrons (Choudhary. 2015; Surabhi & Leelavathi. 2010), prevent the formation of free radical, scavenge, suppress or forms chelates with free radical, repair and eliminate damaged molecules (Cesquini et al., 2003; Gutteridge. 1994; Maxwell. 1995; Raj et al.). Antioxidants produced by the body are crucial for the preservation of healthy cellular processes. The three most effective enzymatic antioxidants are superoxide dismutase, catalase, and glutathione peroxidase. Non-enzymatic antioxidants encompass vitamins E and C, thiol, melatonin, carotenoids, natural flavonoids, and other substances. The standard antioxidant compounds derived from plant sources include vitamin C, vitamin E, carotene, quercetin, and tocopherol. Various plant-derived antioxidants are effective free radical scavengers that are utilized as a nutraceuticals in combination to treat a variety of diseases (Indradi et al., 2017; Raj et al.)

Oxidative stress is caused by an imbalance between antioxidants and reactive oxygen species, which leads to cellular damage, cancer, aging, ischemic injury, inflammation, and neurodegenerative diseases (Erdemoglu et al., 2006; Surabhi & Leelavathi. 2010). Due to the low toxicity and side effect, most of the plants has been using as a natural antioxidant to cure different diseases condition (Baral et al., 2021).

The revival of interest in plant derived drugs is mainly due to the current widespread belief that “green medicine” is safe and more dependable than the synthetic drugs, many of which have adverse side effects (Jigna et al., 2005). Plants are used medicinally in various countries and are the source of numerous potent and powerful drugs (Srivastava et al., 1996). A wide variety of medicinal plant parts such as root, stem, flower, fruit, and some other parts are being used as raw drugs having varied medicinal properties. Some community and folk healers collect a small quantity of plants for local use, while others collect a large amount and supply to the herbal industries (Atanassova et al., 2011; Sharma and Kumar. 2011; Uniyal et al., 2006). Plants produce diverse types of bioactive molecules making them a rich source of different types of medicines. This revival of worldwide interest in medicinal plants reflects recognition of the validity of many traditional claims regarding the value of natural products in health care (Bagewadi et al., 2014). The most important bioactive component of the plants are alkaloids, tannins, flavonoids, and phenolic compound. About 85% of the rural population of Nepal are said to use herbal remedies which is mainly due to the indigenous beliefs and lack of alternatives in rural areas. Acquaintance with different ethnic groups contributes to the development and research on natural products which as a result increases the knowledge about the close relationship between the chemical structure of a compound and its biological properties (Atanassova et al., 2011; Kunwar & Bussmann. 2008).

Natural species of the Rubus genus are not only a source of food, but they are also used as medicine (Sharma & Kumar. 2011). Rubus species are said to be the world's best-known infusion herbarium (Rojas-Vera et al., 2002). According to ethnomedicine, the leaves and fruit of *R. ellipticus* are used to treat bronchitis, nausea, ulcer, antimicrobial,

diabetes, and as a carminative and tonic (Subba et al., 2019; Vadivelan et al., 2009). The fruit extract of *R. ellipticus* has been shown not to be cytotoxic to normal cells, but rather to have a stimulatory effect on their proliferation, with cervical cancerous cells being particularly sensitive (Saini et al., 2014).

R. ellipticus root paste has been reported to be used as a poultice for bone fracture, severe headache , colic pain (Castleman. 1991; Patel et al., 2004). The root bark is also used in diarrhea, dysentery, as abortifacient, emmenagogue and in fractured bones (Kirtikar et al., 2001). It has been reported that its shoot was chewed to relieve stomach upset and that a root decoction was given to warm the stomach (Bhakuni et al., 1987; Yadav et al., 2011). In traditional Tibetan medicine, the inner bark of the *R. ellipticus* plant is prized for its therapeutic properties, which include uses as an anti-diuretic and renal tonic (Pfoze et al., 2012) . The juice of *R. ellipticus*, which has an appealing color and flavor, can be maintained in its natural form and is also useful for preparing squash and edible jam (Vadivelan et al., 2009).

This study was conducted to discover the scientific evidence of *R. ellipticus* using ethnomedicinal uses as a guide. The primary goal of this study is to perform and compare the phytochemical screening, total phenolic and total flavonoid content, and free radical scavenging activity of different parts of *R. ellipticus* Sm.

Table 1. List of the parts of plant with their voucher number

Scientific Name	Family	Local Name	Parts Used	Crude Drugs Voucher Number	Sample Number
Rubus ellipticus Sm.	Rosaceae	Ainselu (Himalayan Raspberry)	Root	PUCD-2020-CR16	CR16
			Stem	PUCD-2020-CR17	CR17
			Leaves	PUCD-2020-CR18	CR18

2. Material and Methods

2.1. Chemicals and Reagents

1,1 Diphenyl-2 Picryl Hydrazyl Radical (DPPH) was purchased from Tokyo Chemical Industry Co. Ltd., Japan. Benedict's reagent, sodium hydroxide, sodium nitrite, sodium carbonate, ethanol, lead acetate, ammonium hydroxide, sodium hydroxide pellets, mercuric chloride, ferric chloride, and hydrochloric acid were supplied from Thermo Fisher Scientific, Pvt. Ltd. India. L-ascorbic acid was purchased from Himedia Laboratory, India. Qalinges Fine Chemicals supplied the sulphuric acid while Ethyl acetate, copper sulphate pentahydrate pure, sodium anhydrous pure, and 1-napthol were purchased from Merck Specialties Pvt. Ltd., Germany.

2.2. Collection and Identification of Plant Material

The experimental plant parts (listed in **Table 1)** were collected (July 2019) from the Annapurna Rural Municipality in Kaski, Western Nepal, at an elevation of 4528 feet above sea level. Plant parts were collected and identified at the National Herbarium Kathmandu, (voucher specimen no. PUH-2022-08) while crude samples were stored at the Pharmacognosy Laboratory of Pokhara University's School of Health and Allied Sciences. The collected parts of the plant were chopped properly and subjected to shed drying. Hot air oven (40°C) was used to remove the moisture in the sample which was regularly monitored by weight variation test at various time interval. After complete

drying, it was finely powdered using a grinder.

2.3. Preparation of Plant Extract

The crude drug was extracted using successive maceration, as described by Pandey and Tripathi (2014) with minor modifications. Ethyl acetate and ethanol were selected as an extracting solvent was based on their polarity and availability of the solvent in the laboratory. 50-100 gm of the crude extract were macerated for 48 hours with enough (1:5 w/v) ethyl acetate to extract the plant sample. Following 48 hours, the filtrate was collected and concentrated using vacuum evaporator. The residue was then again macerated for 48 hours with enough ethanol (1:5 w/v ratio), and the final filtrate was collected and concentrated using vacuum evaporator again.

2.4. Phytochemical Screening

Phytochemical screening was measured according to the previous report explained by Yadav and Agarwala (2011) and Okoduwa, Umar et al. (2016) for qualitative determination of phytochemical compounds with slight modification.

2.5. Total Phenolic Content

The total phenolic content of the extract was determined using the Folin Ciocalteu Method, as described by Fattahi et al., (2014) and Kaur and Kapoor (2002) with minor modifications. 1mL of crude extract (1 mg/mL) was combined with 5 mL of distilled water and 1mL of the Folin-Ciocalteu reagent. After 5 minutes, 1mL of distilled water and 1mL of 10% (W/V) sodium carbonate were mixed and thoroughly shaken. Following 60 minutes interval, the absorbance at 725 nm was measured. Gallic acid is used as a positive control for phenolic compound as a standard. Gallic acid at concentrations ranging (15.63 mg/mL, 31.25 mg/mL, 62.5 mg/mL, 125 mg/mL, 250 mg/mL, and 500 mg/mL) were prepared in ethanol. The calibration curve was plotted using gallic acid as the standard as shown in **Figure 1.** The calibration curve was then used to calculate the total phenolic content of *R. ellipticus* (Different parts), and the results were presented as mg of gallic acid equivalent per gram dry extract weight.

2.6. Determination of Total Flavonoids Content

The flavonoid content in the extract was determined using the aluminum chloride colorimetric method given by Chang et al., (2002) and Chun et al., (2003) with slightly modification. In ethanol, different concentrations of quercetin (15.63 mg/mL, 31.35 mg/mL, 62.6 mg/mL, 125 mg/mL, 250 mg/mL, and 500 mg/mL) were prepared as a standard. 1 mL of each extract solution (1 mg/mL) was combined with 4 mL of distilled water and 0.3 mL of 5% sodium nitrite. After 5 minutes, 1.3mL of 20% aluminum chloride was added and left for another 6 minutes. The absorbance at 510 nm was immediately measured using a UV spectrophotometer after addition of 2 mL of 1 M sodium hydroxide. Total flavonoid content was determined with the help of calibration curve (**Figure 3)** and results were expressed as mg quercetin equivalent per gram dry extract weight as shown in **Table 5.**

2.7. DPPH free radical scavenging method

The free radical scavenging activity of different parts of the R. ellipticus extract was determined using the diphenyl-picrylhydrazyl (DPPH) assay, as described by Akter et al., (2010) and Jabbari and Jabbari (2016). The stock solution (1 mg/mL) was diluted with methanol to a dilution series (50 g - 1000 g/ml). An aliquot of each dilution (2 mL) was mixed with a methanolic solution of DPPH (0.06 nM) and

shaken at room temperature for 30 minutes. Followingly, a control containing a methanolic solution of DPPH (2 mL, 0.06 mM) and ethanol (2 mL) was run. The absorbance was measured at 512 nm against a blank of methanol. Ascorbic acid was used as a standard of comparison. **Equation 1** was used to calculate the percentage of free radical scavenging.

DPPH radical scavenging activity (%) = (Ao-A1)/Ao*100 ……**Equation 1**

[Where Ao = Control Absorbance (Ascorbic acid), A1 = Absorbance when a test sample is present].

3. Results and Discussion

3.1. Yield Value Determination

The extraction yield value of *R. ellipticus* stem, root, and leaves in ethyl acetate and ethanol was calculated and listed in the **Table 2.**

Table 2. Yield value of diffrent plant parts in different sovents

Plant	Common name	Parts	% Yield (Ethyl Acetate extract)	% Yield (Ethanol extract)
Rubus ellipticus Sm.	Aiselu	Leaves	1.66	6.95
		Root	1.68	10.78
		Stem	0.86	3.83

Table 3. Phytochemical screening of ethyl acetate(EA) and ethanolic extract

Compounds	Test	Parts of Plants					
		Leaves		Root		Stem	
		EA	Ethanol	EA	Ethanol	EA	Ethanol
Alkaloids	Mayer's Test	-	-	+	-	+	-
Carbohydrate	Molish Test	+	+	+	+	-	-
	Ferric Chloride test	+	+	+	+	+	+
Phenol	Lead Acetate Test	+	+	-	-	+	+
Flavonoid	Alkaline Test	+	-	-	-	-	-
Glycoside	Legal's Test	+	+	+	+	+	-

3.2. Phytochemical screening

The phytochemical screening of different parts extracts of *R. ellipticus* in different solvents showed the presence of alkaloid, carbohydrate, phenol, flavonoid and glycoside which is mentioned in the **Table 3.**

Ethyl acetate extract of *R. ellipticus* leaves contained phenol, glycosides, and flavonoids, while the root and steam contained carbohydrate and alkaloids as well. In the case of ethanolic extract, leaves contained carbohydrate, phenol, and glycoside, whereas root extract was negative in the lead acetate test. Meanwhile, the stem extract showed positive phytoconstituent result with phenol, flavonoid, and alkaloid.

3.3. Total Phenolic Content

The total phenolic content of all extracts was determined using the FC method with gallic acid as a standard. As shown in **Table 4**, all results were expressed as µg gallic acid equivalent per mg of extract. Among the extracts, ethanolic extract of *R. ellipticus* (Stem) had the highest phenolic content (342.75 ± 2.21 µg GAE/mg of extract), while ethyl acetate extract had the lowest phenolic content (40.92 ± 4.25 µg GAE/mg of extract). depicts the calibration curve for gallic acid. All determinations were made in triplicate, and the results were expressed as mean ± SD as shown in **Table 4** and **Figure 2.**

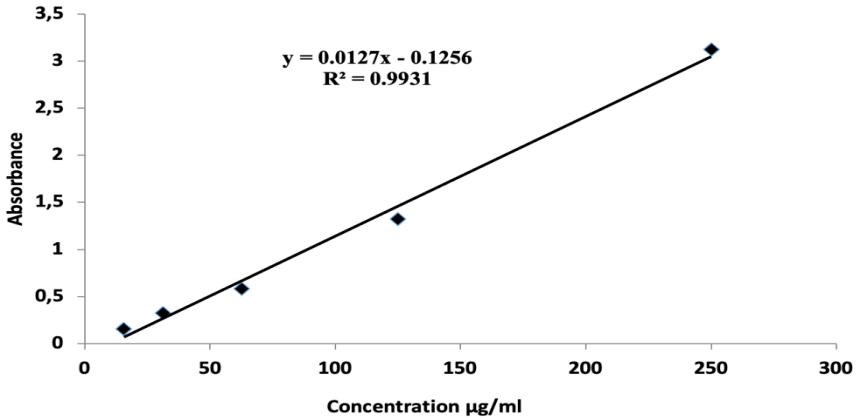

Figure 1. Standard gallic acid calibration curve for total phenolic calculation

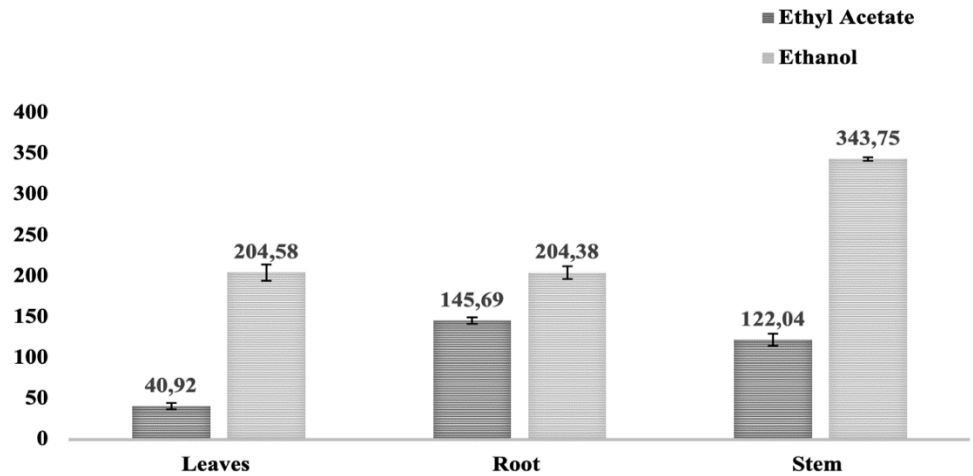

Figure 2. Graphical presentation of total phenolic content expresses as mg GAE/mg of extract

Table 4. Total phenolic content expresses as µg GAE/mg of extract

S.N.	Parts of *Rubus ellipticus* Sm.	Ethyl Acetate (µg GAE/mg of extract)	Ethanol (µg GAE/mg of extract)
1	Leaves	40.92 ± 4.25	204.58 ± 9.82
2	Root	145.69 ± 4.1	204.38 ± 7.95
3	Stem	122.04 ± 7.34	343.75 ± 2.21

3.4. Total Flavonoid Content

The flavonoids were quantified using the Aluminum Chloride colorimetric method, with quercetin as the reference standard. The results were expressed as quercetin equivalents per milligram of extract. Among the experimental extracts, the ethanolic extract of stem had the highest amount of flavonoid content (1563.17±10.79 µg QE/mg of extract) compared to the others (Root and Leaves), whereas all the ethyl acetate extracts, i.e., leaves, root, and steam, had almost comparable amounts of flavonoid, as depicted in **Table 5.**

Table 5. Total flavonoid content of expressed as µg QE/mg of extract

Solvent	Leaves	Root	Stem
Ethyl Acetate (µg QE/mg of extract)	68.06 ± 36.39	66.20 ± 15.34	84.84 ± 16.80
Ethanol (µg QE/mg of extract)	529 ± 27.45**	648 ± 39.56**	1563 ± 10.79**

*Note: ** represents that the original concentration of the extract falls out of the range so they were reduced to its half and final value was calculated equivalent to the original concentration.*

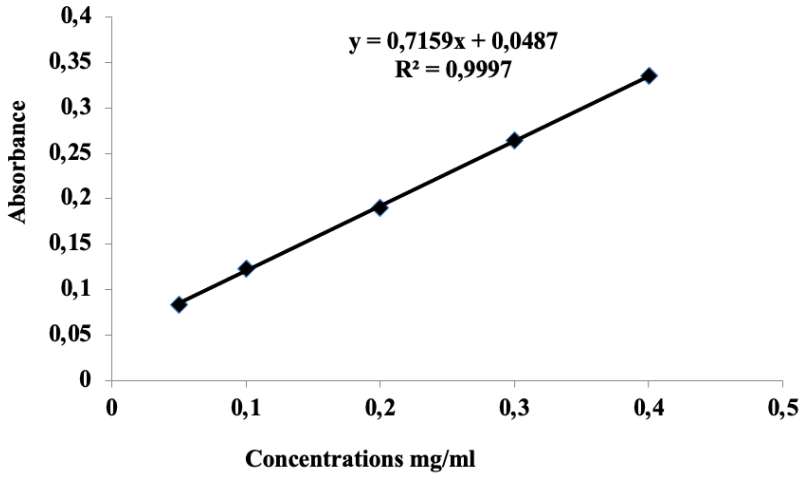

Figure 3. Calibration curve of standard quercetin for the quantification of total flavonoid content

Table 6. Percentage scavenging activity of extract and ascorbic acid

S.N.	Parts of *Rubus ellipticus*	Solvents	% DPPH scavenging activity				
			0.1 µg/mL	1 µg/mL	10 µg/mL	100 µg/mL	IC_{50}
1	Leaves	Ethyl Acetate	1.97±0.15	0.71±3.3	18.90±2.40	81.01±1.29	58.61
		Ethanol	7.84±4.5	16.87±5.92	91.60±0.75	92.33±0.29	5.03
2	Root	Ethyl acetate	1.50±0.47	2.13±2.54	56.09±1.13	94.30±0.57	36.72
		Ethanol	2.32±4.59	15.50±3.08	87.90±2.01	88.61±1.52	5.47
3	Stem	Ethyl acetate	9.01±1.30	39.66±16.20	53.79±17.34	90.34±1.33	34.56
		Ethanol	7.33±1.95	18.29±2.05	90.29±1.13	92.19±1.19	5.08
4	Ascorbic Acid		13.26±0.5	44.28±0.55	95.37±0.21	96.47±0.21	3.562

3.5 Antioxidant Activity Assay

To assess antioxidant activity, the DPPH free radical scavenging activities of various parts *of R. ellipticus* were measured and shown in **Table 6.** The leaves ethanolic extract demonstrated the most potent free radical scavenging activity, with an IC_{50} of 5.03 g, which is comparable to the IC_{50} of standard ascorbic acid, as shown in **Figure 4.**

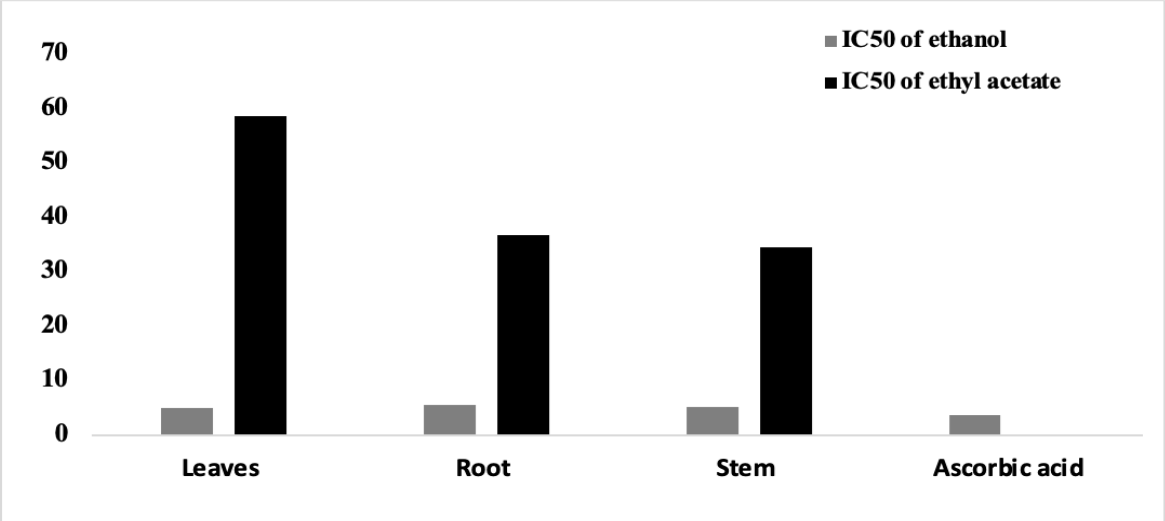

Figure 4. Graphical representation of IC_{50} values of all the extracts along with the standard ascorbic acid

4. Discussion

Plants contain a wide range of natural compounds with different molecular families which show various medicinal properties. Plants are the important source of potentially useful structures for the development of new chemotherapeutics agents (Mukherjee et al., 2001). In this study, we collected the ethnomedicinally used *Rubus ellipticus* Sm. and compared phytochemicals content, free radical scavenging effect, total phenolic, and total flavonoid content of different parts (leaves, stem, root).

The phenolic compounds are one of the largest and most prevalent groups of plants metabolites. They have therapeutic benefits in part because of their antioxidant capabilities, which include metal chelation, scavenging and suppression of reactive oxygen species, and scavenging of electrophiles. Phenolic compounds have been linked to antioxidative effects on living things because they scavenge singlet oxygen and free radicals (Barros et al., 2007).

Among all the extract of *R. ellipticus*, ethanolic root extract have maximum yield value of 10.78% followed by the ethanolic extract of the leaves and stem, whereas all the three extract of ethyl acetate have a minimum yield value compared to ethanolic extract as depicted in **Table 2**, which indicates that the plants parts contain more polar compound so that they were extracted in polar ethanol (Majidaee et al., 2020; Raman et al., 2005). The order of different parts of *R. ellipticus* based on the quantity of yielded extraction was root>leaves>stem.

In the qualitative phytochemical study, extract of *R. ellipticus* leaves, stem and root showed the presence of alkaloids, phenols and flavonoids which is even supported by the previously reported study (Sharma &

Kumar. 2011; Subba et al., 2019) . In our study, during qualitative phytochemical screening to determine the phenolic compounds, all the extract shows the positive ferric chloride test while only the root extracts show negative lead acetate test, that may be due to the absence of sulfur containing amino acids (Sulfhydryl/thiol group) on the root of *R. ellipticus* (Anup. 2020/04/16).

Based on **Table 4,** the ethanolic and ethyl acetate extract of the stem had the highest amount of phenolic content, followed by the root and leaves, which is supported by Öztürk, M., et al (2004), (Öztürk et al., 2007). According to the previous reported study of Saini, R., et al. 2014 (Saini et al., 2014), the extraction process of fruits of *R. ellipticus*, showed that the highest amount of phenols were content in an polar solvent compare to that of other less- polar solvent which is parallel to the results of our study as all ethanolic extract shows higher phenol content compared to ethyl acetate extract. Ethanolic extracts of all *R. ellipticus* samples contained more flavonoids than ethyl acetate extracts as per **Table 5.**

The stem of *R. ellipticus* has the highest flavonoid content in ethanolic as well as ethyl acetate extract which are comparable to each other as shown in **Table 5.** Thus, we can conclude that the flavonoid concentrations are highest in the harder portion of *R. ellipticus*. These above findings clearly showed that ethanol recovered more total phenolics and flavonoids than ethyl acetate extracts, indicating that ethanol is a better solvent solution for the most effective extraction of polyphenols from *R. ellipticus*.
Based on the free radical scavenging activity, all the ethanolic extract of the plant showed comparable radical scavenging properties to each other and showed the IC50 value almost similar to the standard ascorbic acid. Also, among the ethyl acetate extract, stem shows the maximum inhibition of the free radical followed by the leaves and root. According to Subba et al., (2019) half-maximal inhibitory concentration value of methanol extract of leaves of *R. ellipticus* was 31 ± 0.2641 mg/mL depicting more potency as compared to our study.

In our study, the potent IC50 value of ethanolic extract compared to ethyl acetate extract could be attributed to the presence of more phenolic and flavonoid compounds in ethanolic extract, which is mentioned in previous study as well (Duh et al., 1999; Öztürk et al., 2007; Raman et al., 2005; Saxena et al., 2013). Mostly DPPH assay scavenge neutral and cation free radicals which are extracted in higher proportion in a polar solvent (Saini et al., 2014). According to Badhani et al., (2015) and Karuppusamy et al., (2011), there are various types of bioactive polyphenolic and flavonoid components found in different parts of *R. ellipticus* like Anthocyanin, Ascorbic acid, Chlorogenic acid, Gallic acid, Catechin etc. which are known for their antioxidant properties (Badhani et al., (2015); Karuppusamy et al., (2011); Saini et al., (2014); Schulz et al., (2019); Shikha & Kashyap. (2020)). May be because of the presence of such bioactive components, most of the ethanolic extracts showed the potent free radical scavenging effect.

5. Conclusion

The result revealed the presence of medicinally important bioactive constituents in the different parts of *R. ellipticus* with the potency order of leaves>stem>roots. Eventually, this study might also set a milestone to support and give the scientifically evidence of using *R. ellipticus* for free radical scavenging properties. Moreover, isolation of the active hit and lead molecules, further evidence-based determination other pharmacological properties could benefit the mankind and assist in the discovery of various novel compounds.

Authors' contributions

Ananda Lamichhane, Susmita Khatri, Mamata Dhungana, Bijaya Tripathi and Namrata Bhattrai collected the plant, prepared the samples, performed all the experiments, and analyzed the data. Ananda Lamichhane wrote the manuscript. Rishiram Baral, and Dr. Nirmala Jamarkattel reviewed the manuscript. The authors read and approved the final manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

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Contribution to the Microscopic Study of Three Plant Species (Parsley, Spanish Scolyme and White Marrube) Commonly Used in Traditional Algerian Medicine

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Abstract

The present work aims to study the anatomical characteristics of three plant species used in Algeria for their diuretic and antilithiasic properties. Also, to summarize the results obtained in detailed drawings of each part studied as well as diagrams with conventional tissue signs. Therefore, *Petroselinum crispum* (Mill.) Fuss (Apiaceae), *Scolymus hispanicus* L. (Asteraceae) and *Marrubium vulgare* L. (Lamiaceae) were obtained from the Tlemcen (northwestern Algeria) region. In the study, stems and leaves of *P. crispum* and *M. vulgare*, and leaves of *S. hispanicus* were used. Once the species was identified and examined, cross-sections were taken and stained using the double staining technique, then observed under an optical microscope. It was generally observed that the parsley leaf did not have any secretory trichomes, and the secretory duct in the midrib was located between the phloem and the lower epidermis. It was also noted that there were no secretory trichomes in the Spanish scolyme. In addition, branched covering trichomes and secretory trichomes with octacellular head were observed in the leaf of white horehound. These findings are certainly going to help enrich the rare bibliographic data available on the anatomy of these three species.

Keywords: Algerian flora, *Marrubium vulgare*, *Petroselinum crispum*, Plant anatomy, *Scolymus hispanicus*.

1. Introduction

Parsley, Spanish scolyme and white horehound are among the most widely used plants in traditional Algerian and North African medicine (Belakhdar, 1998; Elyebdri et al., 2017) for their diuretic and anti-lithiasic properties (Lahsissene et al., 2010; Tahri et al., 2012; Zeggwagh et al., 2013; Ouis et al., 2014).

According to Bentham (Agyare et al., 2017), Algeria is among the countries where parsley originated. This plant is widely used in the country's cuisine. It should be noted that its fresh leaves are an excellent anti-inflammatory and a very good antioxidant. In addition, its seeds are often used for disinfection and relief from insect bites (Stitou, 2016). Parsley is an excellent anti-allergic plant because it inhibits the secretion of histamine (Pharmacopée française, 2012). Several studies have also shown that parsley has an anti-cellulite action, as it promotes the decrease in fat reserves accumulated in the

body. On the other hand, according to the results from recent research, it could even limit the risk of cancer, by helping the body to get rid of its toxins on a daily basis (Pharmacopée française, 2012). Furthermore, *Scolymus hispanicus* L. is widely distributed in the country (Vázquez, 2000). Its extract is also believed to have antispasmodic and antioxidant effects (Benchiheub, 2015). In Algeria, white horehound is very widespread; it grows mostly in rubble and hedges. Its ethanolic extract was shown to possess mild anti-inflammatory properties. As for marrubin, it would have expectorant and bronchial fluidifying properties (Djahra, 2014). In tachyarrhythmias and arrhythmias, white horehound is used for its cardiosedative effect (Aït Youssef, 2006).

Despite the wide availability of these species and the numerous studies conducted on the chemical composition and biological activity of their extracts (Sahpaz et al., 2002; Zhang et al., 2006; Boudjelal et al., 2012; Snoussi et al., 2016; Aboukhalaf et al., 2020; Kandil et al., 2020), it was not easy to find sufficient data on their anatomy, which made their microscopic identification difficult. Thus, the present study aims to highlight the anatomical characters of the used parts of these species in order to facilitate their identification and avoid confusion with other similar species.

2. Material and Methods

The aerial parts of *Petroselinum crispum*, *Marrubium vulgare* and *Scolymus hispanicus* were obtained from herbalists in the wilaya of Tlemcen (northwestern Algeria). They were then identified according the data published in The Flora of Algeria (Quezel et Santa, 1963). Samples were stored in the Pharmacognosy laboratory – Pharmacy department of Tlemcen. The scientific names of the species were verified against the database (http://www.theplantlist.org/).

The studied parts were stored in a mixture of equal volume of ethanol and glycerin (Ruzin, 1999).

Afterwards, several cross-cuts were made by performed on these samples, using a blade, in the thinnest possible way. They were then put in distilled water to prevent them from drying out, then in sodium hypochlorite to empty the cells of their contents. They were subsequently placed in 10% acetic acid to allow fixation of the dyes (Langeron, 1949) .

Once they were washed with distilled water, they were stained using the Mirande double staining technique (Langeron, 1949) and placed successively in two dyes, i.e. iodine green and alum carmine, for 1 min and 20 min, respectively. It was made sure that they were carefully washed after each step with distilled water.

The sections were then placed between slide and coverslip with a drop of glycerin so they can be observed using a Leica DM300 optical microscope. The diagrams have been made with the conventional tissue signs.

3. Results and Discussion

3.1. Parsley; *Petroselinum crispum* (Mill.) Fuss

3.1.1. Leaf transverse section

The midrib protrudes on both sides, forming a triangle at the top and bottom. It is characterized, from the outside to the inside, by:

- Two upper and lower epidermises formed of more or less rounded cells, with a thin cellulose wall (figures 1- c, 1 - b). It is covered by a thick cuticula. The round sub-epidermal collenchyma is formed by several layers of thick cellulose-walled cells in the protruding parts of both sides (Fig. 1- a, b, c). The cortical parenchyma is found next. It is made up of several layers of large, rounded to polygonal thin-walled cells, leaving inter-cellular spaces between them. It is rich in

chloroplasts. A single secretory duct is located between the phloem and the lower epidermis (1-c) which is characteristic of Apiaceae (Perrot, 1944). This position is an important taxonomic feature(Akpulat et al., 2014). The rounded vascular bundle is in the middle (1-d). In the leaf blade, the two epidermises consist of thin-walled rectangular cells, larger than those of the midrib, like those found in other Apiaceae (Akpulat et al., 2014). The mesophyll is dorsiventral, criteria common to Apiaceae according to Perrot (1944). It is very rich in chloroplasts, with a single layer of palisade parenchyma (Figure 1 - e). The absence of secretory trichoms is also common to Apiaceae (Em. Perrot, 1944) . These results are similar to those found in the same species collected in Brazil in the Alta Floresta region (Larocca, 2013).

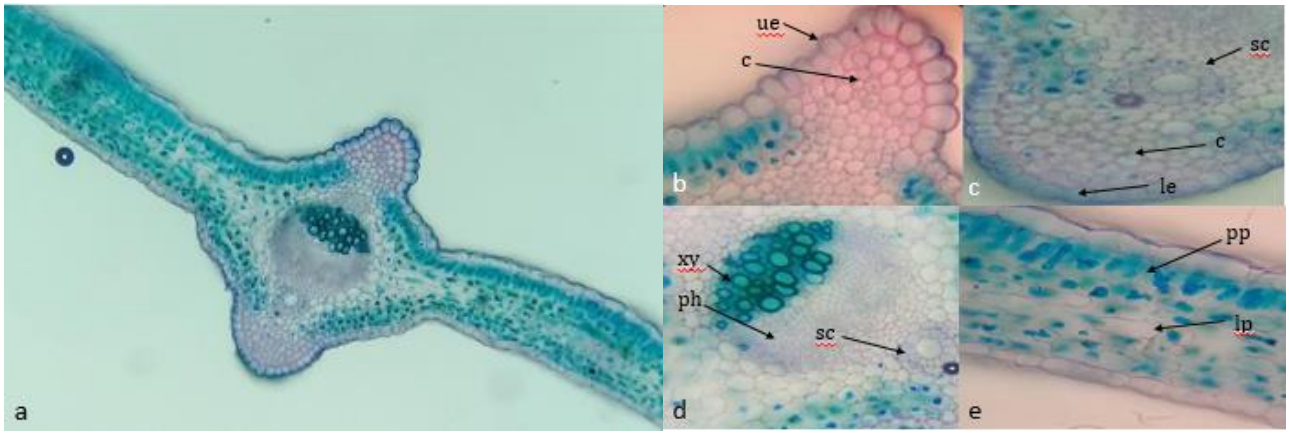

Figure 1. Anatomical characterization of the transverse section of the leaf of *P. crispum*

1- a: General view at magnification (4 x10); 1- b: Upper epidermis with collenchyma; 1- c: Lower epidermis with collenchyma and secretory canal; 1-d: Vascular bundle; 1-e: Dorsiventral mesophyll.ue: upper epidermis; c: collenchyma; sc: secretory canal; le: lower epidermis; xy: xylem; ph: phloem; pp: palisade parenchyma; lp: lacunar parenchyma.

3.1.2. Stem transverse section

This part presents a pentagonal shape with bilateral symmetry (figure 2 - a). It includes, from the outside to the inside, a cortical part that is delimited by an epidermis formed of cells, more or less rounded, with a cellulose wall.

After the angular sub-epidermal collenchyma at the angles (Figure 2 - b), there is the cortical parenchyma with intercellular spaces. It is formed by several layers of round to polygonal shaped cells, with a large size thin wall, in which several secretory ducts are found (Figure 2 - c). It is a feature that is common to Apiaceae (Perrot, 1944).

In addition, there are seven vascular bundles in the central cylinder, where the phloem and the xylem are superimposed. Below the xylem, there is an inner phloem (figure 2 - e). The rest is filled with a medullary parenchyma that is composed of polygonal to round shaped cells with a thick wall, leaving spaces between them. Although a hollow pith in the stems of species belonging to the family Apiaceae is often found (Metcalfe et al., 1950), no stem void has been observed in this species. The medulla is devoid of secretory ducts. These features have also been found in another species of the Apiaceae family: *Peucedanum graminifolium* Boiss. (Akpulat et al., 2014).

In this context, Svoboda et al, (2000) reported that Apiaceae have secretory ducts extending from the roots through the stem, leaves and fruits. This was found in cross sections of the stem and leaf of *P. sativum*. Furthermore, there is a single vascular bundle and secretory duct in the center of the leaf of the species studied, a characteristic feature of the Apiaceae (Mavi et al., 2019).

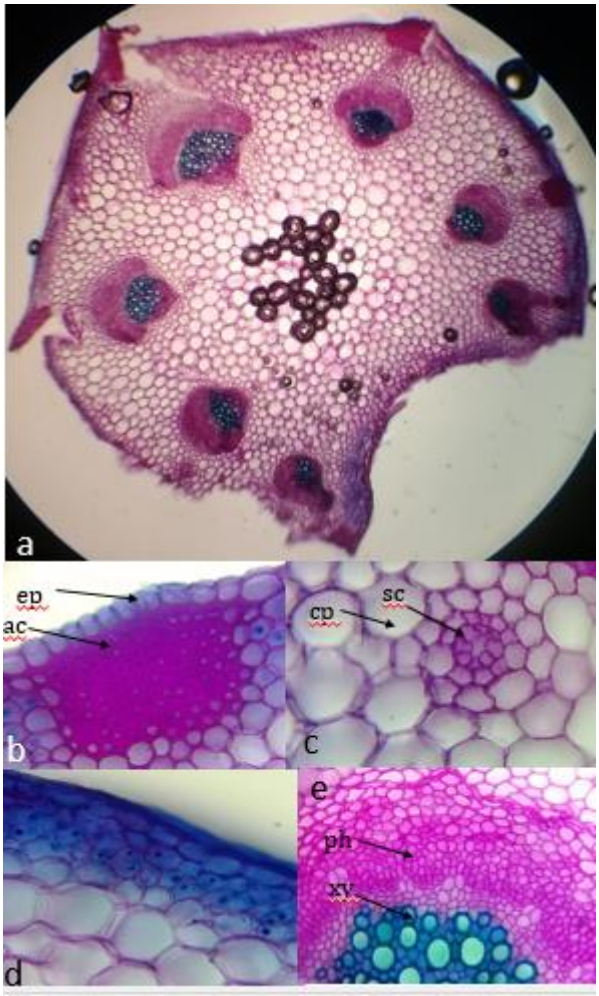

Figure 2. Anatomical details of the transverse section of the stem of *P. crispum*

1- a: General view at magnification (4 x10); 2 - b: Epidermis with angular collenchyma; 2 - c: Cortical parenchyma with secretory canal; 2 - d: Parenchyma rich in chloroplasts; 2 - e: Details of the vascular bundle ep : epidermis ; ac : angular collenchyma ; sc : secretory canal ; cp: cortical parenchyma; ph: phloem; xy: xylem.

3.2. Spanish Scolyme; *Scolymus hispanicus*

The transverse section of the leaf of *S. hispanicus* exhibits a triangular midrib that is prominent on the upper part, and very prominent on the lower part (Figure 3 - a). Under the two epidermises comprising polygonal cells with a thick cellulose wall, there are two to three layers of angular collenchyma (Figure 3 - b). Next, we find the cortical parenchyma formed by more or less round cells with a thin wall (Figure 3 - c). In the middle of this parenchyma, each one of the three vascular bundles is surrounded by a sheath of angular collenchyma (Figures 3-d, 3-e). It was clearly noticed that the glandular trichomes are absent, contrary to what is usually found in the Asteraceae (Perrot, 1944). While the covering trichomes are abundant on both sides of the leaf. These trichomes are uniseriate, multicellular, and smooth-walled. Sometimes the basal cells are biseriate (Figure 3 - f). In the leaf blade, between the two epidermises with flattened cells, there is a dorsiventral mesophyll with a palisade parenchyma layer and lacunar chlorophyll parenchyma layers (Figures 3 - g, 3 - h). This mesophyll is common of the Asteraceae (Kadereit et al., 2007; Ozcan, 2015; Metcalfe et al., 1950). Many researchers have reported the existence of accessory vascular bundles in Asteraceae species (Ozcan, 2015). Their presence meets the requirements for translocation under unfavorable conditions (Sidhu et al., 2011).

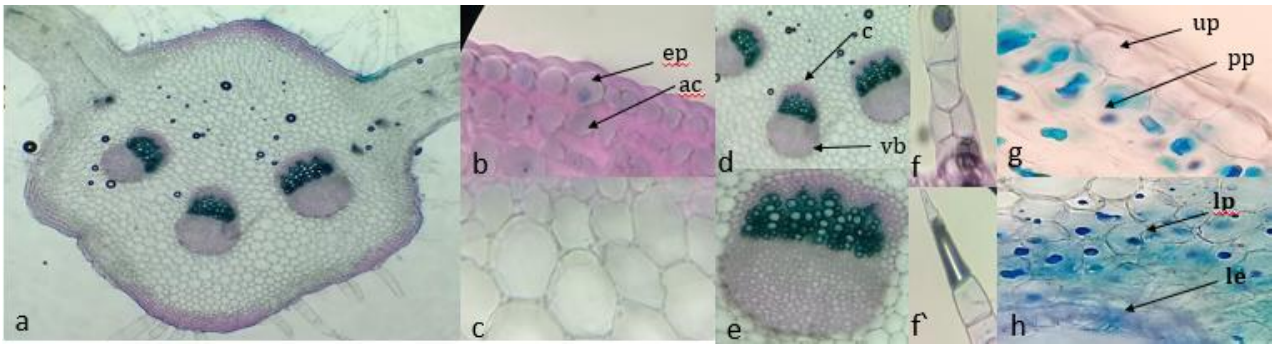

Figure 3. Anatomical characteristics of the transverse section of the leaf of *S. hispanicus*

3 - a: General view at magnification (4 × 10); 3 - b: Epidermis with angular collenchyma; 3 - c: Cortical parenchyma; 3 - d: Collenchyma surrounding the vascular bundle; 3 - e: Vascular bundles; 3 - f: Tector trichomes; 3 - g: Upper epidermis of the leaf blade and palisade parenchyma; 3 - h: Lower epidermis of the leaf blade and lacunous parenchyma; ep: epidermis; ac: angular collenchyma; c: collenchyma; vb: vascular bundle; up: upper epidermis; pp: palisade parenchyma; lp: lacunar parenchyma; le: lower epidermis.

3.3. White horehound; *Marrubium vulgare*

3.3.1. Leaf transverse section

The midrib is very prominent on the lower face and depressed on the upper face. The leaf blade in not very thick (Figure 4 - a). In the midrib, the two upper and lower epidermises, formed by thin-walled contiguous rectangular and rounded cells, and covered by a thin cuticula, surround the round collenchyma, thus occupying a larger area in the upper part (Figures 4 - b, 4 - c). Oval or rectangular epidermal cells were also encountered in species of the same genus as *M. trachyticum* (Akçin et al., 2018). A cortical parenchyma with intercellular spaces, rich in chloroplasts, and made up of several layers of more or less rounded cells with thin walls is found just below (Figure 4 - c).

This parenchyma contains the vascular bundle (Figure 4 - d). The epidermal cells in the leaf blade are larger than those in the midrib. In contrast, the cells of the lower epidermis are smaller than those of the upper one. There is also a bifacial mesophyll, usually found in Lamiaceae (Perrot, 1944), with a single palisade parenchyma layer (Figure 4 - e). The presence of trichomes was observed on both sides of the leaf blade. They were more frequent on the underside and more numerous than in the midrib. These were secretory trichomes with octacellular head and unicellular foot (Figure 4 - g) common to Lamiaceae (Gul et al., 2019). They are embedded in the epidermis, which is in accordance with the findings of Gul et al. (2019).

This type of trichomes was also found by Haratym et al. in a study conducted on the species *M. vulgare* from the Lublin region in Poland. Studies conducted on the chemical composition of their secretory contents have shown the presence of lipids, polysaccharides, polyphenols and terpenes (Haratym et al., 2017).

Also secretory trichomes with bicellular head and unicellular foot (Figure 4 - f), uniseriate and unicellular smooth-walled covering trichomes (Figure 4 - i), and branched covering trichomes (Figure 4 - h) which have previously been reported in other genera of Lamiaceae such as *Stachys* L.(Salmaki et al., 2009), *Nepeta* L. (Jamzad et al., 2003), *Lavandula* L. (T. Upson et al., 2004), and in species of the same genus as *M. trachyticum* (Akçin et al., 2018), *M. friwaldskyanum* and *M. peregrinum* (Gyuzeleva et al., 2022). It is worth noting that the stomata were present on both sides of the leaf.

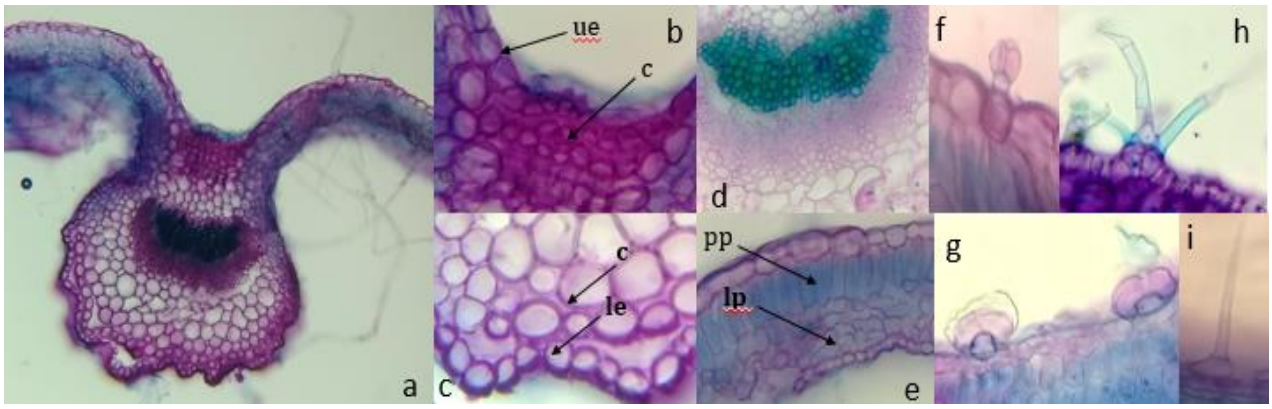

Figure 4. Anatomical features of the transverse section of the leaf of *M. vulgare*

4 - a: General view at magnification (4 × 10); 4 - b: Upper epidermis and collenchyma of the midrib; 4 - c: Collenchyma and lower epidermis; 4 - d: Vascular bundle; 4 - e: Leaf blade (palisade and lacunous parenchyma); 4 - f: Glandular trichome with unicellular foot and bicellular head; 4 - g: Glandular trichome with octacellular head; 4 - h: Branched tector trichome; 4 - i: Unicellular uniseriate tector trichome.
ue: upper epidermis; c: collenchyma; le: lower epidermis; pp: palisade parenchyma; lp : lacunar parenchyma.

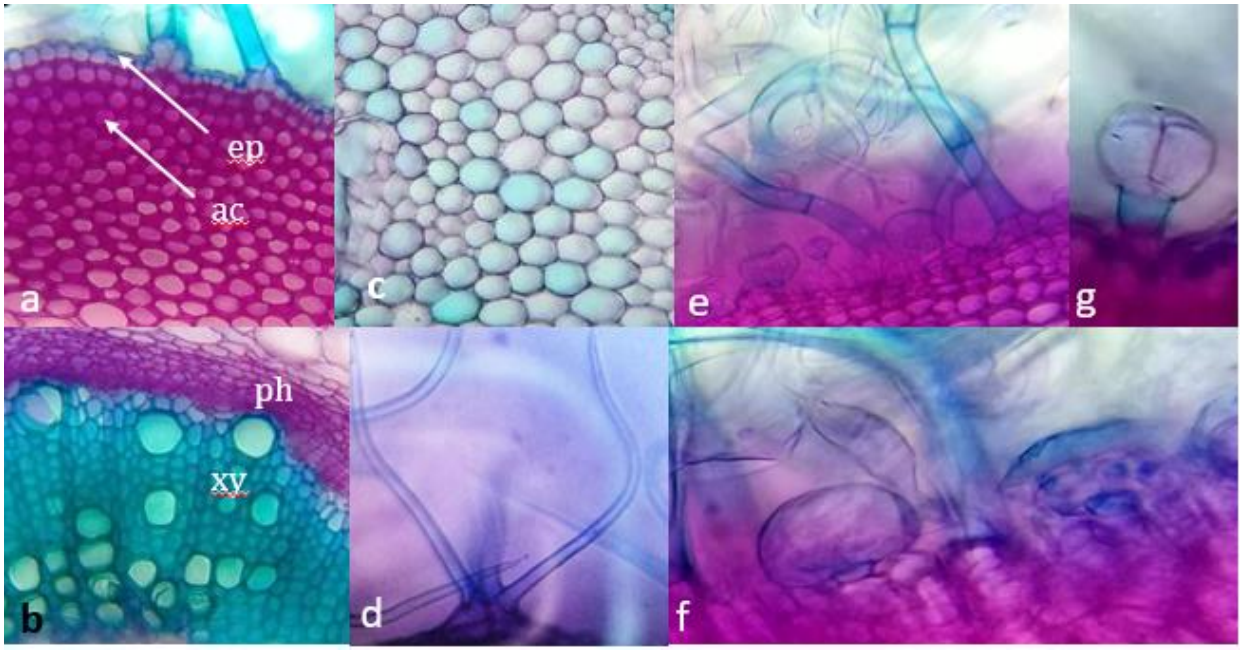

Figure 5. Anatomical features of the transverse section of the stem of *M. vulgare* at magnification (4 × 10)

5 - a: Epidermis and angular collenchyma; 5 - b: Vascular tissues; 5 - c: Parenchyma with intercellular spaces; 5 - d: Branched covering trichome; 5 - e: Multicellular uniseriate covering trichome; 5 – f: 5-g: Glandular trichome with unicellular foot and octacellular head;
Ue: upper epidermis; c: collenchyma; le: lower epidermis; pp: palisade parenchyma; lp: lacunar parenchyma.

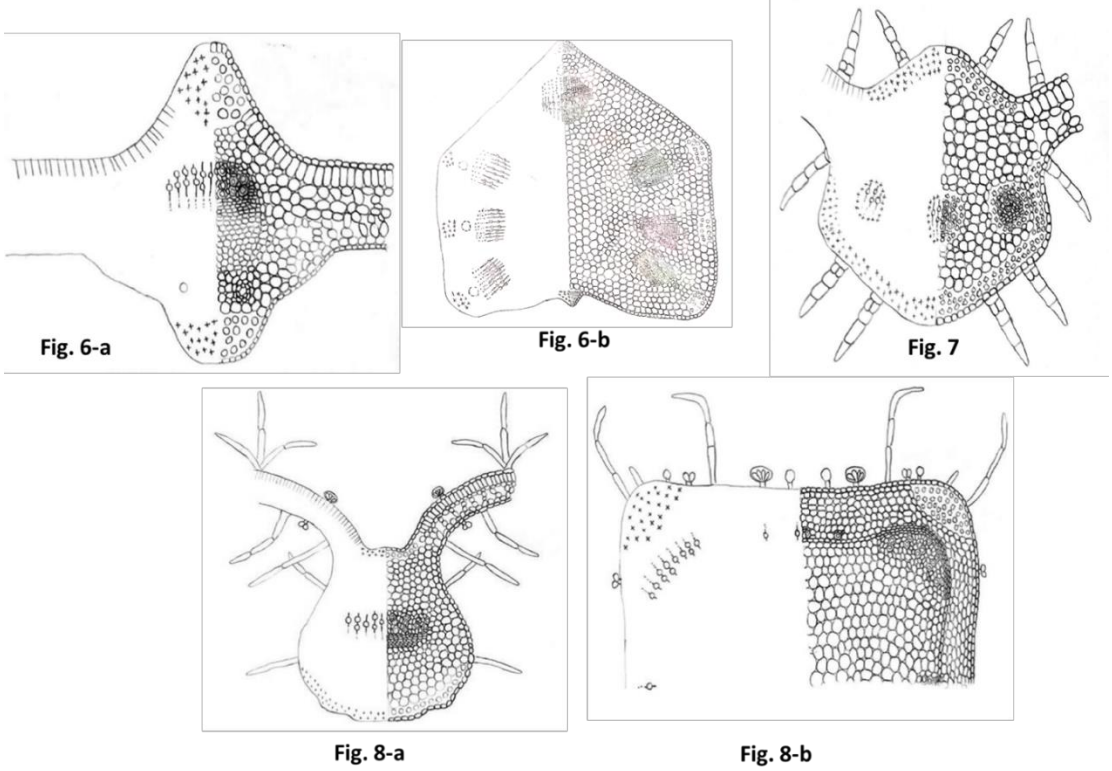

Figure 6- a: Drawing and general diagram of the transverse section of the leaf of *P. crispum* (Apiaceae) at magnification (40 × 10); **Figure 6-b:** Drawing and general diagram of the transverse section of the stem of *P. crispum* (Apiaceae) at magnification (40 × 10); **Figure 7:** Drawing and general diagram of the transverse section of the leaf of *S. hispanicus* (Asteraceae) at magnification (40 × 10); **Figure 8-a:** Drawing and general diagram of the transverse section of the leaf of *M. vulgare* (Lamiaceae) at magnification (4 × 10); **Figure 8-b:** Drawing and general diagram of the transverse section of the stem of *M. vulgare* (Lamiaceae) at magnification (4 × 10)

Table1. Anatomical characters of the three species studied

Character	Localisation and description
P. sativum **Leaf**	
Trichomes	Absent
Calcium oxalate crystals	Absent
Secretory duct	Only one located between the phloem and the lower epidermis
Stem	
Trichomes	Absents
Secretory canals	In cortical parenchyma
Vascular bundle	In the number of seven
Internal phloem	Below the xylem
S. hispanicus **Leaf**	
Trichomes	Abundant on both sides
Vascular bundles	In the number of three
M. vulgare **Leaf**	
Trichomes	-Secretory trichomes with octacellular head and unicellular foot -Secretory trichomes with bicellular head and unicellular foot -Uniseriate and unicellular smooth-walled tector trichomes -Branched tector trichomes
Stem	
Trichomes	-Branched tector trichomes -Uniseriate multicellular tector trichomes -Secretory trichomes with unicellular foot and bicellular head -Secretory tricomes with unicellular foot and octacellular head
Vascular bundles	Continuous ring

3.3.2. Stem transverse section

The cross section of the white horehound stem is quadrangular, which is characteristic of both Lamiaceae (Perrot, 1944) and the genus *Marrubium* (Buyukkartal et al., 2016). It has a cortical part on the outside with an epidermis formed by a single layer of more or less rounded cells with a thin cellulose wall (Figure 5 - a). The epidermis is rich in branched tector trichomes (Fig. 5 - d) and uniseriate multicellular tector trichomes (Figure 5 - e). It also includes secretory trichomes with unicellular foot and octacellular head (Figures 5-g ; 5 - f).

- Several layers of angular collenchyma are located at the angles of the stem, followed by the cortical parenchyma formed by large size round to polygonal cells with a thin wall (Figures 5 - a, 5 - c). Unlike the species *M. bourgaei* and *M. heterodon*, in which a

lamellar collenchyma is found (Büyükkartal al., 2016).

- In the middle of the stem the vascular tissus form a continuous ring with several layers of phloem surrounding those of the xylem. In the middle, the medullary parenchyma with meats and a hollow in the center of the stem can be seen.

The main characteristics of each species are summarized in Table 1. Detailed drawings of each part as well as diagrams with conventional signs are grouped in the appendix (Fig. 6-a; Fig. 6-b; Fig. 7; Fig. 8-a; Fig. 8-b).

4. Conclusion

To our knowledge, no publication presenting microphotographs of the anatomy of *P. crispum, M. vulgare* or *S. hispanicus* from Algeria is available to date. Our work, therefore, provides additional knowledge on these species, which are particularly interesting from a medicinal point of view.
Some of the anatomical characteristics obtained could be guidelines for future micromorphological and phytochemical investigations, necessary to complete this study in order to better define the characteristics of these three species and provide relevant data to facilitate their identification.

Authors' contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [Hasnia Medjahed], [Khawla Yazid],[Sara Hassaïne] and [Nassima Elyebdri]. The first draft of the manuscript was written by [Sara Hassaïne] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest

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The Antioxidant Capacities of Leaf Extracts from *Salvia viridis* L.

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Abstract

This research was conducted at Yozgat Bozok University to define the antioxidant activity of *Salvia viridis* L. grown in the field and *in vitro* conditions. The leaves of the plants grown under field conditions were collected in the pre-flowering period. The leaf extracts prepared with methanol were used in the analysis. DPPH (1,1-Diphenyl-2-picrylhydrazyl) free radical screening activity was used to identify antioxidant activity. The total phenolic, flavonoid, and DPPH IC50 values of *S. viridis* L. grown in the field and under *in vitro* conditions were found as 184.15 ± 36.70 mg GAE g^{-1} and 66.46 ± 0.19 mg GAE g^{-1}, 212.92 ± 11.18 mg QE g^{-1} and 212.92 ± 11.18 mg QE g^{-1}, 117.51 mg $ml^{-1}$and 185.40 mg ml^{-1}, respectively. According to the findings of this study, it was determined that leaves of *S. viridis* L. grown in field conditions exhibited more antioxidant activity than *in vitro* conditions.

Key Words: *Salvia viridis* L., Sage, DPPH, Phenolic, Tissue Culture

1. Introduction

Most taxa in the family Labiatae/Lamiaceae are important sources of secondary metabolites (essential oils, alkaloids, saponins, etc.). For this reason, they have a high potential to be used in different sectors such as food, medicine, and cosmetics (Carović-Stanko et al., 2016). Sage, the largest member of the Lamiaceae family, is the general name of the species in the genus *Salvia*. It is reported that there are over 1000 species of the genus *Salvia* worldwide. In the Flora of Turkey, ninety-nine *Salvia* species are naturally distributed and fifty-one of these species are endemic *Salvia viridis* L., one of these species, is an annual plant that grows erect (11-34 cm in height), has a hairy structure, and has lilac-purple flowers. This species shows spread throughout Turkey except for Eastern Anatolia Region (Celep and Kahraman, 2012). While the seeds and leaves of this plant are used in fermentation barrels to increase the quality of the liquor, the powdered leaf of the plant is also used in the remedy of gum and throat infections (Cosge Senkal, 2019).

In the food industry, mainly synthetic antioxidants (butyl hydroxytoluene (BHT), butyl hydroxy anisole (BHA), tertiary butyl hydroxyquinone (TBHQ), and propyl gallates (PG), etc.) are used to protect foods from oxidative degradation and to extend their

storage periods. Although these synthetic antioxidants are highly effective, stable, and cheap, they have potential side effects such as mutagenic, carcinogenic, and teratogenic (Mammadov, 2014). During the functioning of normal metabolic activities in both humans and animals, free radicals occur under the influence of various external factors or environmental factors. Reactive oxygen species are short-lived and unstable. Therefore, they can easily react with various biological molecules (nucleic acids, lipids, carbohydrates, proteins, etc.) in organisms. As a result, they cause the development of many diseases (cancer, liver diseases, immune system diseases, etc.), especially aging in humans (Halliwell and Gutteridge, 1990).

It is known that antioxidant substances destroy these free radicals. For this reason, consumer preferences have led the industry to seek natural sources of antioxidants, and medicinal and aromatic plants have become increasingly important. Also, flavonoids, antioxidant substances and phenolic compounds obtained from natural plants are effective in protecting from these free radicals formed in the human body (Kahkönen et al.,1999). Plants are a major source of natural antioxidant compounds. Therefore, plants are considered important antioxidants. Among natural antioxidants, phenolic substances (flavonoids, coumarins, tocopherols, phenolic acids, cinnamic acid, etc.) are the most common. Under normal conditions, the damage caused by oxygen radicals is kept under control by the organism's effective antioxidant systems. Studies have shown that certain phenolic antioxidants prevent cell death caused by oxidative stress. Studies have shown that phenolic and flavonoid substances are abundant in *Salvia* taxa. It is stated that various phenolics (caffeic acid and chlorogenic acid etc.) and flavonoids (apigenin and luteolin etc.) are found in the extracts obtained from aerial parts of *S. viridis* (Rungsimakan and Rowan, 2014).

Due to limited quantities of production, difficulties in obtaining standard quality products, being far away from industrial areas of production areas, and high extraction and purification costs, some medicinal and aromatic plants with economic and industrial value in certain countries of the world are produced with tissue or cell techniques under controlled conditions as an alternative method. Micropropagation is the obtaining of new plants from plant parts (seed, leaf, root, stem, shoot, embryo, callus, single-cell or pollen grain, etc.) taken from a plant and have the potential to form a complete plant, in artificial nutrient environments and under aseptic conditions (Baydar, 2013).

The aim of this study was to compare the antioxidant capacities of the leaves obtained in the vegetative period of the plants *in vitro* culture and in field-grown of *S. viridis* L.

2. Material and Methods

2.1. Plant Material

In this research, *S. viridis* L. seeds collected from the collection plots in Yozgat Bozok University Gedikhasanlı Application and Research Station (35 ° 09 '34 "E-39 ° 35 '13" N, Altitude: 1135 m) in 2017 were used as material. The collected seeds were placed in a paper bag and stored in the shade in room condition.

2.2. Viability and Germination Tests

The viability of the seeds was determined with the Tetrazolium (2, 3, 5- Triphenyl Tetrazolium Chloride-TTC) test and 50 seeds were used (AOSA, 2000). Standard germination tests were set up in Petri dishes with 4 replicates using 20 seeds. The average germination rate was determined on the 21st day (Subasi and Guvensen, 2010).

2.3. Growing of Plants in Field Conditions

The research was carried out in the Yozgat Bozok University Research and Application Area/Turkey. Seeds were sown in vials

containing peat. The seedlings that grow well under greenhouse conditions and reach sufficient size were planted in the collection plot at 70x70 cm intervals (30.05.2018). The parts of *S. viridis* used in folk medicine are the leaves, flowering stems, and seeds. In this study, we used leaves for comparison with in vitro culture. Leaf samples before flowering were taken from the plants to do the necessary maintenance (hoeing, irrigation, etc.) on 15.08.2018. According to the soil analysis results, the clay, silt, and sand content of the experimental area soil is 476 g kg^{-1}, 138 g kg^{-1}, and 386 g kg^{-1}, respectively, pH is neutral (7.09), slightly salty (0.178%), lime content and ($CaCO_3$, 7.15%), organic matter content is moderate (2.49%), total nitrogen is sufficient (N, 0.15%), phosphorus (P, 78 µg g^{-1}) and potassium (K, 728 µg g^{-1}) amounts are high has been determined (Yakupoglu, 2018). During the vegetation period (June, July, and August) in the experimental area, the total precipitation was 41.8 mm, the average temperature was 22.23 ^{o}C, and the average relative humidity was 53.6% (MGM, 2019).

2.4. Production under *in vitro* Conditions

S. viridis L. seeds, which will be produced under aseptic conditions, were sown in Caisson brand MS medium on 27.08.2018. All operations during the study were carried out in an ESCO brand laminar flow sterile cabin. First of all, MS (Morishige and Skoog) medium was prepared where seeds will be sown and the pH was fixed to 5.7-8. For the surface sterilization of the seed, the first 20% sodium hypochlorite solution was prepared and 2 drops of Tween 20 for every 100 ml were dropped into it. Seeds were soaked in this solution for 10 minutes and then washed 3 times with sterilized distilled water. The seeds planted in glass plates were placed in a climate chamber operating under a 16/8-hour photoperiod at 4.000 lux light intensity during the day and 20 ° C at night. Plants germinated in Petri and then were taken into glass test tubes and their development was regularly monitored. Plants that reached the required maturity for the determination of antioxidants were taken from the tubes and taken to a shaded place to dry. Later, the samples, dried as desired, were ground and made suitable for analysis.

2.5. Preparation of Extracts

The leaves of *S. viridis* L., both grown in field (a) and in vitro conditions (b), were dried in the shade after harvest (Figure 1). The leaves of the plants grown under field conditions were collected in the pre-flowering period. Dry leaf samples were ground using a blender. This powdered sample was weighed at 0.5 g and 5 mL of methanol (1/10 w v^{-1}) was added to each sample. The incubation time and temperature of the samples in the oven (Elekto-mag M 5040 P) were set as 24 h and 40 ^{o}C, respectively. The samples removed from the oven were filtered through filter paper (Whatman No1). Methanol was removed from the samples with a rotary evaporator (Heating Bath B-491, BUCHI). 2 ml methanol was added into the balloon flasks that were dried in the oven for 24 h and vortexed. The extracts were preserved at 4 °C until the analysis began (Zakaria et al., 2019).

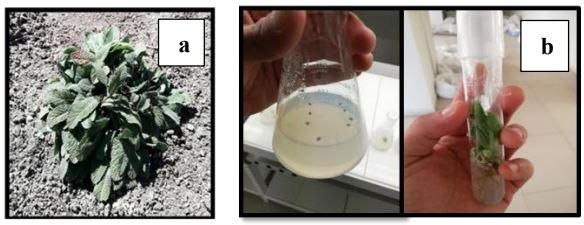

Figure 1. *Salvia viridis* in field (a) and *in vitro* (b) conditions

2.6. Determination of DPPH Radical Scavenging Activity

The antioxidant capacity of the extracts was detected using the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical (Frezzini et al., 2019). In order to be able to compare the samples, first, the amount to neutralize a

certain amount of DPPH radical was determined. 16 mg of DPPH was dissolved in 100 ml of CH_3OH (methanol) and adjusted to 0.1 µM. DPPH readings were made in the spectrophotometer at 517nm. Dilution with methanol was done until the absorbance value was 1000. Samples prepared at six different concentrations (10, 20, 40, 80, 160, 320) were incubated for 30 minutes. The prepared samples were incubated for 30 minutes in the dark. In the study, in which BHT (butylhydroxytoluene) and BHA (butyl hydroxyanisole) were used as controls, analyzes were carried out with four replications. The equation used to calculate the %DPPH radical scavenging activity of the samples: "% DPPH scavenging efficiency = [(A control - Extract) / A control] x100". PerkinElmer Lambda 25 UV / VIS spectrophotometer device was used for spectrophotometric measurements.

2.7. Determination of Total Phenolic Content

The total phenolic content of the extracts was determined using the Folin-Ciocalteu Reagent (FCR) method (Gezer et al., 2006). 100 ml (Na_2CO_3) solution was prepared for the study. 40 µL of the extract was added to glass tubes containing 2.4 mL of distilled water. In the control application, the same amount of methanol was added instead of the extract. The samples were added 200 µL of folin and 600 µL of saturated Na_2CO_3, and then 760 µL of distilled water was added and vortexed. The absorbance measurements of the samples incubated for 2 hours at room temperature were made at 765 nm. Gallic acid was used for standard phenolic control. The values obtained are expressed as Gallic acid equivalents. To prepare the Gallic acid solution, 3 mg of Gallic acid was dissolved well in 15 mL of methanol. Then, by dilution, 100, 125, 150, 175, and 200 µg mL^{-1} control groups were prepared and the Gallic acid curve was drawn. The procedures were carried out in four replications. The total phenolic contents of the samples were determined as a result of the measurements in the PerkinElmer Lambda 25 UV/VIS spectrophotometer device.

2.8. Determination of Total Flavonoid Content

The total flavonoid compound amounts of the extracts were determined by optimizing the aluminum chloride colorimetric method of Biju et al. (2014). 50 µL of the previously prepared 1mg mL-1 extract was put into glass tubes. 950 µL methanol was added to it. Then 4 mL of distilled water was added and mixed for thorough dissolution. Subsequently, 0.3 mL sodium nitrate $NaNO_2$ of 5% was added and incubated for 5 minutes. Then 0.3 mL of 10% aluminum chloride (AlCl**3**) was added and incubated for 6 minutes. After incubation, 2mL of 1mol L^{-1} sodium hydroxide (NaOH) was added. 2.4 mL of distilled water was added to the obtained solution and it was completed to 10mL. The solution was incubated for 15 minutes and then absorbance was measured at 510 nm. For the Quercetin standard, 1 mg mL-1 was prepared as the main stock, and 6 different concentrations (10, 20, 40, 60, 80,100 µg mL^{-1}) were obtained by dilution. The analysis was carried out in four replications. The total flavonoid content of the samples was determined according to the measurements made in the PerkinElmer Lambda 25 UV/VIS spectrophotometer device.

2.9. Statistical Analysis

All studies were done in 4 repetitions. DPPH results of the samples were evaluated by analysis of variance and the LSD (Least Significant Difference) test was applied for the differences between the averages, and the extraction yields, phenolic, and flavonoid content were compared with the t-test. For analysis used the TARİST statistics program, and the results were presented as mean ± standard deviation

(SD). Also, the bilateral relationships were detected using the MINITAB 19 package program (Acikgoz et al.,2004).

3. Results and Discussion

3.1. Viability and Germination in Seeds

Some physical properties were determined as well as the vitality and germination values of the seeds. The width, length, and 1000 seed weight of seeds were determined as 1.05 mm, 1.96 mm, and 2.56 g, respectively. Yilar and Altuntaş (2017) reported that they recorded the length, width, and thickness values of *S. viridis* seeds between 2.57-2.88 mm, 1.52-1.64 mm, and 1.04-1.24 mm, respectively. It was observed that there was no germination problem in the seeds of the plant and the germination value was 93% on average. According to the results of the Tetrazolium test; the viability rate of seeds belonging to the species was determined as 76%. Seeds belonging to the genus Salvia have a mucilaginous seed coat. For this reason, seeds either do not germinate at all or germinate very little (Hayta and Arabaci, 2011, Ozcan et al., 2014). However, a high germination value was recorded in the seeds of *S. viridis* in our study.

3.2. Extraction Yield of Samples

The extract yields of the samples are presented in Table 1. When these data are examined, it is seen that the extract yield of the plants grown in the field is higher than those grown under in vitro conditions.

Table 1. Extract yield of *S. viridis* leaves

	Field-Grown	***in vitro***
Dry Matter Amount (g)	0.5	0.5
Extract Amount (g)	0.0685±0.216	0.0509±0.0056
Extract Ratio (%)	13.03±4.0284	9.45±1.0401
t-value	1.229^{ns}	

^{ns}: not significant

3.3. Antioxidant Capacities of the Samples

This test is based on the spectrophotometric determination of the characteristic purple discoloration by scavenging the stable free radical 2,2-diphenylpicrylhydrazyl (DPPH) by these chemicals in the presence of electron or hydrogen atoms-donating antioxidant chemicals (Cuendet et al., 1997, Esmaeili et al., 2015). The % scavenging activity obtained as a result of the determination of DPPH free radical scavenging activities of extracts obtained from the samples were subjected to linear regression analysis and the IC50 value was calculated. Accordingly, the IC50 values obtained in the analysis were determined in terms of mg extract mL^{-1}. The concentration density required to scavenge half of the DPPH radical from the medium is expressed as the IC50 value. A low IC50 value indicates that the antioxidant activity of the sample is high, while a high value indicates that the activity of the sample is low (Aqil et al., 2006). In terms of DPPH values in our study, the difference between samples was statistically significant at the 1% level. Considering that the antioxidant activity increases as the DPPH radical scavenging activity value decrease, it was observed that the antioxidant activity of the plants grown in the field is higher than the plants grown under in vitro conditions. On the other hand, it was determined that the DPPH radical scavenging activity of both samples included in the study was much lower than the synthetic antioxidants used as control (Table 2).

While the phenolic substance contents of the samples were found statistically significant at a 1% level, the flavonoid substance contents were not significant. However, the leaves of plants grown in the field had 51.163 mg QE g-1 higher flavonoid content than in vitro conditions. While the phenolic substance contents of the samples were found statistically significant at a 1%

level, the flavonoid substance contents were not significant.

Table 2. Antioxidant capacities of the samples

	Total Phenolic (mg GAE g^{-1})	Total Flavonoid (mg QE g^{-1})	DPPH IC_{50} (mg ml^{-1})
Field-Grown	184.15±36.70	212.92±11.18	117.51±6.60 [b]
in vitro	66.46±0.19	161.76±56.13	185.40±4.78 [c]
BHA (uq ml^{-1})			19.662 [a]
BHT (uq ml^{-1})			13.818 [a]
F-value			1275.76**

** Statistically significant at the 0.01% level

However, it was recorded that the leaves of plants grown in the field had 51.163 mg QE g-1 higher flavonoid content than in vitro conditions (Figure 2, Figure 3).

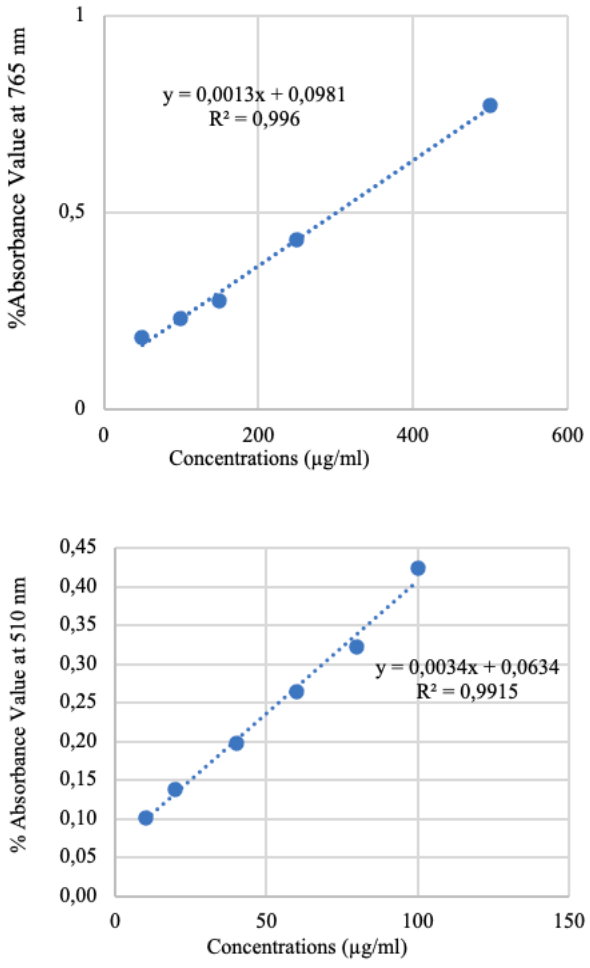

Figure 2. Gallic acid (on top) and Quercetin (bottom) standard curves

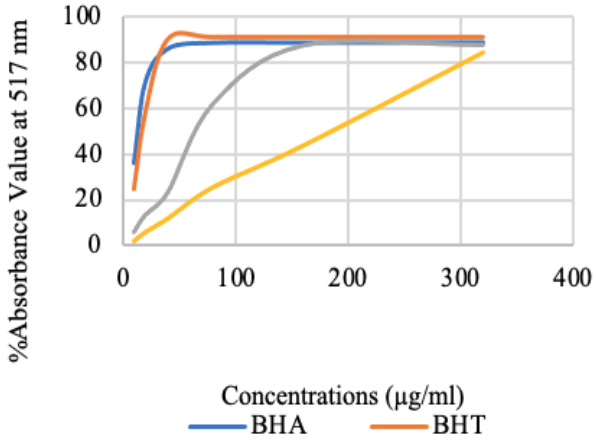

Figure 3. DPPH radical scavenging activity capacities of samples

Similarly, in this study that antioxidant activity was investigated in both wild plants and callus cultures of three *Ephedro* spp. was observed that both wild plants and callus of all three *Ephedro* species had antioxidant activity, but callus exhibited lower activity than wild species. In addition, the phenol content of wild plants was found to be higher than callus (Parsaeimehr et al., 2010).

Due to its reproducibility and low cost, the DPPH test is the most commonly used method to evaluate the antioxidant potential of plant materials. It has been used to test the activity of extracts obtained from aerial parts of a large number of Salvia species (*S.officinalis, S.aethiopis, S.candidissima, S. verticillate, S.virgata, S. hypargeia, S.sclarea, S. limbate, S. microstegia,* and *S.glutinosa*, etc.) (Tosun et al.,2009; Tepe et al., 2006; Veličković et al. 2011). Bayan and Genc, (2016), in their research to define the antioxidant capacity of methanol extract on *S. verticillata* species, determined the IC50 value of DPPH activity as 11.47±0.30 mg ml^{-1}. They also recorded total phenolic and total flavonoid contents as 140.18±8.73 mg GAE g^{-1} extract and 51.56±1.18 mg QE g^{-1} extract, respectively. The antioxidant capacities, total phenolic and flavonoid contents of *Salvia* species exhibit broad variation. For example, in the study carried out with different *Salvia*

species, the lowest and highest antioxidant capacity values were found in *S. dichroantha* (73.855 mg GAE $g^{-1)}$ and in *S. heldreichiana* (80.207 mg AAE g^{-1}), respectively. The highest total phenolic content was found in *S. tomentosa* (13.316 mg GAE 100ml^{-1}), while the lowest total phenolic content was recorded in *S. halophila* (6.168 mg GAE 100 ml^{-1}) (Er, 2012)

Phenols are very important plant components that have free radical scavenging ability due to their hydroxyl groups (Hatano et al., 1989). Therefore, the phenolic content of plants directly contributes to their antioxidant effect and flavonoid biosynthesis (Bendini et al.,2006; Dlugosz et al.,2006; Wojdylo et al.,2007; Parsaeimehr et al.,2010). In our study, it was recorded that there was a positive correlation between the DPPH activities of the extracts and the total phenolic and flavonoid amounts (Figure 4). Similar findings were also expressed by Tosun et al. (2009).

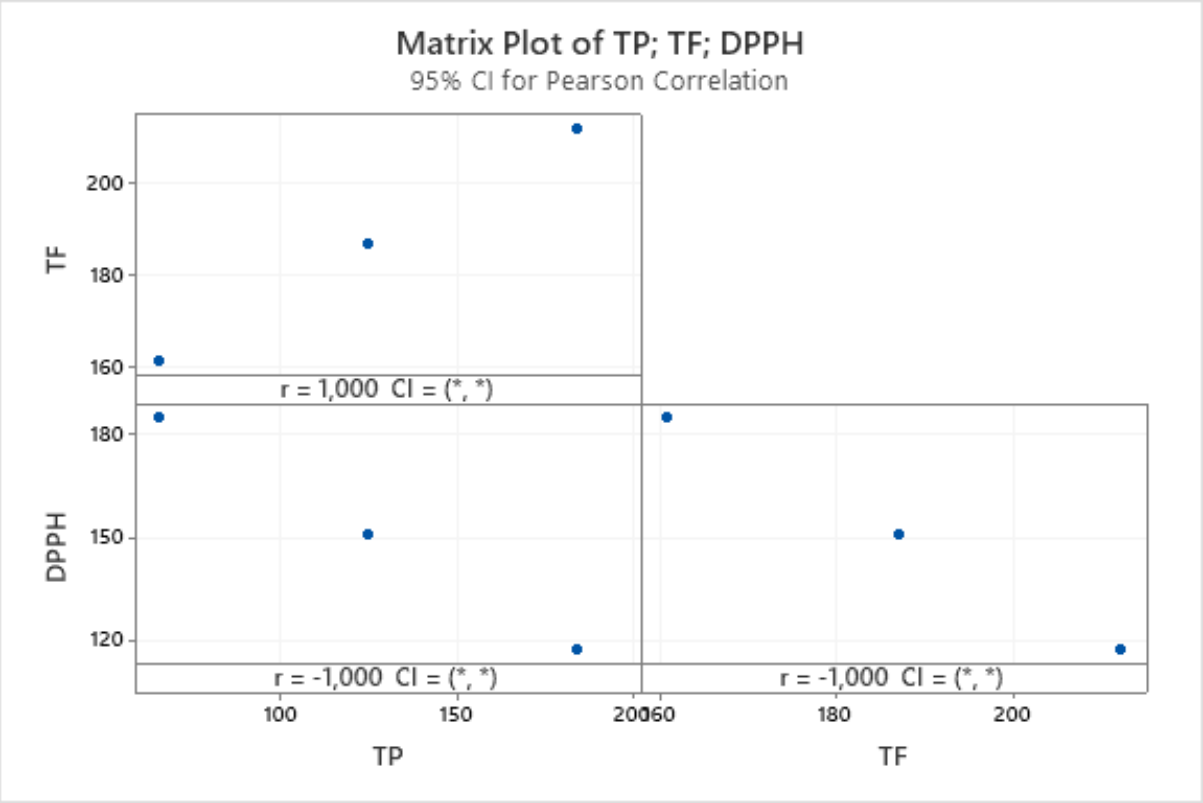

Figure 4. Bilateral Relations among TP (Total Phenolic), TF (Total Flavonoid) and DPPH

Plants that are members of the Lamiaceae family contain many polyphenolic compounds. Rosmarinic acid ($C_{18}H_{16}O_8$), carnosic acid ($C_{20}H_{28}O_4$), and salvianolic acid ($C_{26}H_{22}O_{10}$), are the main phenolic components identified in extracts from *Salvia*. Rosmarinic acid is present in the above-ground parts and roots of *S. viridis*, but the rosmarinic acid concentration in its leaves was very low (Fotovvat et al., 2019, Zengina et al.,2019) We only used leaves in our study. And it was determined that the antioxidant capacity of the leaves was low.

4. Conclusion

In this research, the antioxidant activity of leaves obtained from in vitro and field-grown plants of *S. viridis* was compared for the first time. Our findings showed that the antioxidant capacity determined in the leaves of field-grown *S. viridis* was higher than in vitro conditions. As it is known, various environmental stresses often cause an increase in plant secondary metabolites. In previous studies, it has been explained that aerial parts and roots of *S. viridis* show strong

antioxidant activity. Therefore, economically important raw materials from this species can be produced. This study showed that desirable bioactive substances for pharmaceutical purposes can be obtained from plants grown in vitro. However, in order to bring this species to the economy, its natural compounds and biological activity should be clarified with comprehensive studies.

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Author Contribution

This manuscript has been prepared from the master thesis.

KK: Supply of materials required for research, field, and laboratory. He contributed to the establishment and execution of the experiments, the acquisition and evaluation of data, and the writing of the Master's Thesis.

BCS: Planning the research, supplying the plant materials required for the research, establishing and conducting field and laboratory trials, obtaining data and making statistical analyzes. He contributed to the writing of the Master Thesis and the conversion of the Thesis into an article.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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Anti-Acetylcholinesterase and Synergistic Antifungal Activities of Selected *Salvia* Species: Correlation with Metabolic Profiles

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Abstract

Salvia species are known for their anti-fungal and anti-acetylcholinesterase (AChE) activity. Metabolomics is defined as a comprehensive quantitative and qualitative analysis of large-scale metabolites. In this study, besides determining anti-acetylcholinesterase and synergistic antifungal activities of selected *Salvia* species (*S. cryptantha*, *S. tchihatcheffii*, *S. officinalis*, *S. virgata*), the metabolite profiles were clarified and correlation analyzes between the activity results and profiles were carried out. Aerial parts of all plant materials were extracted by methanol to determine the metabolic profile by using GC-MS (Gas Chromatography-Mass Spectrometry) and LC-QTOF-MS (Liquid Chromatography Quadrupole Time of Flight Mass Spectrometry). Also, Ellman's spectrophotometric method for anti-acetylcholinesterase activity and the checkerboard method for synergistic antifungal activity between extracts and fluconazole were performed. 295 known and 1408 unknown metabolites were detected in GC-MS while 346 known and 69008 unknown metabolites in LC-QTOF-MS. *S. cryptantha*, *S. tchihatcheffii*, *S. officinalis*, and *S. virgata* demonstrated inhibitory activities on AChE with the ratio of 32.72%, 5.9%, 43.96%, and 12.1% at the concentration of 200 µg/m and a synergism was found for all *Salvia* sp. against *Candida tropicalis* ATCC 750 strain with the FICI values of 0.28, 0.15, 0.10, 0.5 respectively. In addition, as a result of correlation analysis, 73 metabolites positively and 31 metabolites negatively correlated with anti-acetylcholinesterase activity, while 18 metabolites positively, and 117 metabolites negatively correlated with synergistic activity were found. All *Salvia* species exhibited synergic activity with fluconazole while having weak AChE inhibitory activity. Combining natural products with synthetic drugs is important in order to increase efficacy. At the same time, herbal products must be used carefully at the point of herb-drug interactions. Further studies are needed to investigate active metabolites according to correlation results.

Keywords: Acetylcholinesterase inhibitory activity, Metabolomics, *Salvia,* Synergism, *Candida*

1. Introduction

Since ancient times, people have utilized medicinal plants for their therapeutic effects. Previous studies have revealed that these plants have a definite impact on several human biological systems, including the

nervous, urinary, and respiratory systems, and are significant sources of potential therapeutic agents [Kumar et al., 2013; Mamun et al., 2014]. Current methods among which omics technology has an important place, are being developed in the research of effective compounds in the content of medicinal plants [Verpoorte et al., 2005; Nemutlu et al., 2012].

Metabolomics (phytomics for plants) is an omics step used to determine the metabolite profile in plant extracts. GC-MS (Gas Chromatography-Mass Spectrometry) and LC-QTOF-MS (Liquid Chromatography Quadrupole Time of Flight Mass Spectrometry) are the chromatographic methods used effectively in metabolomics studies [Gonulalan et al., 2020].

In plant extracts, biological activity may sometimes be attributed to a single molecule, but two or more metabolites may contribute to the effect through synergistic or antagonistic mechanisms. Determination of which metabolites are effective and how they contribute to the activity has some challenges [Williamson, 2001].

Nowadays, metabolite-activity correlation studies are a beneficial approach for determining the effective metabolites and contribution levels [Gonulalan et al., 2020].

Recently, the increase in the prevalence of *Candida* infections has made them a substantial public health problem. Candidemia is shown as the fourth most common hospital-acquired disease in the United States due to its high mortality rates [Bibi et al., 2021]. Especially in hospitalized and immunocompromised patients, candidiasis is a major cause of fungal infections. It is especially common in individuals suffering from diabetes, cancer, AIDS, and transplant patients as well as those with other severe diseases [Ng'uni et al., 2022]. In order for antifungal medication to be effective, new solutions are required as the epidemiology of these fungal infections is continually changing [Meirelles et al., 2017].

Candida tropicalis often ranks third or fourth as a cause of candidemia but is a significant contributor to invasive candidiasis with a 30-day mortality rate as high as 40–50%. However, it is one of the leading causes of candidemia in parts of the Asia Pacific and Latin America. Although *C. tropicalis* is known to be susceptible to many antifungal drugs, resistance to the azoles has been increasing reported [Kighley et al., 2022]. However, recent multicenter studies in Turkey have shown an increase in the prevalence of azole-resistant and azole-tolerant isolates, especially in blood isolates [Arastehfar et al., 2020].

The need for novel, alternative treatments is urgent due to the widespread use of antifungals and the rise in infections brought on by emerging species. Natural medicines have been utilized for centuries in Turkey and other parts of the world. Checkerboard method can be used for synergistic antifungal activity between extracts and FLZ [Tullio et al., 2019].

Alzheimer's can be short-defined as an irreversible neurodegenerative disease that causes severe memory loss, unusual behavior, personality changes, and cognitive dysfunction [Rao et al., 2012]. There is no cure for Alzheimer's and the drugs available are inadequate. Acetylcholinesterase inhibitors are effective and used in the treatment of Alzheimer's [Björkholm and Monteggia, 2016].

The genus *Salvia*, which belongs to Lamiaceae family, is important in terms of its richness of species, its traditional use and, its preference as a cultivated plant. Some *Salvia* species are reported to be effective in the treatment of conditions such as coronary heart disease, hepatitis, dysmenorrhea, and insomnia [Lu and Foo, 2002].

Monoterpenes, diterpenes, triterpenes, and flavonoids are the major ingredients of *Salvia* species, which are abundant in essential oils [Amaro- Luis et al., 1998].

Aim of this study is to elicit both concerned activities and metabolite profile of *Salvia* species known for their anti-fungal and anti-acetylcholinesterase (AChE) activities [Rus et al., 2015; Kobus-Cisowska et al., 2019].

2. Material and Methods

2.1. Plant Materials

Aerial parts of *S. cryptantha* Montbret & Aucher ex Benth. (HUEF20028), *S. tchihatcheffii* (Fisch. & C.A.Mey.) Boiss. (HUEF20035), *S. virgata* Jacq. (HUEF20036) were harvested during the flowering season from Ankara Beytepe region. Only *S. officinalis* L. (TBÇ-S-001) was purchased from the Selçuk University.

2.2. Extraction of Plant Materials

Each of the 5 g of powdered crude drug materials was extracted with 50 mL of methanol over a 30-minute period in a reflux cooler at 40 °C. The residue was then extracted for 15 minutes with 30 mL of methanol and then filtered once more. The filtrates were combined, dried with an evaporator, and then lyophilized. Quantity of extracts were obtained as 856.2, 939.7, 820.3, 690.1 mg for *S. cryptantha*, S. *tchihatcheffii*, *S. officinalis*, and *S. virgata* respectively.

2.3. Sample Preparation

2.3.1. Sample Preparation for Activity Assays

Stock solutions were prepared in methanol as 4000 ppm and diluted to final concentrations, which were 200, 100, 50, 25, and 12.5 μg/mL for extracts and 20, 10, 5, 2.5, and 1.25 μg/mL for galanthamine. The concentration ranges applied in the MIC assay were 0.5-256 μg/mL for FLZ and 2-1024 μg/mL for each *Salvia* species. For the checkerboard assay, the ranges for FLZ and *Salvia* species extracts were 0.25 to 128 g/mL and 2 to 256 g/mL, respectively.

2.3.2. Sample Preparation for GC-MS

10 mL of methanol was used to dissolve 1 mg of lyophilized extract. Myristic acid was added as an internal standard and centrifugated. 200 mL of this solution was separated and vacuum-dried. 20 μL of 20 mg/mL methoxyamine in pyridine was used for methoxyamination (30 °C for 90 min). 80 µL of MSTFA (N-Methyl-N-(trimethylsilyl) trifluoroacetamide with 1% TMCS (trimethylchlorosilane) was used for derivatization (37 °C for 30 min). And then solutions are placed in vials with 200 µL silanized inserts for GC-MS analysis.

2.3.3 Sample Preparation for LC-QTOF-MS

1000 μg/mL plant extracts were diluted from the stock solution and 10 μg/1000 mL phenylalanine was added as an internal standard. Lyophilized plant materials were dissolved in 10 mL methanol. The samples were transferred into LC-QTOF-MS vials.

2.4. Antifungal and Checkerboard Assays

2.4.1. Strain, Culture Condition and Chemicals

The reference strain *Candida tropicalis* ATCC 750 was sub-cultured on Sabouraud Dextrose Agar (SDA, Merck) and incubated for 24 h at 37 °C. Periodically at -80°C, strains were replenished from frozen stocks. Experiments were conducted in RPMI 1640 medium supplemented with l-glutamine but lacking sodium bicarbonate (Sigma) and buffered with 0.165 M morpholinepropanesulfonic acid (MOPS) [Alkhalifa et al., 2022].

The stock solution of FLZ powder that was purchased from Abcr was prepared in dimethyl sulfoxide (DMSO) (1024 µg/mL). The stock solutions of the extracts were

prepared in DMSO at a concentration of 2048 µg/mL. Stock solutions were kept at -20 °C until use. All experiments were conducted in duplicate. It was previously done to control using DMSO 2%.

2.4.2. Antifungal Activity

C. tropicalis were assayed for susceptibility to four *Salvia* sp. and to FLZ by a broth microdilution method (MIC), in accordance with the guidelines of CLSI M27-A3 document [CLSI, 2008]. The concentration ranges used were 0.5–256 µg/mL for FLC, 2-1024 µg/mL for each extract. Plates were incubated at 37 °C for 24 h. The MIC was established as the lowest concentration of FLC and *Salvia* extract at which no visual turbidity was observed in *C. tropicalis* ATCC 750.

2.4.3. Checkerboard Microdilution Assay

The most used technique for evaluating antibacterial combinations in vitro is the checkerboard approach. Synergic interactions between FLZ and the extracts were determined by the checkerboard microtiter assay. Checkerboard synergy testing was performed by the microdilution method as previously described [Canturk, 2018]. The final concentrations for FLZ and the extract varied from 0.25 to 128 μg/mL and 2 to 256 μg/mL, respectively, from the initial concentration of fungal suspension in RPMI 1640 medium of 10^3 CFU/mL. Microtiter plates were incubated at 37°C for 24 h. The fractional inhibitory concentration index (FICI) was figured out using the first non-turbid well in each column and row at 96 well U-bottom microplate. FICI ≤ 0.5 is considered synergistic; >0.5 to <4 indifference, and ≥ 4 antagonistic.

2.5. Anti-AchE Assay

The *in vitro* modified Ellman's spectrophotometric assay was used to determine the anti-AChE activities of the samples [Ellman et al., 1961]. Briefly, potassium phosphate buffer (pH:7.5), 5,5'-dithio-bis-(2-nitrobenzoic acid) (1.25 mM), acetylcholinesterase enzyme (electric eel, Type VI-S) and the different concentrations of test samples were incubated in a 96 well plate for 10 min. The enzymatic reaction was initiated by adding the substrate acetylthiocholine iodide (7.5 mM). The changes in the absorbance were read at 412 nm for 4 min. Galantamine was used as the reference compound.

2.6. Metabolomics Analysis

2.6.1. LC-QTOF-MS Analysis

Conditions of the LC-QTOF-MS analysis system have been previously described in Gonulalan et al., [2020].
Metabolites' auto MS-MS data were recorded above the 200-count threshold between 100 and 1700 m/z. The plant metabolites were fragmented by MS/MS using a collision energy of 20 eV.

The recorded raw MS data was processed using MS-Dial 3.96 for deconvolution, peak identification, and alignment [Tsugawa et al., 2015]. The minimum amplitude for peak detection was set at 2000 amplitude. Tolerances for MS1 and MS2 were switched to 0.01 and 0.025 Da.

The RIKEN tandem mass spectral database (ReSpect), a plant-specific ms/ms-based database, was used to predict the molecular formula and structure. A positive mode was used for the identification of metabolites. The identification socer cut off value was set to 60%, and the mass tolerances for the MS1 and MS2 were modified to 0.01 and 0.05 Da. Microsoft Excel was used for correlation analysis.

2.6.2. GC-MS Analysis

Metabolomic analysis based on GC-MS was carried out as previously described [Nemutlu et al., 2015]. Microsoft Excel was used for correlation analysis.

3. Results and Discussion

3.1. Antifungal Activity

Salvia species are well-known herbal therapeutic medicines that have been utilized for centuries. Because of their antioxidant, antibacterial, antifungal, and anti-leishmanial characteristics, they are popular medicines both inside and outside of European nations [Nikmehr et al., 2014]. Due to the increase in yeast resistance, there is a need to search for new strategies in the treatment of candidiasis. In this study, a potential pharmacological strategy in anti-candidal therapy was investigated, specifically the combination of FLZ which is one of the conventional antifungals with *S. cryptantha, S. tchihatcheffii, S. officinalis* and *S. virgata* extracts of the Lamiaceae family.

In our study, we initially determined the minimum inhibitory concentrations of the FLZ and the *Salvia* species extracts in a liquid culture medium. **Table 1** show antifungal effects of FLZ and *Salvia* species extracts against to *C. tropicalis* ATCC 750. MIC results were evaluated as susceptible to *C. tropicalis* ATCC 750 in terms of CLSI standards.

Candida tropicalis strains is frequently corporate with candidiasis in neutropenic patients. Antifungal therapy can be challenging, and the yeast cellular wall's thickness has been described as a shield to polyene antifungal drugs [Pozzatti et al., 2008]. All *Salvia* species had modest antifungal activity in this research. The best results were determined as *S. virgata* extract with 32 µg/mL against *C. tropicalis* ATCC 750 strain. Recently, the antifungal activity of *Salvia officinalis* has been studied by Martins et al., [2015] and the MICs found for the methanol extract of *Salvia officinalis* were higher than those found by us.

3.2. Checkerboard Microdilution Assay

Antifungal drug resistance problems approaches propose several possible ways of preventing and overcoming drug resistance. The efficacy antifungal drugs can be developed by using combination therapy [Samber et al., 2015]. The effect of antifungals in combination with Lamiaceae species extracts may contribute to the reduction of pathogen resistance to drugs thus making the treatment more effective [Mirghani, 2022]. This is the first time that antifungal activity of the aforementioned *Salvia* species methanol extracts in combination with FLZ has been studied.

Checkerboard microdilution assay was performed to study the synergistic activity between the antibiotic FLZ and the methanol extracts of all *Salvia* species on *C. tropicalis* ATCC 750. **Table 2** provides a summary of the findings of the checkerboard analysis. Combinations of FLZ and all of the *Salvia* species extracts which used in this study showed synergetic effects against *C. tropicalis* ATCC 750.

Table 1. Determination of MICs of *Salvia* species methanol extracts and FLZ against *C. tropicalis* ATCC 750.

	MIC (µg/mL)	CLSI M27-A3 Breakpoints		
		S	SSD	R
S. cryptantha	64	-*	-	-
S. tchihatcheffii	64	-	-	-
S. officinalis	128	-	-	-
S. virgata	32	-	-	-
FLZ	2	≤ 2.0	4	≥ 8.0

*(-) breakpoints not provided by CLSI documents M27-A3.

Table 2. Interaction of FLZ with *Salvia* sp. methanol extracts against common *C. tropicalis* evaluated by checkerboard and interpretation by fractional inhibitory concentration index.

Strain	Checkerboard MICs of Combination		FICI	Outcome
	FLZ (µg/mL)	*Salvia* sp. extracts (µg/mL)		
C. tropicalis ATCC 750	1	2[a]	0.28	Synergy
	2	2[b]	0.15	Synergy
	1	4[c]	0.10	Synergy
	1	16[d]	0.5	Synergy

a *S. cryptantha Montbret & Aucher ex Benth*, b *S. tchihatcheffii (Fisch. & C.A.Mey.) Boiss.*c *S. officinalis L.* d *S. virgata Jacq.*

The combinations of studied *Salvia* species extract and FLZ gave very pronounced synergistic effects regarding *C. tropicalis* ATCC 750 with FICI values between 0.10 and 0.5.

There is a lack of studies demonstrating the synergistic effect of Salvia species, synergy studies with different components have been discussed. In a study conducted by Nóbrega et al. (2019). *C. tropicalis* ATCC 13803 strain sensitive to the carvacrol/FLZ combination resulted in additive effects (FICI=1.25). Another study using thymol and carvacrol in combination with FLZ showed synergistic effects against the *C. tropicalis* ATCC 750 strain [Ahmad et al., 2013]. The findings of our research provide evidence of a synergistic effect in terms of the results compared to those obtained from other studies. The results presented here thus support the design of clinical studies to assess the effectiveness of this combination therapy. Natural medicines with intrinsic antimicrobial activity or substances that promote the action of extensively used antibiotic/antifungal treatments may be employed as new ways to treating multi-resistant pathogens and preventing the interactions of these microorganisms with synthetic drugs. Natural substances can also be used with conventional antimicrobials to increase the antimicrobial effectiveness of both [Ermenlieva et al., 2022]. However, the cases such as overdose or side effects that may occur in the use of these *Salvia* species, which are widely used for traditional treatment, with antifungals should be investigated in further studies.

3.3. Anti-AChE Assay

The anti-AChE activity of four *Salvia* species and galanthamine are shown in **Table 3** as the percentages of inhibition. Among these four species, the most active one was determined to be *S. officinalis.* None of the *Salvia* species has significant anti-AChE activity.

Table 3. Anti-acetylcholinesterase (AChE) activity results of *Salvia* species and galanthamine.

	200 µg/mL	**100 µg/mL**	**50 µg/mL**	**25 µg/mL**	**12.5 µg/mL**
S. cryptantha	32.72	17.93	11.27	3.87	-
S. tchihatcheffii	5.90	1.47	-	-	-
S. officinalis	43.96	23.88	12.1	1.19	0.86
S. virgata	12.10	4.68	1.21	0.96	0.33
	20 µg/mL	**10 µg/mL**	**5 µg/mL**	**2.5 µg/mL**	**1.25 µg/mL**
Galanthamine	80.56	69.53	57.50	37.93	24.48

3.4. Metabolomics Analysis

Metabolomics analyses (Phytomics in plants) is a new and effective holistic approach to clarifying the metabolic profile of plant extracts, as well as providing some unique opportunities to identify active metabolic compound(s) when combined with correlation analysis [Waris et al., 2022].

After the GC-MS chromatograms were deconvolved and aligned, 1703 mass spectral features were found, of which 295 were analyzed using retention index libraries. Major compound groups and quantities of the metabolites are listed in **Table 4**.

Table 4. GC/MS Based Metabolomic Profiling (Major metabolites).

Super Class	Class / Sub Class / Parent	Quantity of Metabolites
Organic acids and derivatives	Amino acids, peptides, and analogues	52
	Hydroxy acids and derivatives	7
	Dicarboxylic acids and derivatives	6
	Keto acids and derivatives	6
Organic oxygen compounds	Carbohydrates and carbohydrate conjugates	56
Lipids and lipid-like molecules	Fatty Acyls	33
	Prenol lipids	10
Benzeneoids	Benzene and substituted derivatives	19
	Phenols	11
Organoheterocyclic compounds	Azoles, Indoles and derivatives, Pyridines and derivatives, etc..	27
Phenylpropanoids and polyketides	Cinnamic acids and derivatives, Flavonoids, Phenylpropanoic acids, etc..	24

69354 peaks were discovered in the LC-qTOF-MS data, 346 of which were recognized by the MS/MS spectrum. **Table 5** lists the major compound groups and quantity of metabolites.

Table 5. LC-QTOF-MS Based Metabolomic Profiling (Major metabolites).

Types of Metabolites	Quantity of Metabolites
Flavonoid-7-*O*-glycosides	15
Flavonoid-3-*O*-glycosides	10
Anthocyanidin-3-*O*-glycosides	10
Anthocyanidin-5-*O*-glycosides	5
Flavonols	5
Methoxyphenols	5
Hydroxycinnamic acids	4
Hydroxybenzoic acid derivatives	3
7-hydroxycoumarins	2

3.5. Correlation Analysis

Correlation analysis can be used to find active ingredients of plant materials. For this purpose, total compound groups are usually selected [Muflihah et al., 2021]. In our study different and more spesific compound groups are correlated with the activity. Also the number of the correlated metabolites and the correlation coefficients has determined.

The LC-QTOF-MS analysis's correlation studies revealed that 30 metabolites positively correlated ($r \geq 0.85$) with the anti-AchE activity, while 22 metabolites were negatively correlated ($r \leq - 0.85$).

The GC–MS analysis's correlation studies revealed that 43 metabolites positively correlated ($r \geq 0.85$) with the Anti-AchE activity, while 9 metabolites were negatively correlated ($r \leq - 0.85$).

The LC-QTOF-MS analysis's correlation studies revealed that 14 metabolites had a positive correlation ($r \geq 0.85$) with the synergistic effects with FLZ, while 66 metabolites displayed a strongly negative correlation ($r \leq - 0.85$).

The GC–MS analysis's correlation studies revealed that 4 metabolites had a positive correlation ($r \geq 0.85$) with the synergistic effects with FLZ, while 51 metabolites displayed a strongly negative correlation ($r \leq - 0.85$).

4. Conclusion

The present study provides new information regarding the anticandidal potential of *S. cryptantha*, *S. tchihatcheffii*, *S. officinalis, S. virgata* methanol extracts and their synergistic effects with FLZ. The results indicate that all *Salvia* species studied possess anticandidal activity as well as combined high anticandidal effect and high synergistic interaction with the FLZ. We selected a relatively small number of samples because we regarded it as a form of a pilot study. Additional studies are necessary to determine the mechanism of these synergistic antifungal associations.

According to our results, FLZ and *Salvia* species extracts combination may be a potential therapeutic option for the treatment of *C. tropicalis* related infections.

According to the anti-AChE activity results, four *Salvia* species has no significant anti-AChE activity. Only *S. officinalis* showed moderate effect. Further studies are needed to clarify metabolite/metabolites as determined by the correlation analyses may have higher activity or not.

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Author Contribution
EMG contributed Anti-Acetylcholinesterase activities and correlation analysis, considered and designed the research, drafted the manuscript, and approved the final version of the manuscript. CK performed anti-Acetylcholinesterase activities and also contributed to the draft of the manuscript. NBB contributed the antifungal and synergic activities by using the broth microdilution method, and checkerboard microdilution assay, respectively, and also contributed to the draft of the manuscript. GBY performed the antifungal and synergic activities by using the broth microdilution method and checkerboard microdilution assay, respectively, and also contributed to the draft of the manuscript.

Conflicts of Interest
The authors have no conflicts of interest to declare and disclose any financial field.

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Effect of chronic administration of aqueous extract of Neem (*Azadirachta indica*) leaves on Paracetamol-induced hepatotoxicity in Wistar albino rats

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Abstract

Hepatoprotective drugs are not available for use in modern medicine and different parts of medicinal plants like Neem (*Azadirachta indica*) are used as hepatoprotectants in traditional medicine. Although there are scientific reports of its hepatoprotective activity on acute administration, we found only one study which had evaluated its hepatoprotective effect on chronic administration. **Objectives:** To evaluate the effect of chronic oral administration of Neem on paracetamol-induced hepatotoxicity in Wistar rats. **Methods:** We randomly assigned 72 male and female Wistar albino rats to four groups of 18 animals each and orally administered Distilled water 5ml/kg body weight/day to Groups A (Normal control) and B (Experimental control), 500 mg/kg aqueous Neem leaf extract (Test) to Group C and Silymarin suspension (Standard) 100mg/kg/day to Group D for 30 days. On the 8th day, we induced hepatotoxicity with Paracetamol 2g/kg body weight single dose to groups B, C and D. We performed liver function tests, recorded liver weights and examined liver histology of six rats from each group on 10th, 20th and 30th days. **Results:** We observed significant difference (P<0.05) in Mean ± SEM values of serum bilirubin, ALP, AST, ALT, liver weights, total protein and albumin: globulin in Group B compared to Group A, whereas these changes were significantly less in groups C and D compared to B. Histopathological examination of liver showed hepatic necrosis on 10th day in Groups B, C and D, but on 20th and 30th day in Group B and on 10th day in Groups C and D, we observed decrease in sinusoidal congestion and cloudy swelling along with small areas of regenerative changes. All abnormal histological changes decreased by the 30th day. **Conclusion:** Thus, aqueous extract of *Azadirachta indica* leaves shows hepatoprotective activity on chronic oral administration in Wistar rats.

Key Words: *Azadirachta indica*, Neem, Hepatoprotective, Wistar rats, Paracetamol, Hepatotoxicity

1. Introduction

The liver is a major organ which plays a central role in many essential physiological processes, thus maintaining the metabolic homeostasis of the body. It is also responsible for the metabolism and detoxification of

many endogenous and exogenous compounds (xenobiotics). Hepatic damage may result from primary or secondary liver disease as well as exposure to miscellaneous drugs and environmental chemicals. Although its regenerative capacity is well known, this makes the organ particularly prone to damage from these pharmaceutical and environmental chemicals.[1]

In conditions where liver function is compromised or at risk of toxic effects, hepatoprotective drugs can be administered to the patients. But such medicines are deficient in allopathic medicine. Due to the absence of reliable drugs for the management of liver ailments in modern medicine, plants and natural products are being relied upon as a source of hepatoprotectants. Natural sources of hepatoprotective agents are widely used in traditional medicine. Currently, Silymarin which is derived from Silybum marianum (Milk thistle) is available as a hepatoprotective drug formulation.[2,3] Silymarin is a flavolignan and is a mixture of structural components silybin A, silybin B, isosilybin A isosilybin B, silydianin, silychristin, isosilychristin and taxifolin.[4] Many other phytochemicals present in different parts of plants, like *Allium sativum, Andrographis paniculata, Ocimum sanctum, Solanum nigrum, Phyllanthus niruri, Mangifera indica, Magnolia officinalis, Nigella sativa, Gingko biloba,* etc have been found to have hepatoprotective effect.[5,6]

Neem, known by its botanical name *Azadirachta Indica* A. Juss, belonging to the mahogany family Meliaceae, is an evergreen tree, traditionally cultivated in various parts of South east Asia (Indian subcontinent), Africa but nowadays it is grown in Central and South America also.[7,8] The US National Academy of Sciences published a report in 1992 entitled, "Neem—a tree for solving global problem," underscoring the value of Neem.[9] It is a commonly used traditional medicinal plant in India. Various parts of the Neem tree (leaves, bark, fruit, flowers, oil and gum derivatives) have been reported to possess analgesic, anti-inflammatory, antipyretic, hypoglycaemic, hypolipidaemic, antihypertensive, immunostimulant, hypoglycaemic, antiulcer, antifertility, antioxidant, anxiolytic, antimicrobial, anthelminthic, antimalarial, antiviral, antifilarial and anticarcinogenic, properties.[10-53]

The hepatoprotective effect of different parts of Neem (*Azadirachta indica*), including leaf extract has been reported by previous researchers.[52-76] However, it has been observed by other researchers to be hepatotoxic.[5] Moreover, we found only one study[75] which has evaluated the effect of chronic administration of Neem leaves on the liver.

Thus, our objective was to evaluate the hepatoprotective effect of chronic oral administration of aqueous extract of leaves of Neem (*Azadirachta indica*) on Paracetamol-induced hepatotoxicity in Wistar albino rats

2. Material and Methods

2.1. Collection of Plant material

The study was undertaken after our experimental research protocol was granted permission of Institutional animal ethics committee (IAEC Reg. 634/02/a/CPCSEA).

Fresh leaves *of Azadirachta Indica* (Neem) were collected from local gardens of Dibrugarh, Assam. We collected the leaves once a week on sunny days during spring season. Before collection, the plant was authenticated by Ms. Belinda Lahon, PhD in Botany from North Bengal University, West Bengal, India.

2.2. Drugs and chemicals

Preparation of *Azadirachta indica* aqueous extract (ANLE)

We mixed one Kg of freshly collected, shade dried, powdered leaves of *Azadirachta Indica* (Fig.1) with four liters of distilled water and allowed the suspension to soak overnight. Thereafter, it was centrifuged at 5000 rpm for 20 minutes and filtered through a Whatman's No 1 filter paper. The supernatant fluid was kept in glass petri dishes under tube light to provide heat for

facilitating evaporation and to prevent dampness and avoid contamination by organisms. After drying, we scraped off the powder (total yield 5.5%). We then prepared stock solution by dissolving 500mg of the extract in distilled water.[56]

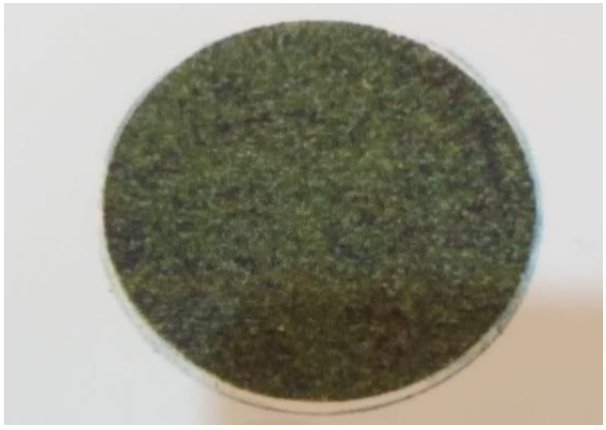

Figure 1. Neem leaf powder

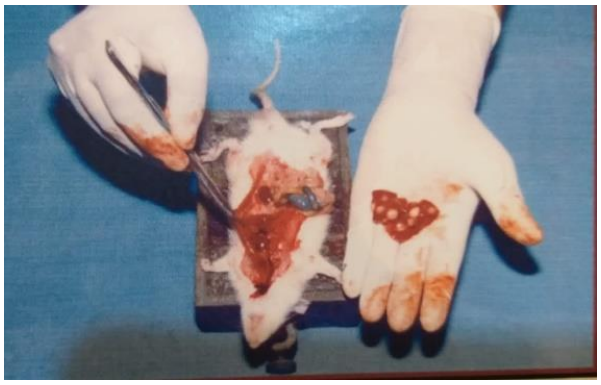

Figure 2. Dissection of rat liver

Silymarin

Silymarin powder was obtained from Micro Labs Ltd., Bangalore, India. The stock solution was prepared by dissolving 100mg of Silymarin powder in 5ml of distilled water and used as a standard drug in the dose of 100mg/Kg body weight/day, following the method of Mansour *et al*.[2]

Paracetamol

Paracetamol powder was obtained from Quality Pharma Pvt. Ltd. (Kolkata, India).

All drugs and chemicals were of analytical grade.

2.3. Experimental animals

Seventy-two healthy adult Wistar albino rats of either sex, weighing 100-150gms were procured from the Central Animal House of Assam Medical College & Hospital, Dibrugarh, Assam. Before starting the experiment, animals were allowed to acclimatize to the laboratory environment for one week. They were housed in a well ventilated room at 23.0±2°C, humidity of 65-70% and 12hr light/dark cycle. They were fed with standard diet along with water in sufficient amount. All animals were looked after ethically, as per the principles of laboratory animal care prescribed by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).[77]

2.4. Experimental Design

The experiment was carried out for 30 days. Animals were weighed and their weights were recorded. They were numbered and divided into four groups randomly, each group containing eighteen animals.

Grouping and treatment schedule:

Group A: Normal control received Distilled water 5ml/kg body weight/day X 30 days. Group B: Experimental control received Distilled water 5ml/kg body weight/day X 30 days. Group C: Received Aqueous Neem leaf extract (ANLE) 500mg/kg body weight/day X 30 days.

Group D: Received Silymarin suspension (SILY) 100mg/kg body weight/ day X 30 days. On the 8th day, Paracetamol 2g/kg body weight single dose was given to animals of Groups B, C and D (experimental control, test and standard drug groups).

Dose selection of *Azadirachta indica* (Neem) leaf aqueous extract and standard drug Silymarin was based on previous studies.[56,2]

The drugs were administered to the animals orally by an intragastric feeding tube. All animals were observed for any physical and/or behavioural features of toxicity following administration of the extract throughout the experiment.

Six animals from each group were randomly selected for estimation of liver function on the 10th, 20th and 30th day of the experiment. Blood was collected directly by cardiac puncture under light ether anaesthesia, serum was separated and sent for laboratory estimations.

Laboratory parameters for measurement

Liver function tests:

Serum bilirubin, Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), Total serum protein, Albumin:Globulin ratio were estimated by the following methods:

ALT - Modified IFCC/UV kinetic method, AST - Modified IFCC/UV kinetic method, ALP – Total protein - Biuret method, Albumin - BCG Dye binding method.[78-81]

Histopathological examination:

Six animals from each group were humanely sacrificed under Inj. Sodium pentobarbitone 150mg/kg i.p. anaesthesia on 10th, 20th and 30th days respectively to collect the liver specimens for histopathological examination (Fig.2). The gross physical appearance and weight of the livers were recorded and they were preserved in 10% formalin after washing initially with normal saline. Paraffin embedding technique was performed. Liver sections of 5-mm thickness were obtained and stained with hematoxylin and eosin. Thereafter, we examined the liver sections for histopathological changes under a light microscope.[82]

Statistical analysis:

We used GraphPad Instat statistical software for data analysis. The quantitative variables were expressed as Mean ± SEM. Statistical significance of the results of quantitative variables (LFT values) between groups was analysed using one-way ANOVA followed by Bonferroni's multiple comparison test. $P < 0.05$ was considered as statistically significant at 95% confidence level.

3. Results and Discussion

We evaluated the effect of chronic oral administration of aqueous leaf extract of Neem (*Azadirachta indica*) in Wistar albino rats over 30 days.

The animals did not show any physical or behavioural symptoms of toxicity after chronic oral administration of *Azadirachta indica* (Neem) aqueous leaf extract. The non-toxic nature of Neem leaf extract and Neem leaf glycoprotein to mice and rats was reported by Haque *et al.*, Mallick, *et al.* respectively[83,84]

Table 1: Mean ± SEM of LFTs and liver weights of control and drug-treated albino rats

Gr	Serum Bilirubin (mg/dl)	Alkaline Phosphatase (IU/L)	AST (IU/L)	ALT (IU/L)	Serum Protein (g/dl)	Albumin: Globulin	Weight of liver (g) (% B.W)
A (DW 5ml/kg/day)	0.48±0.02	212.00±2.04	36.33±1.28	36.50±1.12	5.58±0.06	1.35±0.01	5.98±0.60
B (DW 5ml/kg/day)	0.57±0.02[a]	529±3.170[a]	216.33±3.07[a]	190.67±3.85[a]	5.32±0.07[a]	1.27±0.01[a]	7.28±0.14[a]
C (ANLE 500 mg/kg/day)	0.42±0.02[b]	315.67±3.20[bd]	78.00±2.48[b]	65.83±2.09[bd]	5.52±0.06[b]	1.39±0.02[bd]	6.50±0.04[b]
D (SILY 100mg/kg/ day)	0.45±0.02[b]	335.33±12.21[b]	82.00±2.44[b]	73.50±1.93[b]	5.50±0.06[b]	1.31±0.01[b]	6.61±0.07[b]

Statistical analysis by One-way ANOVA followed by Bonferroni's test with significance at $P<0.05$. a: $P<0.05$ when compared with Group A; b: $P<0.05$ when compared with Group B; c: $P>0.05$ when compared with Group A; d: $P<0.05$ when compared with Group D; e: $P>0.05$ when compared with Group E. DW = Distilled water, ANLE = Aqueous Neem leaf extract, SILY= Silymarin suspension. Paracetamol 2g/kg single dose administered to groups B,C and D on 8th day. (10th day of experiment; n=6).

Table 2. Mean ± SEM of LFTs and liver weights of control and drug treated albino rats

Gr	Serum Bilirubin (mg/dl)	Alkaline Phosphatase (IU/L)	AST (IU/L)	ALT (IU/L)	Serum Protein (g/dl)	Albumin: Globulin	Weight of liver (g) (% B.W)
A (DW 5ml/kg/day)	0.48±0.02	212.00±2.37	29.33±2.51	32.00±2.25	5.67±0.05	1.29±0.01	6.00±0.07
B (DW 5ml/kg/day)	0.83±0.02[a]	376.67±5.62[a]	171.67±4.05[a]	168.33±3.24[a]	4.97±0.05[a]	1.31±0.02[c]	6.86±0.08[a]
C (ANLE 500 mg/kg/day)	0.36±0.02[bd]	240.67±2.25[bd]	58.50±2.23[bd]	56.33±1.69[b]	5.78±0.06[b]	1.36±0.02[bd]	6.40±0.04[b]
D (SILY 100mg/kg/ day)	0.41±0.01[b]	251.33±5.53[b]	63.67±3.04[b]	59.67±2.44[b]	5.73±0.05[b]	1.32±0.01[e]	6.53±0.04[b]

Statistical analysis by One-way ANOVA followed by Bonferroni's test with significance at $P<0.05$. a: $P<0.05$ when compared with Group A; b: $P<0.05$ when compared with Group B; c: $P>0.05$ when compared with Group A; d: $P<0.05$ when compared with Group D; e: $P>0.05$ when compared with Group E. DW = Distilled water, ANLE = Aqueous Neem leaf extract, SILY= Silymarin suspension. Paracetamol 2g/kg single dose administered to groups B, C and D on 8th day. (20th day of experiment; n=6).

Table 3. Mean ± SEM of LFTs and liver weights of control and drug treated albino rats

Gr	Serum Bilirubin (mg/dl)	Alkaline Phosphatase (IU/L)	AST (IU/L)	ALT (IU/L)	Serum Protein (g/dl)	Albumin: Globulin	Weight of liver (g) (% B.W)
A (DW 5ml/kg/day)	0.47±0.02	169.33±3.04	34.50±0.96	35.00±1.70	5.63±0.04	1.35±0.02	5.99±0.06
B (DW 5ml/kg/day)	0.54±0.02[c]	270.33±2.94[a]	58.67±1.84[a]	52.00±1.55[a]	5.25±0.06[a]	1.32±0.03[a]	6.40±0.06[a]
C (ANLE 500 mg/kg/day)	0.37±0.02[b]	202.00±6.18[b]	28.33±0.95[b d]	38.00±1.15[b]	5.59±0.05[b]	1.40±0.03[bd]	6.04±0.05[bd]
D (SILY 100mg/kg/ day)	0.40±0.02[b]	221.67±4.80[b]	43.00±1.12[b]	39.00±1.98[b]	5.62±0.05[b]	1.36±0.02[b]	6.21±0.07[b]

Statistical analysis by One-way ANOVA followed by Bonferroni's test with significance at $P<0.05$. a: $P<0.05$ when compared with Group A; b: $P<0.05$ when compared with Group B; c: $P>0.05$ when compared with Group A; d: $P<0.05$ when compared with Group D; e: $P>0.05$ when compared with Group E. DW = Distilled water, ANLE = Aqueous Neem leaf extract, SILY= Silymarin suspension. Paracetamol 2g/kg single dose administered to groups B,C and D on 8th day. (30th day of experiment; n=6).

LFT and Liver weights

The results of LFT estimation and liver weights are shown in Tables 1-3.

On 10th day of the experiment, the mean levels of serum bilirubin, ALP, AST, ALT and liver weights were significantly elevated ($P<0.05$) in Group B (Paracetamol treated) when compared with Group A (Normal control) whereas serum protein levels and albumin globulin ratio were significantly decreased ($P<0.05$). The pretreated groups with aqueous Neem leaf extract (Group C) and Silymarin (Group D) however showed a significantly lesser increase ($P<0.05$) in the level of serum bilirubin, alkaline phosphatase, AST, ALT and liver weights and a significantly lesser decrease ($P<0.05$) in total protein level and albumin globulin ratio when compared with Group B (Table 1).

On 20th day of the experiment, mean values of serum bilirubin, ALP, AST, ALT and liver weights decreased and total protein and aalbumin globulin ratio increased compared to values on 10th day (Table 2).

Similar significant differences ($P< 0.05$) were observed in all parameters except for albumin globulin ratio, where we found no significant difference in values between Group A and B and between B and D. However, Group C mean values of LFT and liver weight showed significant difference compared to those of Group B.

On 30th day of experiment, mean values of serum bilirubin, liver enzymes and liver weights decreased and total protein and albumin globulin ratio increased compared to values on 20th day (Table 3). The same pattern of significant differences ($P<0.05$) were observed between Groups A and B except for serum bilirubin values and between C, D compared to B.

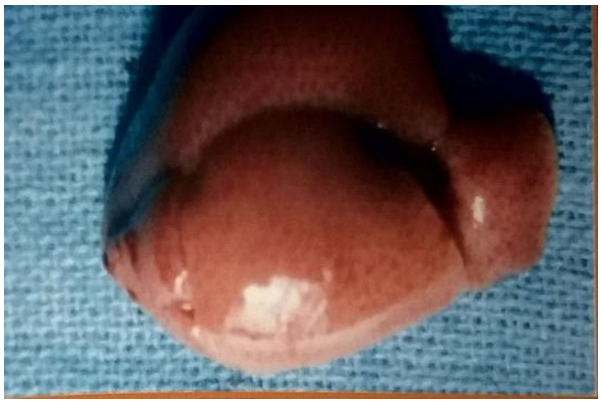

Figure 3. Liver of Normal control group

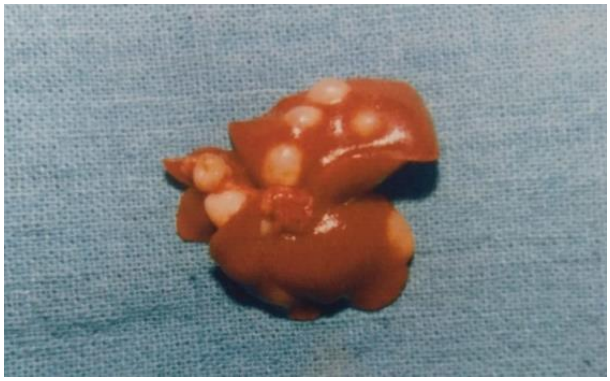

Figure 4. Liver of Paracetamol treated group

Thus, serum bilirubin, ALP, AST, ALT and liver weights were significantly ($P<0.05$) raised in experimental control and total serum protein, albumin globulin ratio (except on 20th day) were significantly decreased ($P<0.05$) compared to normal control on 10th, 20th and 30th days (except serum bilirubin on 30th day) following Paracetamol administration.

In ANLE and Silymarin treated groups also, we observed significant increase ($P<0.05$) in LFT parameters and liver weights and decrease in total protein, albumin globulin ratio on 10th, 20th and 30th days compared to normal control. But the observed increases in liver enzymes and decrease in protein and albumin globulin ratio were less compared to observed values in untreated group (Group B).

Gross appearance of Liver

Gross appearance of the livers of normal control group (Group A) showed normal architecture having red colored smooth regular undersurface (Fig.3). Paracetamol treated (Group B) liver showed multiple white nodules indicating necrotic areas and increase in the weight particularly on the 10th day of experiment. (Fig.4) Thus, gross appearance of liver changed with increase in size and presence of nodules in all Paracetamol treated groups (B,C,D), but by the 30th day, livers of ANLE and Silymarin treated groups showed normal appearance.

Liver Histopathology

Histology of liver from normal control group showed normal arrangement of hepatocyte with clearly brought out nuclei, central vein and portal triad throughout the experimental period (Fig. 5).

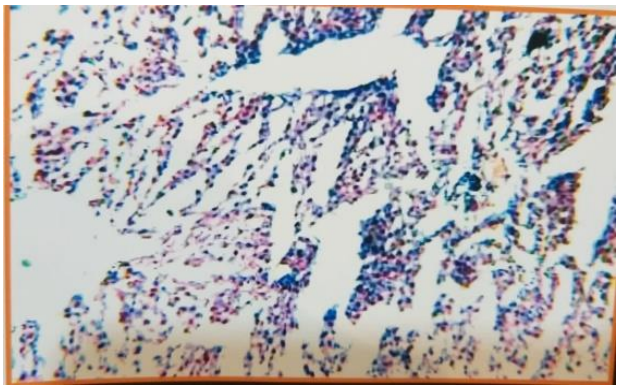

Figure 5. Photomicrograph of normal rat liver (H&E; Low power 10X10)

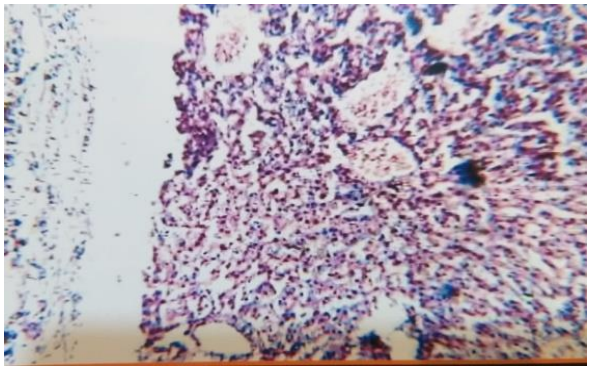

Figure 6. Photomicrograph of liver cell injury by Paracetamol (H&E; Low power 10X10)

The Paracetamol treated group showed congestion of sinusoids, cloudy swelling, central vein congestion, centrilobular fatty changes and necrosis of hepatic cells on the 10th day of experiment (Fig. 6). But on the 20th and 30th day, small areas of focal degeneration and sinusoidal dilatation evidenced the regenerative activity. The groups pretreated with ANLE and Silymarin showed a marked reduction in the congestion of sinusoids and cloudy swelling of liver on the 10th day of experiment (Fig. 7 & 8). Almost all changes were diminished by the 30th day of the experiment.

Thus, histology showed evidence of hepatic necrosis which was partly reversed by ANLE and Silymarin as early as the 10th day, but we observed spontaneus reduction in necrotic areas and appearance of regenerative areas in experimental control group also by the 20th day.

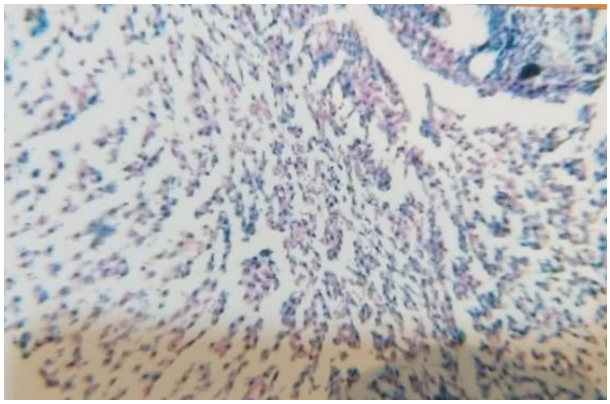

Figure 7. Photomicrograph of regenerative area in liver by Neem leaf extract (H&E; Low power 10X10)

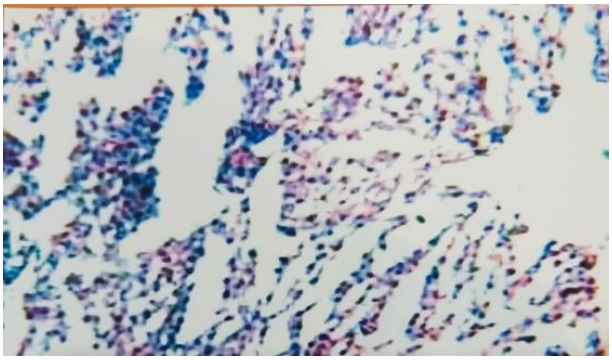

Figure 8. Photomicrograph of regenerative area in liver by Silymarin (H&E; Low power 10X10)

3.1. Hepatotoxicity

The liver can be damaged by disease, infection, during detoxification of endogenous substances or exposure to toxins, which can cause impairment of its function and sometimes, even structural damage at gross and microscopic (histological and ultrastructural) level. Zone 3 of the liver cell acinus is more vulnerable to toxic damage. Liver stem cells are found on the border of the portal system and hepatocyte lobules in zone 3 of liver parenchyma. These stem cells can differentiate into hepatocytes and bile duct cells.[85]

Common biomarkers of liver damage are raised levels of the enzymes AST, ALT and ALP, although ALT is more specific for liver damage. Aminotransferase levels > 75 times the upper reference limit indicate ischemic or toxic liver injury in more than 90% of cases of acute hepatic injury. In hepatocellular damage or death, ALT released from damaged heptocytes increase serum ALT levels.[86] The enzymatic parameters are part of the liver function tests which are more indicative of hepatocyte integrity rather than liver function.[87]Marked release of transaminases into the circulation reflects severe damage to hepatic tissue membranes during paracetamol intoxication.[64]A decrease in serum albumin may indicate decrease in functioning liver mass. Increased protein catabolism in drug - induced hepatitis might have a direct adverse effect on the

synthesis and secretion of albumin. Previous studies have documented hypoalbuminemia during hepatic dysfunction.[64,88-91]
Thus, we can infer that Paracetamol induced hepatotoxicity which led to raised liver enzymes, serum bilirubin and decrease in total serum protein and albumin globulin ratio as well as hepatic inflammation and necrosis.

3.2. Hepatotoxic mechanism of Paracetamol

Paracetamol induced hepatotoxicity results from an unstable toxic metabolite.[92]In normal therapeutic doses, paracetamol is primarily metabolized in the liver by glucuronidation and sulphation; however, a small proportion is converted to N-acetyl-p-benzoquinoimine (NAPQI) after bioactivation by cytochrome P450. This minor metabolite can react with sulphydryl groups such as glutathione[93]and protein thiols to form mercapturic acid.[94] When paracetamol overdose occurs, elevated levels of the toxic NAPQI metabolite are generated, which extensively deplete hepatocellular glutathione (GSH) especially in centrilobular hepatocytes. Thus, NAPQI covalently modifies cellular proteins by binding to their cysteine groups to form 3-(cystein-S-yl) paracetamol adducts; this results in a chain ultimately causing hepatocyte necrosis.[95-97]Cytochrome P450-dependent bio activation of paracetamol is thus the main cause for hepatic necrosis upon administration or intake of lethal doses of paracetamol.[98,99]Hepatotoxic dose of Paracetamol depletes the endogenous glutathione level to below a threshold value (<20% of control), therefore permitting interaction of NAPQI with cell macromolecule.[100]In an adult man, a minimum of 7.5-10 gm of the drug produces hepatic necrosis.[101]But, in alcoholics, as little as 4-8gm Paracetamol /day may produce liver damage. In patients with underlying liver diseases, a much lower dose of Paracetamol can produce liver damage.[102] Gujral, *et al.* reported that mode of hepatocyte cell death in Paracetamol overdose is mostly oncotic necrosis and less apoptosis.[103]

3.3. Silymarin

Silymarin and aqeuous extract of Neem leaf exhibited hepatoprotective activity, which was evidenced by significantly (P<0.05) lower levels of serum bilirubin, alkaline phosphatase, AST, ALT, liver weight and significant rise in total protein and albumin globulin ratio compared to untreated experimental control group. The histopathological examination of Silymarin and Neem leaf extract treated groups revealed hepatoprotection, as shown by marked reduction in congestion of sinusoids, cloudy swelling and congestion of central vein during the experimental period.
Silymarin has multiple mechanisms by which it exerts hepatoprotective effect. Silymarin stabilizes the lipid structures in the hepatocellular structure [102] and has anti-lipid peroxidative effect which helps in preservation of membrane integrity. Silymarin can chelate transition metal ions such as iron and copper, rendering them effective antioxidants. In addition, it increases GSH content in liver and protects it against toxicity of GSH depletors such as paracetamol. Silybinin component of Silymarin inhibits the function of Kupffer cells which are involved in hepatic fibrosis.[104,105]. It also has an inhibitory effect on NF-kB/Rel activity in a human hepatoblastoma-derived cell line and human histiocytic lymphoma cells; this results in partial inhibition of IL- Iß production (anti-inflammatory effect).[64]

3.4. Hepatoprotective activity of Neem (*Azadirachta indica*) leaf extracts

The hepatoprotective activity of Neem was earlier reported by Chattopadhyay, *et al.*, Nahed, *et al.*, Bhanwra, *et al.,* Yanpallewar, *et al.*, Johnson, *et al.*, Nwobodo, *et al.*, when aqueous Neem leaf extract was administered in Paracetamol-induced hepatotoxicity in rats.[53,55,56,58,64,73] Hepatoprotective activity

of aqueous Neem leaf extract and of Azadirachtin-A and Nimbolide compounds (present in Neem leaves) in Carbon-tetrachloride induced hepatotoxicity was also reported by Mukherjee, *et al.*, Kalaivani, *et al.*, Baligar, *et al.,* Idu *et al.*[54,59,66,67] Baligar *et al*. stated that nimbolide hepatoprotective activity was comparable to that of Silymarin.[67] Hepatoprotective effect of aqueous Neem leaf extract was also reported by Kale, *et al.* against anti-tubercular drugs-induced hepatotoxicity, Akinola, *et al.* against Streptozotocin induced hepatotoxicity in diabetic rats, Ezz-din D, *et al.,* Abdel-Moneim, *et al.* and Dkhil, *et al.*, against cisplatin-induced hepatotoxiciy, Essien, *et al.* against Alloxan-induced hepatotoxicity in diabetic rats, Koul, *et al.* against DMBA-induced hepatotoxicity, Sani, *et al* against snake venom induced hepatoxicity, Althaiban against Rifampin induced hepatotoxicity.[57,61,60,67,62,63,65,75]

In an in-vitro model involving *Azadirachta indica* gold nanoparticles (GNP) linked with the anti-HIV drug Azidothymidine, Kesarkar et al. observed that *A.indica* conferred hepatoprotective activity to the gold nanoparticles. The authors made this inference from observing that IL-10 (anti-inflammatory) activities were enhanced, whereas there was controlled secretion of pro-inflammatory IL-6 and downregulation of TNF-alpha in primary co-cultures of rat liver Kupffer cells by ELISA.[68]

Neem comprises of various ingredients such as liminoids, and nimbosterol in different parts of the plants. Important bioactive principles of Neem are flavonoids, alkaloids, tannins, saponins, steroids namely, quercetin, gallic acid, (+)gallocatechin, (-)epicatechin, (+)catechin and epigallocatechin, nimbin, 6-desacetylnimbinene, nimbandiol, 17-hydroxy azadiradione, ascorbic acid, nimbolide, nimbiol n-hexacosanol,7-sdesacetyl-7-benzoylazadiradione,7-sdesacetyl-7-enzoylgedunin, and nimbiol, Azadirachtin, Chlorogenic acid, Kaempferol derivatives, Myricetin, Rutin, Scopoletein, Sigmasterol/Beta-sitosterol.[28,76,85,106,107]Leaves contain mixture of compounds including nimbin, nimbanene, 6-desacetylnimbinene, nimbandiol, nimbolide, ascorbic acid, n-hexacosanol and different amino acids, and nimbiol and several other types of ingredients.[11]Many of these components – Azadirachtin, chlorogenic acid, Kaempferol derivatives, Myricetin flavonoids, Nimbin, Nimbolide, Rutin, Scopoletein, Sigmasterol/Beta sitosterol 6-desacetylnimbinene, quercetin, nimbandiol, 17-hydroxy azadiradione, ascorbic acid, nimbolide, n-hexacosanol,7-sdesacetyl-7-benzoylazadiradione,7-sdesacetyl-7-enzoylgedunin, and nimbiol have anti-inflammatory, immunomodulating and/or antioxidant properties.[28,106,107]

3.5. Mechanism of action of Neem (*Azadirachta indica*) leaf extract as a hepatoprotectant

Terpenoids, alkaloids, flavonoids and glycosides are the major classes of constituents found within the neem leaf extract that in isolation or in combination simultaneously work as antioxidants, f r e e radical scavengers and anti-inflammatory compounds.[28] Chattopadhyay, Chattopadhyay, *et al*., Suhendro, *et al.*, Sithisarn, *et al.*, Manikandan, *et al.*, Al-Hashemi, *et al.* have reported the antioxidant activity of Neem leaf extract.[28,85,108-112])

Exposure to oxidant molecules leads to generation of reactive oxygen species (ROS), like hydrogen peroxide, superoxide, hydroxyl radicals that can readily alter DNA, proteins and/or membrane phospholipids, thus altering their structure and function. Normally, the body's endogenous antioxidants like superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH) and glutathione peroxidase (GSH-Px) act against these ROS. But they get depleted in acute oxidative stress leading to ROS accumulation.

Neem leaf extracts reverse this phenomenon and re-establish antioxidant mechanisms.

Neem extract contains phenol and antioxidant effects of phenolic compounds are related to a number of different mechanisms, such as free radical-scavenging, singlet oxygen quenching, metal ion chelation, hydrogen-donation and their action as substrates for free radicals such as superoxide anion and hydroxyl radical.[64]Azadirachtin and nimbolide exhibited concentration-dependent free radical scavenging activity in cancer models.[113]Antioxidants can also elevate the levels of endogenous antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH) and glutathione peroxidase (GSH-Px), thus decreasing lipid peroxidation.[64]The antioxidant capacity of Neem leaf extracts were evidenced through reduced H_2O_2 mediated lipid peroxidation and DNA damage.[111]Johnson *et al.* reported that paracetamol administration caused increased lipid peroxidation, reduced SOD, reduced CAT and depletion in GSH activity in the liver. Liver tissue contains relatively high content of polyunsaturated fatty acids (PUFAs), which are sensitive to peroxidative damage. Paracetamol caused increase in lipid peroxidation and due to NAPQI formation, GSH stores decreased, as evidenced by elevated levels of malondialdehyde.[64,73]Abdel Moneim, *et al.* observed that flavonoids in neem possess both antioxidant and anti-inflammatory activities via scavenging free radicals and inhibition of lipid peroxidation.[69] In addition, neem leaves are rich in polyphenolics, which are known for their potent antioxidant and free radical scavenging properties. Suhendro *et al.* proposed that neem leaf extract contains quercetin, flavonoids and beta carotene which have free radical scavenging and, anti-lipid peroxidative properties and also increase Glutathione reductase activity (which protects cell membrane integrity).[85]In paracetamol induced hepatotoxicity, antioxidant mechanisms facilitate good regeneration of hepatocytes. Stem cells outside the necrotic area, particularly on central vein area and the portal system multiply and differentiate to become a hepatocyte and replace the necrotic hepatocytes in the absence of free radicals.[85]NF-κB, a redox-sensitive transcription factor that has been proposed to be the sensor for oxidative stress, was induced by cisplatin induced hepatotoxicity and reduced by neem leaf extract.[62]

Neem leaf extracts have also been reported to inhibit TNF-α triggered induction of NF-κB that is linked to inflammation.[114]Anti-inflammatory effects of chlorogenic acid in neem leaf was observed in lipopolysaccharide (LPS) - stimulated RAW 264.7 cells. Chlorogenic acid significantly inhibited not only NO production butalso the expression of COX-2, nuclear translocation of NF-κB, iNOS along with other cytokines (IL-1β, IL6 and TNF-α) in a dose-dependent manner.[115]Bhanwra *et al.*, proposed that hepatoprotective effect of Neem was probably due to its anti-inflammatory effect. Althaiban also supported the antioxidant and anti-inflammatory basis for the mechanism of action of neem leaf extract.[56,74]

Tables and Figures

4. Conclusion

Paracetamol induced a hepatotoxic effect in Wistar rats which caused increase in LFTs and liver weights. The increase in level of serum bilirubin and liver enzymes AST, ALP, ALT as well as liver weights and decrease in total serum protein and albumin globulin ratio following hepatic injury induced by Paracetamol were less in aqueous Neem leaf extract group compared to experimental control group. Chronic administration of aqueous Neem leaf extract also caused decrease in hepatic necrosis, showing regenerative activity on 10th day of the experiment, compared to such regeneration in untreated animals only on 20th day. All inflammatory and necrotic changes disappeared by the 30th day. Hence, we can conclude that chronic oral administration of *Azadirachta indica* (Neem) aqueous leaf

extract shows hepatoprotective activity in Wistar albino rats.

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Author Contribution

Author 1: Conception, design and development of the protocol, data collection and analysis, initial drafting and reviewing the manuscript and final approval of the prepared manuscript. **Author 2:** Conception, design and development of the protocol, supervision of experiments, data analysis and reviewing the manuscript. **Author 3:** Design and development of the protocol, supervision of experiments, data analysis and reviewing the manuscript. **Author 4:** Supporting role in conducting the experiments, re-drafting and reviewing the manuscript and final approval of the prepared manuscript.

Conflicts of Interest: No

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Screening of Biological Potential of *Pleurostylia opposita* (Wall.) Alston. - A Rare Medicinal Plant of Eastern Ghat

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Abstract

The present study aims to evaluate the phytochemical, antioxidant, and antibacterial properties of *Pleurostylia opposite* leaf and bark crude extracts in various solvent. Preliminary phytochemical and antibacterial screening by agar well diffusion method was conducted. Further, the estimation of total phenolic and flavonoid contents along with antioxidants properties by DPPH method was carried out in various solvents extracts. The phytochemical study revealed that the maximum secondary metabolites such as alkaloids, polyphenols, sterols, terpenoids flavonoids, and saponins were present in methanol extracts followed by other extracts. Likewise, antibacterial screening showed the maximum growth of inhibition in methanol leaf and bark extracts against *Escherichia coli* (23mm), *Salmonella typhi* (23mm), *Bacillus subtilis* (24mm), *Staphylococcus aureus* (19mm), and *Pseudomonas aeroginosa* (24mm). The minimum inhibitory concentrations of the leaf and bark extracts were in the range of 19.5 mg/L – 625 mg/L while gentamycin is 9.75 mg/L. The results indicated that the methanol extracts of leaf and bark showed potent antibacterial efficacy. Further, the antioxidant activity by DPPH assay showed that all the extracts exhibited significant antioxidant properties in a dose-dependent manner. Among the tested extracts, methanol exhibited excellent radical scavenging activity which was significant compared to gallic acid. Likewise, each extract also showed excellent sources of phenolic and flavonoid contents. This study reveals that the plant contains a considerable amount of phenols and flavonoids, including antioxidant properties which are also possessing antibacterial efficacy. This study offered a new avenue for the potential use of this species as a source of natural antioxidants.

Keywords: *Pleurostylia opposita*, Phytochemical, Antibacterial, Antioxidants, DPPH

1. Introduction

Nature has provided fruitful opportunities for mankind in many aspects since ancient times. Plants are the major source of many bioactive compounds for thousands of years (Motaleb, 2011; Prakash et al., 2014). Most of the present-day synthetic drugs have been developed from natural sources based on their traditional usage. Since ancient times, innumerable plant species have been used to treat countless diseases all over the world (Sajem et al., 2008; Sofowora et al., 2013). Green plants produce a diverse range of bioactive molecules which enrich the source of active compounds, and they continued to play a dominant role in the maintenance of

the quality of human health (Cragg and Newman, 2013; Nisa et al., 2013). Along scientific lines, biological screening tests are very much crucial to substantiate the therapeutic properties of natural resources. A maximum percentage of drugs originated from natural resources while the plant kingdom represents an extraordinary reservoir of countless novel molecules. The potential usage of higher plants, which are the major source for a new drug and needs to be explored (Girish and Satish 2008). These are the reservoirs of potentially useful chemical compounds which gives hints for the present day's modern drug design (Yadav, and Agarwala, 2011). Most importantly, these bioactive compounds from plant origin are the constituents of alkaloids, tannins, flavonoids, and phenolic compounds. Keeping the above mentioned facts, *Pleurostylia opposita* - an important medicinal plant species that belongs to the family *Celastraceae* also known as the bitter-sweet family was undertaken for the present study. There are about 90-100 genera and 1,300 species have been reported, in India, especially found in the Western Ghats region of Kerala, South Sahyadri, Malabar, and the Eastern Ghats region of Karnataka (Gamble, 1997). The plant contains a large number of chemically complex and biologically active compounds (Neam suvan et al., 2012). Traditionally, the oral administration of the root and bark decoction of this plant is used for the treatment of malaria, toothache and the wound healing process (Uma Maheswari et al., 2012). Apart from the identification of compounds, no reports are available on the comparative biological studies of this plant. Therefore the study was conducted to evaluate the phytochemical analysis, antibacterial activity, and antioxidant properties in bark and leaf various solvent extracts of *P. opposita*.

Materials and Methods

2.1. Plant Collection and Extracts Preparation: The bark and leaf material of *P. opposita* were collected in Chamundi hill, Mysore, Karnataka, India. Plant material was thoroughly washed in sterile distilled water and shade dried. The dried leaves and bark materials were powdered and subjected to crude extraction using hexane, petroleum ether, chloroform, ethyl acetate, and methanol. About 50g of each material was taken in a 250 ml conical flask containing respective solvents and placed on a rotary shaker at 120rpm for 24h and extracts were stored in an airtight glass bottle until further use (Sadika et al., 2012).

2.2. Phytochemical Analysis and Thin Layer Chromatography: The preliminary phytochemical screening was carried out for the detection of secondary metabolites in each crude solvents extracts of leaf and bark (Harborne, 1973). Further, the maximum positive test showed methanol leaf and bark extracts were subjected to thin-layer chromatography (TLC) analysis for the detection of specific compound (Ramaswamy et al., 2013). Each sample was spotted on previously activated TLC plates and placed in a saturated chromatography chamber using a suitable solvent system. Each TLC plate was removed after a successful one-fourth running of solvents. The visualization of the secondary metabolites was done by spraying different reagents such as iodine vapours, Dragendroff's, Benedict's, Liebermann-Burchard's, and Folin- Ciocalteu's (FC) reagents. The plates were activated at 100º C for 10 min. and observed under a UV light (366 - 254 nm) for colour development and the mean Rf values were recorded.

2.3. Antibacterial Activity by Well Diffusion Assay: The antibacterial screening was conducted by agar well diffusion methods (Joshi et al., 2011). Each crude extract was tested against bacterial pathogens such as B. *subtilis* (MTCC121), *Staph. aureus* (MTCC7443), *E. coli* (MTCC7410), *P. aeruginosa* (MTCC1688), and *S. typhi* (MTCC733) procured from Microbial Type of Culture Collection (MTCC), Chandigarh. A 24h. old fresh inoculum (106

CFU/ml) was uniformly spread in Petri dishes containing nutrient medium. Wells were made by using a sterile cork borer (6mm) and each well was filled with 50 µl (50 mg/L) and gentamycin (1mg/L) and respective solvents served as a positive and negative control. Plates were incubated at 37°C overnight and the zone of inhibition was measured. Minimal inhibitory concentration (MIC) was also determined by broth microdilution method using an ELISA multi-plate reader. Each extract was tested at concentrations ranging between 5- 0.002mg mL-1 from the stock solution of 100 mg mL-1 along with controls and the assay was performed in triplicates.

2.4. Total Phenolic Content: Each solvent extract of leaf and bark was subjected to measure the total phenolic content by the method of Folin- Ciocalteu's method (Muthukrishnan et al., 2018). Each sample was tested at 100 µg mL-1 and the absorbance was measured at 765nm using a spectrophotometer. The obtained results were compared with that of the standard drug gallic acid, and expressed as milligrams of gallic acid equivalence (GAE, µg mL-1).

2.5. Determination of Total Flavonoid: The amount of flavonoid contents in leaf and bark extracts were determined by the method of Muthukrishnan et al., (2018). About 0.5 mL of 2% AlCl3 solution was mixed with 0.5 mL (100 µg mL-1) of each extract. The reaction mixture was kept in at room temperature for 1h. and the absorbance was measured at 420nm using spectrophotometer. The yellow colour in the reaction mixture indicates the presence of flavonoids and the quercetin calibration curve (50 - 250 µg mL-1) served as a reference standard. The obtained results are expressed as milligrams of Quercetin equivalence per gram of dry weight.

2.6. Antioxidant Activity: The DPPH (2, 2-diphenyl-1- picrylhydrazyl) free radical scavenging activity was performed using a 96 well plate (Yamasaki et al., 1994). Samples were tested at 20, 40, 60, 80, and 100µg mL-1 concentrations and the gallic acid was used as a standard. The reaction mixture was incubated at room temperature in dark conditions for 30 min. The OD was measured at 517nm using ELISA multi-plate reader. The assay was performed in triplicates and the percentages of free radical scavenging activity and the IC50was calculated by using a formula.

$$\% \text{ scavenging activity} = ((\text{Abs control} - \text{Abs sample}))/(\text{Abs Control}) \times 100$$

2.7. Statistical Analysis: Each assay was performed in triplicates and the data were analyzed using one-way analysis of variance (ANOVA) using SPSS Inc. 16.0. Significant effects of treatments were tested by Tukey's HSD test separated and determined by F values ($p \leq 0.05$).

3. Results and Discussion

3.1. Phytochemical Screening: The preliminary qualitative phytochemical test revealed various classes of secondary metabolites in both leaf and bark extracts of *P. opposita.* The phytochemicals such as sterols, triterpenoids, carbohydrates, glycosides, tannins, flavonoids, resins, and carbohydrates are the major constituents present in the extracts. These phytochemicals have played the most important role in providing various nutraceutical products (Das et al., 2012). The results obtained in this study were summarized (Supp. table 1) and it showed that all the extracts possess several phytochemicals. The comparative phytochemical screening revealed that the maximum tests were positive in methanol bark extract than leaf methanol extract, while the remaining extracts showed presence of less number of phytochemicals. Similar results were reported by Misra et al., (2011) wherein the maximum phytochemicals were present in methanol extracts of Alstonia scholaris.

Further, the maximum phytochemicals showed methanol leaf and bark extracts subjected to TLC analysis. The results revealed the presence of secondary metabolites such as reducing sugars, alkaloids, saponins, and phenols after spraying Dragendroff's, Benedict's, Liebermann Burchard, and FC reagents. The results were presented in figure 1 and the corresponding Rf value of confirmed phytochemicals were 0.4, 0.38, 0.59, 0.98, and 0.84, 0.65, 0.57, 0.56 for reducing sugars, alkaloids, saponins, and phenols appeared orange, light brown, and bluish respectively. Likewise, Ramaswamy et al., (2014) also investigated various classes of phytochemicals in leaves and petioles of Oroxylum indicum through TLC analysis. Cassia et al., 2011 have also reported the presence of phytochemicals in many other species of the Celastraceae family due to their massive pharmacological activities. The study demonstrated that biological interest which is associated with the presence of flavonoids, alkaloids, triterpenes, phenols, and glycosides (Dantanarayana et al., 1982).

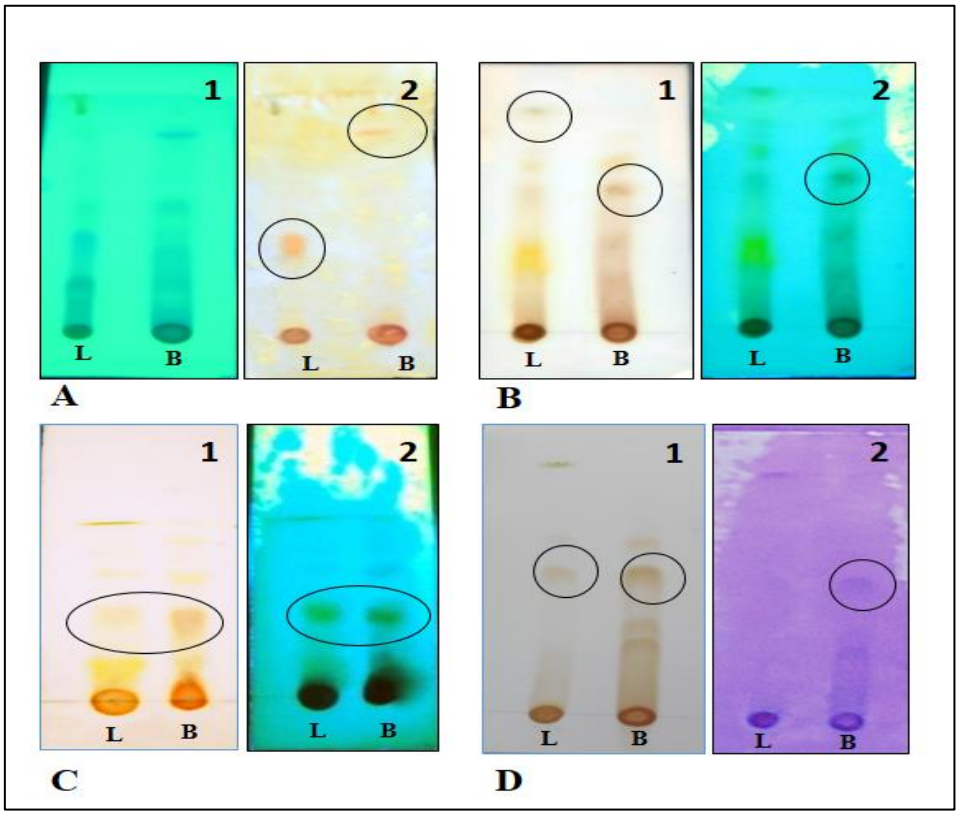


Figure: 1. A-Dragendroff's test for alkaloids, **B-** Benedict's test for reducing sugars, **C-** Liebermann Burchard test for sterols and saponins, **D-** FC reagent test for phenols. (**L-** Leaf extract, **B-** Bark extract, **1-** Before spray, **2-** After spray).

3.2. Antibacterial Activity: The evaluation of the antibacterial activity of the crude extracts of leaf and bark against selected bacteria through agar well diffusion assay. The outcome of this study and the zone of inhibition were presented in supporting table 2 along with minimum inhibitory concentrations (MIC). Among the extracts tested, the methanol extract of both leaves and bark was highly sensitive to tested pathogens. Followed by ethyl acetate, and chloroform extracts also showed growth inhibition against tested pathogens excluding hexane and petroleum ether extracts. Overall, in the comparative study of antibacterial activity, the bark extracts showed more significant antibacterial activity than leaf extracts due to the presence higher number

of phytochemicals in methanol extracts. Similar results were also reported by Moteriya et al., (2014) where they have noticed the remarkable difference in antibacterial properties in Maytenuse marginata leaf and stem extracts. The highest zone of inhibition observed in P. aeruginosa and B. subtilis is 24mm with a MIC concentration of 19.5 µg mL-1, while the standard antibiotic shows a zone of inhibition of 22-28 mm with MIC concentrations of 9.75 µg mL-1 against tested pathogens. The results presented here is in accordance with the earlier report of Selvamohan et al., (2012); Borges et al., (2017) wherein leaf and bark extracts of Brosimum gaudichaudii against Staph. aureus and P. aeroginosa.

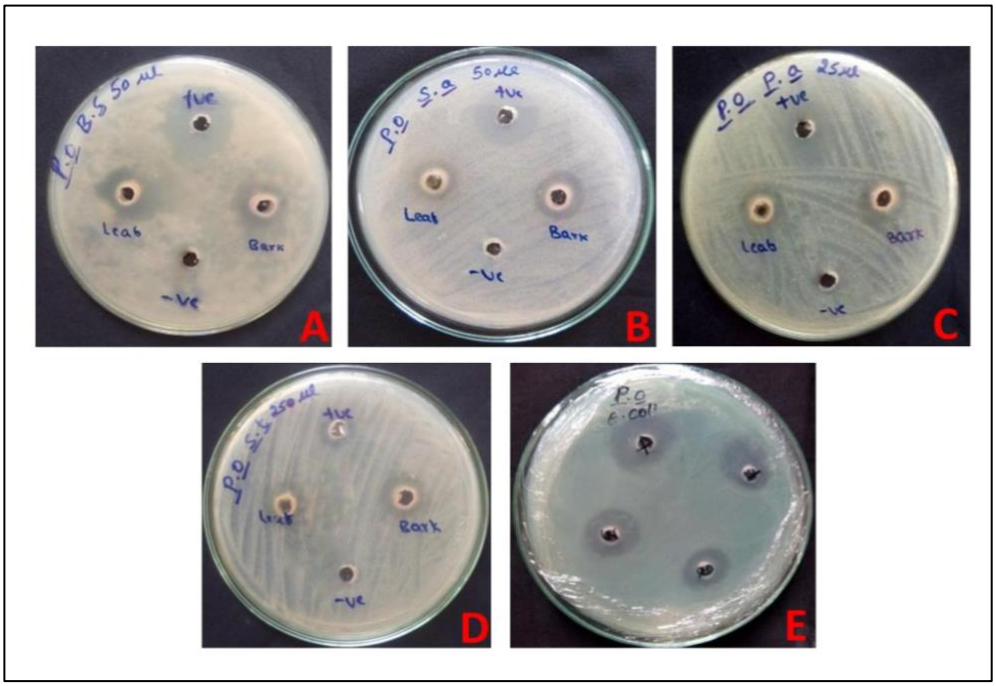


Figure: 2. Antibacterial activity of methanol leaf and bark extracts of *Pleurostylia opposite* against; A – *B. subtilis*, **B –** *Staph. aureus*, **C –** *P. aeroginosa*, **D –** *S. typhi*, **E –** *E. coli.*

3.3. Antioxidant Activity by DPPH Assay: The antioxidant property of each extract was evaluated by DPPH assay. The free radical scavenging activities measured by DPPH assay, it is a widely used method to analyze the antioxidant properties in most of the plant extracts. The test samples are directly involved in the inhibition of the generation of reactive oxygen species (ROS). The results obtained from this experiment revealed that all the tested extracts showed significant antioxidant properties which are in a dose-dependent manner. The outcome of this study was depicted in the figure 3 and 4. The methanol extract of leaf and bark showed excellent antioxidant activity compare to other solvent extracts. The IC50was calculated by plotting the graph of radical scavenging activity against the different concentrations of extracts by using standard gallic acid. It is also observed that bark methanol extract showed the maximum free radical scavenging activity which is nearly equal to the standard gallic acid with the half maximal inhibitory concentration (IC50) value of 16.97µg mL-1 while the IC50 value of gallic acid was 15.95µg mL-1. Likewise, leaf methanol extract also showed significant antioxidant properties with an IC50 value of 18.83 µg mL-1. Therefore the results revealed that the plant possesses excellent antioxidant properties and each extract effectively scavenged free radicals which are varied among the different solvent extracts. Similar results were reported by Jhade et al., (2012); Nisa et al., (2013) where the extracts possess

plant metabolites that effectively inhibit the generation of free radicles. The results obtained are due to the higher number of secondary metabolites in the extracts which are responsible for antioxidant activity. These findings are also supported by earlier reports by Gupta et al., (2011); Yuan et al., (2011); Mayakrishnan et al., (2012) wherein the plant metabolites such as flavonoids, tannins, catechins, and other phenolic compounds are the major constituents which are responsible for good sources of antioxidant activity.

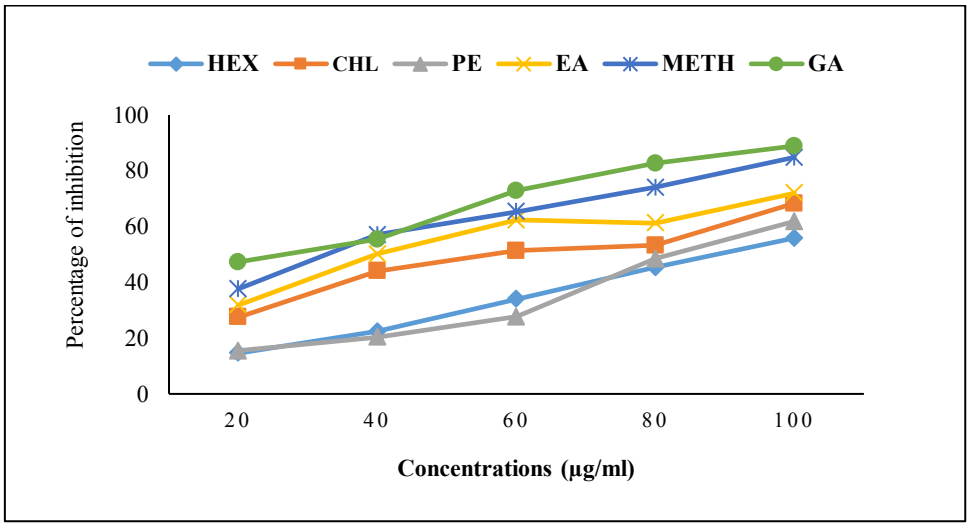


Figure: 3. DPPH antioxidant activity of different solvent extracts of leaf in *P. opposita.*

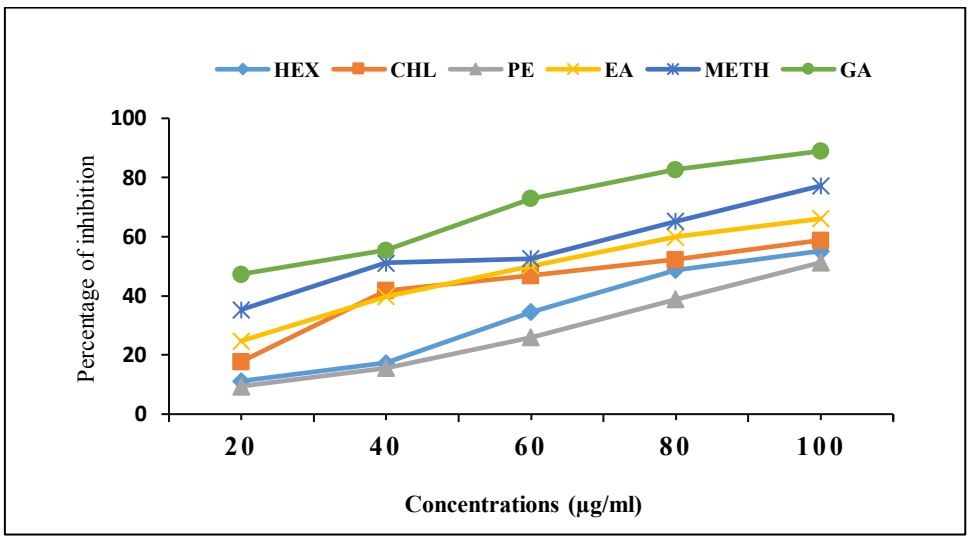
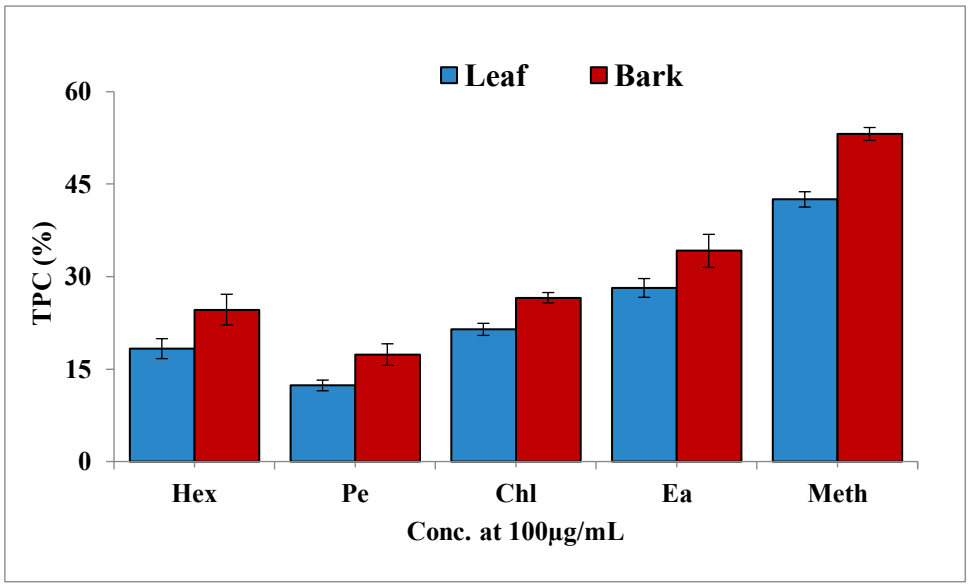


Figure: 4. DPPH antioxidant activity of different solvent extracts of bark in *P. opposita.*

3.4. Total phenolic Content: The summary of the total phenolic content (TPC) of various solvent extracts of leaf and bark was estimated by the Folin-Ciocalteu (FC) method by using standard drug gallic acid. Phenolic compounds are very important secondary metabolites with potent antioxidant activity, which chelate redox-active ions and prevent the conversion of reactive oxygen species (Khorasani Esmaeili et al., 2015). The TPC of P. opposita leaf and bark different solvent extracts is presented in figure 5. Overall the TPC value was higher in methanol extracts of bark (53.14%) and leaf

(42.53%) followed by other solvent extracts. The lowest TPC content was observed in petroleum ether extract and it was also noticed that leaf extracts were found to possess less percentage of phenolic contents than bark extracts. The obtained results were correlated with standard gallic acid by plotting the linear regression (y = 0.0153x; R2= 0.9953). The differences in the phenolic contents between leaf and bark solvent extract is may be due to the presence of complex biopolymers such as carbohydrates and proteins in the leaf extract. The result presented here was in accordance with the recent reports of Phuyal et al., (2020); Muhammad et al., (2012) in Zanthoxylum armatum, where the ethanolic extracts of bark and fruit possess a higher amount of TPC than leaf extracts.



Figure; 5. The total phenolic content of leaf and bark different solvent extracts of *P. opposita.*

3.6. Total Flavonoid Contents: Presence of total flavonoid contents in the leaf and bark extracts of P. opposite was determined spectrophotometrically. The flavonoid contents of each extract was calculated by plotting the linear regression calibration curve of the standard drug quercetin (y = 0.0146x; R^2 = 0.9918) and expressed as mg quercetin equivalent per gram of dry weight (mg/g). The assay revealed that the flavonoid content was much higher in bark methanol extracts (64.58%) than in leaf (51.56%) followed by other extracts and the result is presented in figure 6. Contrary to phenol contents overall, the flavonoid content was comparatively lower in leaf extracts than in bark extracts and the result was compared with that of the standard drug quercetin. The obtained results revealed major variations in the percentage of flavonoid contents may be because of the presence of vast number secondary metabolites present in the plant extracts. The result is in agreement with the previous report of Saeed et al., (2012) wherein the Torilis leptophylla plant extracts, and Sulaiman, and Balachandran, (2012) have also revealed the amount of total flavonoid content present in some medicinal plants inhabited in India. Further, Iqbal et al., (2015) also observed rich flavonoid contents in bark and leaf extracts of Goniothalamus velutinus and justified that the plants manifest themselves as good sources of antioxidants

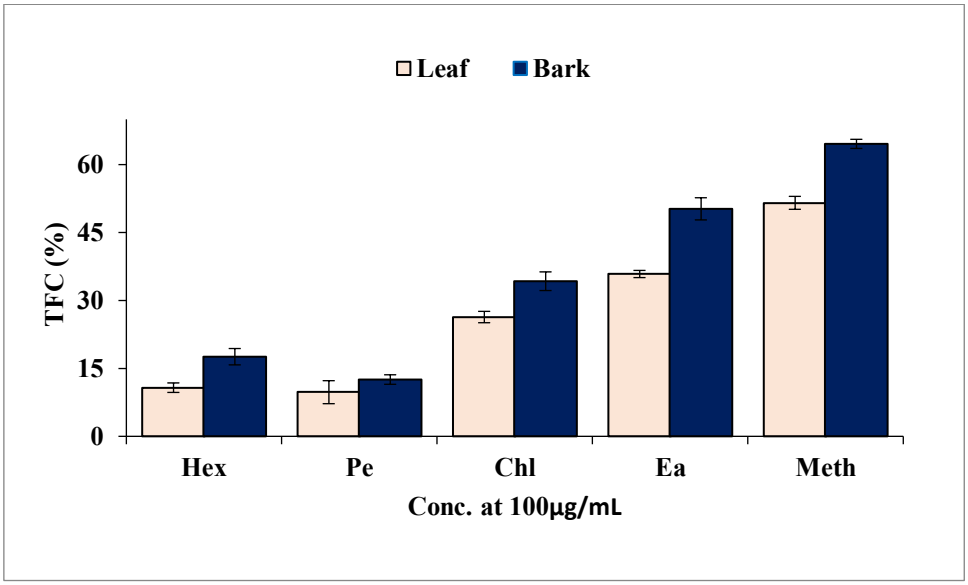


Figure 5. The total flavonoid content of leaf and bark different solvent extracts of *P.opposita.*

3. Conclusion

The present study summarises that this plant possesses a good source of phytochemicals. The difference in the presence of number of phytochemicals between the solvent extracts is remarkably varied. However, these parameter is better in bark extracts than compared to leaf extracts. The plant extracts proved to be potentially inhibit the tested bacterial strains and comparatively methanol extract of both leaf and bark was found to possess the most effective antibacterial properties. The differential total phenolic, flavonoid content, and antioxidants properties from different solvent extracts of bark and leaf may due to the variations in the phytochemical constituents. Our investigation will provide the basis for the selection of potent medicinal plant species for the investigation of desired new bioactive molecules from herbal resources. With this result, we concluded that further studies are needed for insight into the investigation, and isolation of bio-active compounds from this plant for future health applications.

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Conflict of Interest

The authors declare that there are no conflict of interest and agree to publish the data.

Author contribution

Mahendra C., Savitha R. S. and Ravindra K. N. collected the plant, prepared the samples, performed all the experiments, and analyzed the data. All the researchers wrote the manuscript, reviewed it together. Theyt read and approved the final manuscript.

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