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Sincerely,
Prof. Dr. Nazım ŞEKEROĞLU
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Antibacterial Effect of Methanolic Extract of Saffron Petal (*Crocus sativus* L.) on Some Standard Gram Positive and Gram Negative Pathogenic Bacteria *In vitro*

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Abstract

Background: Nowadays, increasing antibiotic resistance of bacteria has provided the opportunity to replace herbal remedies with fewer side effects than conventional medicines; therefore, in this study, the antibacterial effects of methanolic extract of saffron petal against *Staphylococcus aureus*, *Bacillus cereus*, *Pseudomonas aeruginosa* and *Escherichia coli* strains were evaluated. **Materials and Methods:** In this in vitro study, after collecting the saffron plant and confirming its scientific name, the saffron petal extract was prepared in concentrations of 20 mg/ml to 400 mg/ml. Then the antimicrobial effects of this extract were investigated by well diffusion and tube dilution method. **Results:** The results showed that the methanolic extract of saffron petal had antibacterial effects on the tested bacteria in both tube dilution and well diffusion methods. The highest effect was observed in *S. aureus* and the least in *P. aeruginosa.* **Conclusion:** Based on the above results, it can be hoped that Saffron petals extract can be used in the treatment of bacterial infections and can be a suitable replacement for conventional chemical drugs in the treatment of infections.

Key Words: Medicinal Plants, Antimicrobial Effects, Extract, Pathogenic Bacteria

1. Introduction

Increasing use of chemical drugs leading to development of antibiotic resistance to bacterial strains (VandenBergh et al., 1999). The medicinal plant is herbs with organs contain substances that affect living things (Singh et al., 2007). There has always been a close relationship between people and plants during the development of all human civilizations. Although most plant species are known to date, there is still much time left to discover new and valuable plant resources (Digrak et al., 2001; Skaltsa et al., 1999; Jafari-Sales et al., 2019a) which are only partially identified so far. These chemicals can be used as a drug but also as a unique starting point for the manufacture of pharmaceutical analogs, as well as an interesting tool to better understanding biological phenomena (Mobaiyen et al., 2016; Sales, 2014; Jafari-Sales et al., 2019b). One of the most important therapeutic challenges is coping with infectious diseases due to their high prevalence resulting with expansion of the

clinical use of synthetic antibiotics. The overuse of these antimicrobial drugs has led to increased drug resistance against different antibiotics in most bacteria (Sales et al., 2017; Sales et al., 2015; Jafari-Sales et al., 2019b). This has been one of the reasons for the growing use of herbs as low-risk, affordable and inexpensive natural ingredients in the treatment of bacterial infections compared to synthetic antibiotics. leading to increasing number of worldwide studies concerning antibacterial effects of various plants in recent years (Jafari-Sales et al., 2015; Jafari-Sales et al., 2019a).

The saffron plant, scientifically named Crocus sativus L. is a small, perennial plant of the Iridaceae family, which is a traditional medicinal plant that grows in different parts of the world. The dried stigma of this plant is used as saffron in the food industry (as a fragrant spice and for coloring food) and in the pharmaceutical industry (as a sedative and analgesic for asthma, black cough and inflammation) (Mirheidar, 2005). It is officially listed on the Chinese Medicines List and has been used in traditional Chinese medicine to treat hematomas, depression and seizures as a sedative (Tang and Eisenbrand, 2013). Recent studies have shown that this plant has the potential to reduce the risk of various diseases (Poma et al., 2012).

Some metabolites derived from saffron stigma due to hypolipidemic, antiparasitic, antioxidant, and diabetes mellitus function. Many therapeutic effects have shown themselves. The aqueous and alcoholic extracts of saffron are protective of the heart and counteract neurodegenerative disorders. Numerous medicinal properties of saffron are related to its various components such as Crocetin, Crocin and other substances that have potent antioxidant properties and accumulate oxygen free radicals and proinflammatory cytokines (Hepsø et al., 1988). Studies have shown that there are more than 151 different substances in saffron stigma. The strongest components of saffron are carotenoids and monoterpene aldehydes. Studies on the relationship between the function and structure of the molecule have shown that some properties of saffron are due to its deglycosylated derivatives, and others are related to glycosylated derivatives (Kandil et al., 1994).

Saffron petals contain strong antioxidant flavonoids that are bound to albumin in the blood serum and interact with this protein. On the other hand, the different effects of flavonoids on cholesterol-lowering and its anti-radical properties have been proven many times (Catoni et al., 2008). It is native to the Middle East and Southwest Asia, including Iran. Iran is one of the most important saffron production hubs in the world, so the value of saffron exports exceeds 300 billion rials per year. In the process of saffron production, stigma and cream are used as commercial saffron, and other parts of the flower, including petals, are discarded as having a high volume, so that the annual figure of 7257625 kg of saffron petals is obtained as a by-product which is expected to increase even in the coming years due to increasing production (Hemmati Kakhki and Rahimi, 1994).

Therefore, finding a solution to recover this large volume of waste is of great importance. One of these solutions could be the use of saffron petals as a natural antimicrobial agent in the treatment of bacterial infections. The root of this plant has beneficial medicinal properties such as anti-inflammatory, antiviral, antimicrobial and anti-cancer activity along with immune-enhancing effects, cough control and detoxification of the liver. It is also used in Addison's disease, asthma, bronchitis, cough, peptic ulcer and arthritis (Gupta et al., 2008; Gezici, 2019).

Therefore, this study aims to investigate the antibacterial effects of methanolic extract of saffron petal on some standard pathogenic bacteria.

2. Material and Method

In this descriptive *in vitro* study, saffron petals were collected from a field around the town of Benabe Marand in East Azarbaijan province. The petals were placed at ambient temperature for drying in the dark and were subjected to several steps until complete drying. After the samples were completely dried, the petals were prepared for grinding. Soxhlet method was used to extract 60 grams of petal powder with 300 ml of methanol as solvent for 8 hours in Soxhlet extractor. This solvent was slowly evaporated at 40 °C using rotary apparatus.

The concentrated extract was obtained from it. Extracts of solvent concentrated 5% DMSO (dimethylsulfoxide) at concentrations of 20, 30, 50 and 400 mg/ml for use in Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and Agar Well Diffusion experiments was prepared. The microorganisms studied in this study were: *S. aureus* ATCC 25923, *B. cereus* ATCC 1052, *E. coli* ATCC 25922, and *P. aeruginosa* ATCC 27853 (Microbial collection from University of Tehran). A separate culture was performed on the Mueller Hinton Agar medium to allow emerging colonies to be prepared with a half-McFarland turbidity solution (1.5×10^6 cfu/ml). For this purpose, for preparation of microbial suspension, 4-5 colonies of bacterial culture were transferred to Mueller Hinton Broth (MHB) to adjust the microbial suspension turbidity according to standard 0.5 McFarland tube. To reach a concentration of 1.5×10^6 cfu/ml, the microbial suspension was diluted to 0.01. In order to evaluate the antibacterial effect of methanol extract 4 concentrations of 20, 30, 50 and 400 mg/ml of methanolic extract were prepared in 5% DMSO solvent.

In this study, the antimicrobial effect of methanolic extract was investigated by agar well diffusion and dilution test. In the agar well diffusion method, 500 ml of microbial suspension was transferred onto Mueller Hinton Agar (MHA) medium and cultured by sterile swab in 3 directions. Then wells were prepared at 6 mm in diameter and 2.5 cm apart on agar surface. Then 100 µl of 20, 30, 50 and 400 mg/ml concentrations of methanol extract were injected into each well. Negative control was obtained using a solution used to dissolve the extracts (5% DMSO) and chloramphenicol antibiotic was used as positive control. Plates were then incubated at 37 ° C for 24 hours and microbial cultures were measured for the presence or absence of growth zone in millimeters.

The MIC and MBC of methanol extract were determined by tube dilution method. In this method, to determine the MIC of methanolic extract prepared by dilution serial dilutions of 6.25, 12.5, 25, 50, 100 and 200 mg/ml in MHB. Then, 1 ml of 1.5 × 106 cfu/ml active bacterial suspension was added to each dilution. Positive control (culture medium containing no bacterial extract) and negative control (culture medium without bacterium) were added to the tubes. Finally, the tubes were incubated at 37 °C for 24 hours.

After incubation, the tubes were examined for turbidity caused by inoculated bacterial growth and the last dilution in which no turbidity was observed (non-growth) was considered as MIC. Samples were then taken from all tubes in which bacterial growth was observed and MBC was determined by plate culture. Plates were then incubated for 24 hours at 37 °C. The tube containing the lowest concentration of the extract that had no visible bacterial growth on the plate was considered MBC of that material.

Each experiment was repeated 5 times to reduce the error of the experiment. SPSS software version 18 was used for data analysis. Analysis of variance and chi-square test were used to investigate the significant differences between the two groups and the significance level was set at $p < 0.05$.

3. Results

According to Table 1, the antibacterial activity of methanol extract of saffron petal in quantitative and qualitative methods showed that this extract showed a significant inhibitory effect on *S. aureus* and *B. cereus* bacteria. As the concentration of methanolic extract increased, the inhibitory effect increased. This study showed that the inhibitory effects of methanolic extract of saffron petal on Gram-positive bacteria was significantly higher compared to Gram-negative bacteria. MIC and MBC values of methanol extract of saffron petal against the tested bacteria showed that, like the well diffusion method, Saffron petal extract on Gram-positive bacteria has higher bactericidal power than Gram-negative bacteria (Table 2). These results indicated that there was a significant difference between the tested bacteria in the sensitivity of to saffron petal extract ($p < 0.05$).

Table 1. Mean diameter of non-growth zone of methanolic extract of saffron petal against selected bacteria in millimeters (mean ±standard deviation)

Well diffusion method (mm)						
	Concentration of extract (mg/ml)					
Bacterial strain	20 mg/ml	30 mg/ml	50 mg/ml	400 mg/ml	Negative control	Positive control
S. aureus	11.24±1.12	18±1.14	21.8±0.83	27.6±1.14	--	21
B. cereus	9±1.12	12.4±0.83	15.6±0.54	19.2±1.3	--	20
E. coli	0	0	11.6±1.14	16.5±0.83	--	27
P. aeruginosa	0	0	7.8±1.14	14.5±0.83	--	22

Table 2. MIC and MBC values of methanol extract of saffron petal (mg/ml)

Bacterial strain	**Concentration of extract (mg/ml)**	
	MIC	**MBC**
S. aureus	6.25	12.5
B. cereus	12.5	25
E. coli	50	100
P. aeruginosa	100	200

4. Discussion

The rise of pathogenic microorganisms and their resistance to a wide range of antibiotics along with the economic and social problems resulting from them have led to the expansion of studies on the production of herbal medicines. Therefore, screening such plants may lead to the discovery of new effective compounds that are able to inhibit pathogenic microorganisms. Compounds that can inhibit the growth of pathogenic microorganisms or kill them without toxicity to host cells are considered as candidates for the production of new antimicrobial drugs. As a result, there is a critical need for

research on novel antimicrobial agents with promising natural activities to provide alternatives to common antibiotics (Zhang et al., 2016). The results of this study showed that the saffron petal extract has an inhibitory effect on the gram-positive bacteria *S. aureus* and *B. cereus.* By increasing the concentration to 400 mg/ml, it is also effective on Gram-negative bacteria *P. aeruginosa* and *E. coli.* Vahid et al. (2002) examined the antimicrobial effects of the extract of different parts of saffron against E. coli, S. aureus, S. epidermidis and micrococcus showed that the extract of all parts of saffron except for leaves had antimicrobial activity (Vahidi et al., 2010). The results of this study showed that S. aureus and *B. cereus* were the most susceptible bacteria and *E. coli* and *P. aeruginosa* were the most resistant bacteria to the saffron petal extract, which was in agreement with the results of the study by Tayel and El-Tras (2009). Pintado et al. (2011) reported that safranal, crocin, and their associated chemicals are involved in the antimicrobial activity of saffron. Other researchers have pointed to the different susceptibilities of bacterial species to various antimicrobial agents. The difference in susceptibility of different microorganisms to antimicrobial agents is probably due to the different structure of the microorganisms. B. subtilis is one of the Gram-positive bacteria that, unlike Gram-negative bacteria, does not have an outer membrane on its wall, which can cause the active compounds to have a better penetration. Gram-negative bacteria are more impermeable due to the fat layer in the outer layer (Ordog et al., 2004). In a 2003 study by Razaghi et al., the antimicrobial effects of saffron stigma on three microbial strains of *E. coli, S. aureus* and *P. aeruginosa* were investigated and the results showed that safranal in saffron inhibited the growth of *E. coli* and *S. aureus* strains. Vahidi et al., (2010) investigated the antimicrobial effects of different parts of saffron extract including leaves, gynoecium and coralla against *E. coli, S. epidermidis,* micrococcus and fungi and reported their antimicrobial effects. Tajalli (2008), investigated the antioxidant effects of methanolic extract of saffron petal. The results showed that saffron petals were a natural and easy source of antioxidant with the highest concentration of inhibitory extract at 300pM. Islam et al., (2008) showed that plant extracts against Gram-positive bacteria were more effective than Gram-negative bacteria (Islam et al., 2008). Also, differences in the methods of evaluation of antibacterial properties of the extracts can lead to different results in the calculated MIC in different studies. Motamedi (2010.) by examining the antibacterial effect of ethanolic and methanolic extracts of saffron against some pathogenic bacteria showed that *S. aureus, B. anthracis, B. cereus, L. monocytogenes* and *B. melitensis* are the most susceptible species. *P. mirabilis* and *S. typhi* showed resistance to these extracts. In 2012, Azami et al., showed that *S. typhimurium* is the most sensitive bacterium to the petal of saffron petals. Gandomi Nasrabadi et al. (2012.) studying the antibacterial properties of saffron petal extract showed that methanol extract of saffron was effective on *S. typhimurium, B. cereus* and *L. monocytogenes*.

5. Conclusions

One of the reasons for the difference in MIC in different studies is the differences in the composition of the extracts. The composition of the extracts of a plant species can vary based on the region's geography, harvest season, plant age, growth stage, and the method of drying and extraction. In general, the plant extract has the highest antimicrobial activity during flowering or immediately after flowering. In addition, the extracts obtained from different parts of a particular plant have different antimicrobial activity. Also, the sensitivity of different bacteria to different extracts is different. In general, the results of this study showed that the extract of saffron extract has antibacterial

activity against the standard bacteria studied. However, clinical trials on patients after using saffron extract are recommended to confirm this data so that it can eventually be made available to patients in the category of medicinal plants.

Conflict of Interest

The authors have declared that they have no conflict of interest.

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Analysis of Mineral Compound of Plant *Anthemis pseudocotula* Boiss with Different Solvents by Ion Chromatography

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Abstract

This aim of this study was to determine the mineral contents in the plant of *anthemis pseudocotula boiss* with several solvents by Ion Chromatography , that the plant *Anthemis pseudocotula Boiss* commonly native and collected from their natural place from Hawdian's village soran/Erbil, North Iraq.The extract of five solvent as (distilled water, ethanol, distilled water: ethanol, hexane: ethanol and hexane) with both technique of microwave and conventional extraction. It was shown that fifteen mineral ion compounds were identified in the suppressed IC technique. Our results were detected eight mineral ions as (F, Cl-, PO_4+, SO_4+, NH_4, K+, Mg and Ca+) from 15 ions in our plant according to solvent extract and method technique. The concentrations of mineral ion compounds were the results showed high amounts of K+ (71.370ppm), Ca+ (71.420ppm), PO_4+ (24.164ppm), SO_4+ (28.127 ppm), Cl- (17.548 ppm) and Mg (10.279ppm) were found by microwave extraction with distilled water and ethanol with other solvents respectively. Furthermore, the results showed that mineral values of the K+ (43.277 ppm), SO_4+ (26.761 ppm), Cl- (18.028 ppm) were high concentration by conventional extraction with distilled water and water-ethanol solvents, respectively. These results were compatible with quantitative measurements. This study showed that the technique of microwave with distilled water and ethanol: distilled water solvents were important for the minerals content of *Anthemis pseudocotula* Boiss, a precious plant.

Key Words: *Anthemis,* Mineral Compound, Plant, Microwave Extraction

1. Introduction

Herbal plants play a significant role in conventional western medicine. People all over the continents have long utilized poultices and imbibed infusions of hundreds, if not thousands, of indigenous plants, courting returned to prehistory (Barbour et al., 2004). The genus *Anthemis* is belonging to the family Asteraceae and consists of about one hundred thirty species in the world

(Karioti et al., 2009) and this is one of the great phytochemical investigated genera of the family Asteraceae (Vučković et al., 2006). The geographic classification of anthemius extends throughout Europe, Southwestern Asia, Northern, and Northeastern Africa, Southern Arabia, and tropical East Africa (Bremer and Humphries 1993; Bremer 1994), the species of this genus have been in many instances used in traditional medicine healing procedures (Javidnia et al., 2004; Saroglou et al., 2006).

The mineral content material of plant samples is generally determined by analyzing the liquid phase after dry ashing or wet ashing ground dried plant materials. The processes and the problems of fume disposal and special laboratory equipment required for the methods have been mentioned (Johnson and Ulrich 1959). This study aimed to determine the mineral contents in the plant of *Anthemis pseudocotula* Boiss with several solvent extracts.

2. Material and Method

2.1. Plant Collection

The aerial part of plants was collected from their natural place. The species *A. pseudocotula* Boiss was collected from North Iraq, Erbil, Soran, Hawdian's village, which taxonomists at this plant was identified by Dr. (Abdullah Sh.Sardar), Erbil, Iraq: College of Education, Salahaddin University. After collected the plant sample was ground by new modern grinder (Model GI, Capacity/hour 15 Kg, Capacity 5 letter, Speed 13000 rpm, and Cycle 500 gr) has been done at the Forest Faculty, Kahramanmaras Sutcu Imam University, Turkey. After that prepared of powder plant, it was kept in a plastic bag in refrigerator at 4°C.

2.2. Preparation and Extraction Plant

The plant powder was extracted by ten gram with 200 ml of different solvents as (distilled water, ethanol: distilled water, ethanol, hexane: ethanol and hexane), with the using two different of method: Conventional extraction and microwave extraction, after that evaporated by using Fume Hood.

2.3. Analysis Plant Extract by Ion Chromatography (IC)

Ion chromatography (IC) is a speedy and sensitive method for isolating and analyzing solutions containing complex mixtures of mineral ions (Basta and Tabatabai, 1991). The study was carried out with Shimadzu Prominence (HIC-20A Dual IC) Super Ion Chromatograph device brand Dual Flow-Line IC/HPLC.

After prepared extract crude plant, Approximately 0.001 g of the crude plant is weighed and then 10 mL distilled water was added and shaken by vortex instrument for during 10 minutes to precipitate crude plant after that take 4 ml of solution added by micro peptide into vial injected into Ion chromatography technique. A representative isolation the utilization of IC-CD of a standard solution containing the inorganic compounds specified in the crude plant. The optimized columns shim packs IC-SA3 and shim pack IC-C4 were performed (250 mm x 4.6 mm x 0.25 µm). The elution of the mobile phase of 2.5 Mm oxalic acid and 3.6 mM Na_2CO_3 at the 450C Column Oven Temperature, the solvent flow rate was maintained at 0.8 mL/min with sample size 50 uL and injection volume was settled as 1600 µL. the solvent flow rate at a flow rate of this technique was used for the separation of mineral compounds as anions and cation (F, ClO2-, BrO3-, Cl-, NO2-, Br -, NO3-, PO4-, SO4-, Li+, Na+, NH4+, K+, Mg+, and Ca+) (Masson et al., 2005).

3. Results and Discussion

IC was used to representative separation of a standard solution to identified fifteen mineral ion compounds (F, ClO_2-, BrO_3-, Cl-, NO_2 -, Br -, NO_3-, PO_4-, SO_4-, Li+, Na+, NH_4+, K+, Mg+, and Ca+), in our plants. that the Ion chromatography (IC) technique identified just eight mineral compounds (F, Cl-, PO_4-,

SO_4-, NH_4+, K+, Mg+ and Ca+) by using different methods, conventional extraction and microwave-assisted extraction with five different solvents in our plant (Table1,2) and (Figure 1,2). The conventional extraction methods were detected the limit values of ion mineral compounds as K+ (60.697 ppm), SO_4+ (26.380 ppm), PO_4+ (14.759 ppm), Cl- (18.558 ppm), Ca+ (16.891 ppm) and Mg+ (8.157 ppm), with solvent extracts of distilled water-ethanol, water and hexane, respectively.

Table 1. Results of mineral components according convectional extraction in plants

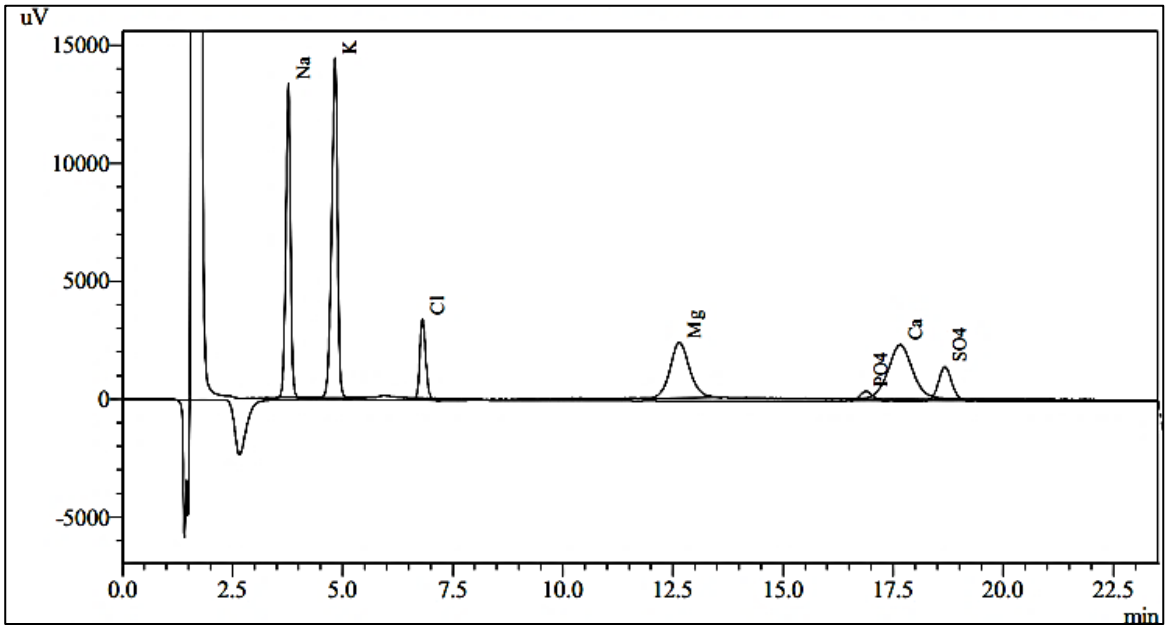
CE/S									
Detector	**Name**	**Ret.Time**	**Area**	**W Conc. (ppm)**	**W: E Conc. (ppm)**	**E Conc. (ppm)**	**H: E Conc. (ppm)**	**H Conc. (ppm)**	**Units**
1.	**F-**	0.000	0	0.000	5.994	5.165	0.000	0.000	mg/L
2.	**ClO_2-**	0.000	0	0.000	0.000	0.000	0.000	0.000	mg/L
3.	**BrO_3-**	0.000	0	0.000	0.000	0.000	0.000	0.000	mg/L
4.	**Cl-**	6.814	31508	12.469	18.558	18.028	12.859	12.373	mg/L
5.	**NO_2-**	0.000	0	0.000	0.000	0.000	0.000	0.000	mg/L
6.	**Br-**	0.000	0	0.000	0.000	0.000	0.000	0.000	mg/L
7.	**NO_3-**	0.000	0	0.000	0.000	0.000	0.000	0.000	mg/L
8.	**PO_4-**	16.904	1684	14.759	13.897	0.000	0.000	6.654	mg/L
9.	**SO_4-**	18.690	36764	20.109	26.761	21.337	16.380	23.206	mg/L
10.	**Li+**	0.000	0	0.000	0.000	0.000	0.000	0.000	mg/L
11.	**Na+**	3.783	135792	N.D	N.D	N.D	N.D	N.D	mg/L
12.	**NH_4+**	0.000	0	0.000	0.000	0.680	0.000	0.000	mg/L
13.	**K+**	4.784	29062	43.277	60.697	16.807	8.452	10.309	mg/L
14.	**Mg+**	12.651	56929	7.616	8.157	6.284	4.989	6.243	mg/L
15.	**Ca+**	17.668	105006	14.700	13.884	14.859	9.606	16.891	mg/L

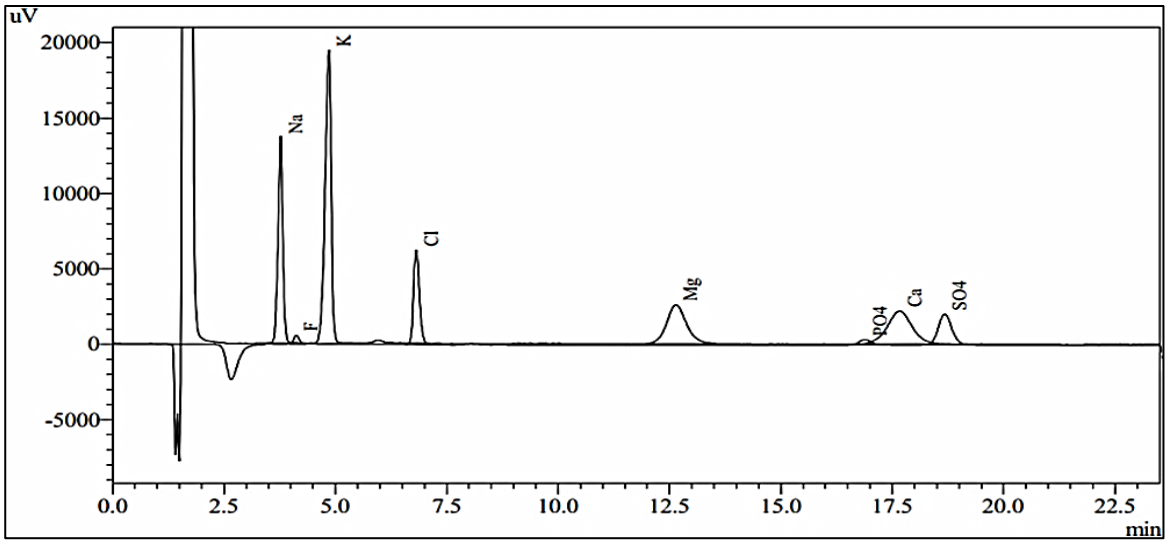
Not detected: N.D,
Method: CE/S Convectional Extraction Solvent
Solvents: DW: Distilled water, DW: E, Distilled water-Ethanol, E: Ethanol, H-E: Hexane-Ethanol, and H: Hexane

On the other hand, the microwave extraction method was detected highly values of K+ (71.370 ppm), Ca+ (71.420 ppm), and PO_4- (24.164 ppm), SO_4- (28.127 ppm), Cl- (17.548 ppm) and Mg+ (10.279 ppm) in solvent extracts of the distilled water-ethanol, water, and hexane, respectively. Furthermore, significant values of the mineral compound in the distilled water solvents were determined by the microwave extraction method. Whereas, compared with the method of conventional extraction that showed a lower concentration. Therefore, the concentration of K+, PO_4+ and Ca+ extracted with water and water: ethanol was similarity to, or larger than, all other extraction solvents extraction.

Our result the Calcium (Ca+) was the highest value in our plant, It is very much required for the normal functioning of the cardiac muscle blood coagulation, milk clothing and the

system of the permeability, also the Ca+ constitutes a large proportion of the bone, human blood and extracellular fluid (Indrayan et al., 2005), and also the Ca+ is an essential component in fibrin formation which types fibrinogen and in consequence fibrin and collagen (Schalm et al., 1975).The Fibrin is a dotting issue responsible for homeostasis. K+ and Na+ ions are acknowledged activators of energy potentials throughout nerve membrane collectively with Ca+, ions may also serve as replenishment in diarrheic conditions, maintenance of ordinary apprehensive function and intestine peristalsis. Mg+ ions are recognized hormone activators in kind 2 diabetes (Schalm et al., 1975). However, the both of Mg+ and Ca+ were indicated a high and limited value in our plant .that is a play a big function in photosynthesis, bio molecules metabolism and binding agents of cell walls. Ca+ is additionally the component of bone and assists in tooth improvement (El Khatib et al., 2003).





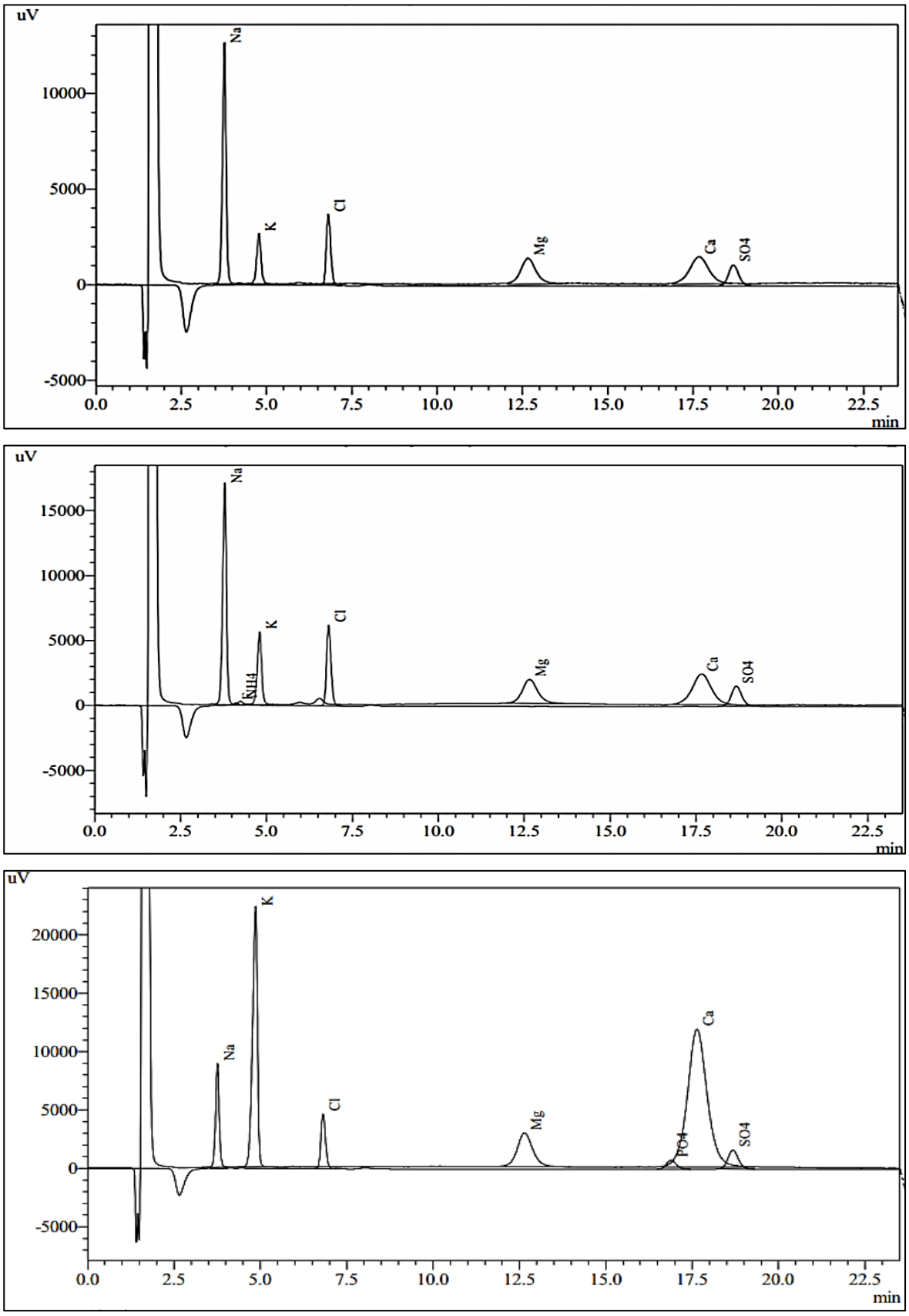
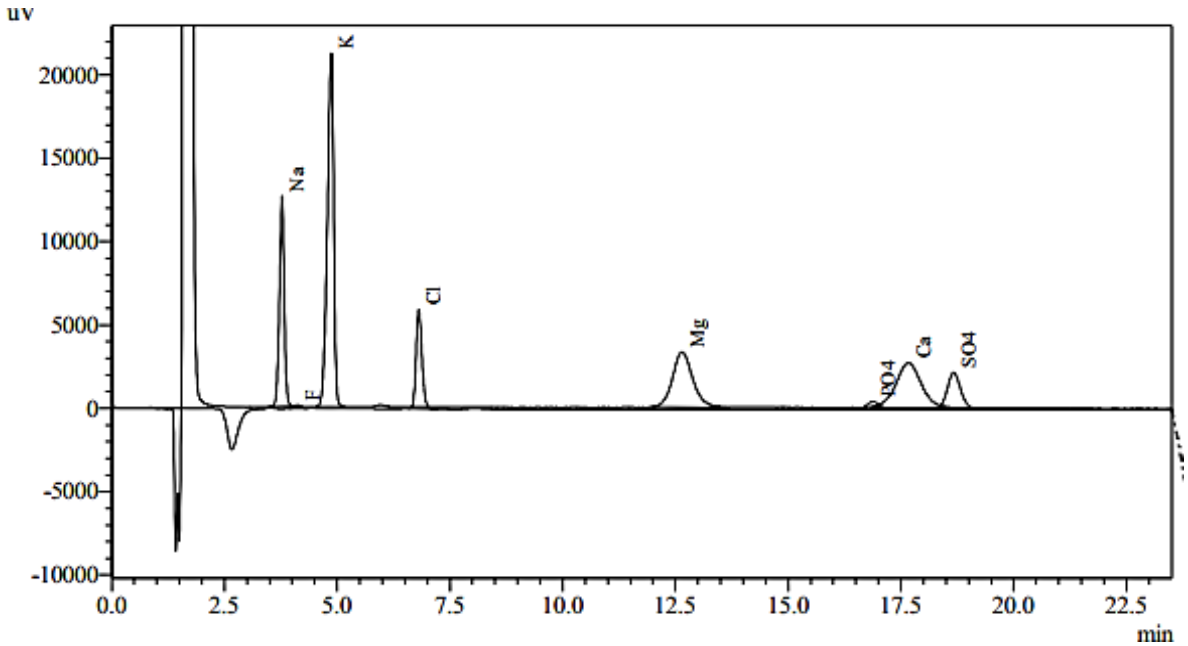


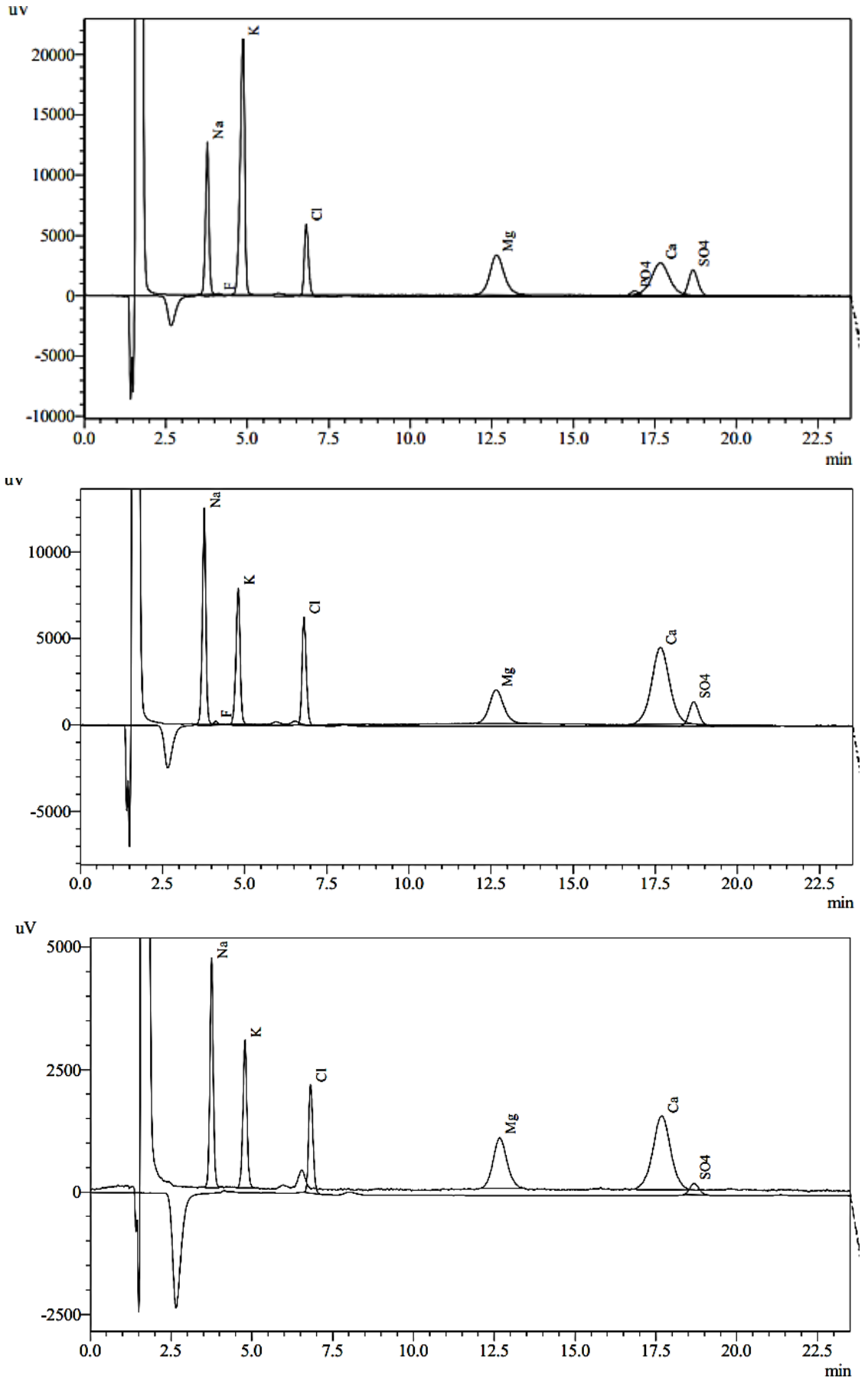
Figure 1. Quantitative result of solvents with conventional extraction by ion chromatography *(Water solvent; Water-ethanol solvent; Ethanol solvent; Hexane-ethanol solvent; Hexane solvent)*

Table 2. Results of mineral components according microwave extraction in plants

ME/S									
Detector	**Name**	**Ret. Time**	**Area**	**W Conc. (ppm)**	**W: E Conc. (ppm)**	**E Conc. (ppm)**	**H:E Conc. (ppm)**	**H Conc. (ppm)**	**Units**
1.	**F-**	0.000	0	0.000	5.203	5.221	0.000	0.000	mg/L
2.	**ClO_2-**	0.000	0	0.000	0.000	0.000	0.000	0.000	mg/L
3.	**BrO_3-**	0.000	0	0.000	0.000	0.000	0.000	0.000	mg/L
4.	**Cl-**	6.809	43978	15.278	17.548	18.096	9.836	6.904	mg/L
5.	**NO_2-**	0.000	0	0.000	0.000	0.000	0.000	0.000	mg/L
6.	**Br-**	0.000	0	0.000	0.000	0.000	0.000	0.000	mg/L
7.	**NO_3-**	0.000	0	0.000	0.000	0.000	0.000	0.000	mg/L
8.	**PO_4-**	16.884	15066	24.164	16.365	0.000	0.000	0.000	mg/L
9.	**SO_4-**	18.674	34633	22.139	28.127	19.683	7.172	8.977	mg/L
10.	**Li+**	0.000	0	0.000	0.000	0.000	0.000	0.000	mg/L
11.	**Na+**	3.755	65152	N.D	N.D	N.D	N.D	N.D	mg/L
12.	**NH_4+**	0.000	0	0.000	0.000	0.000	0.000	0.000	mg/L
13.	**K+**	4.861	221769	71.370	67.166	23.078	9.455	3.691	mg/L
14.	**Mg+**	12.640	88458	8.938	10.279	6.481	4.035	4.423	mg/L
15.	**Ca+**	17.639	472445	71.420	16.967	27.025	9.977	9.328	mg/L

Not detected: N.D
Method: MWE/S Microwave Extraction Solvent
Solvents: DW: Distilled water, DW: E, Distilled water-Ethanol, E: Ethanol, H-E: Hexane-Ethanol, and H: Hexane

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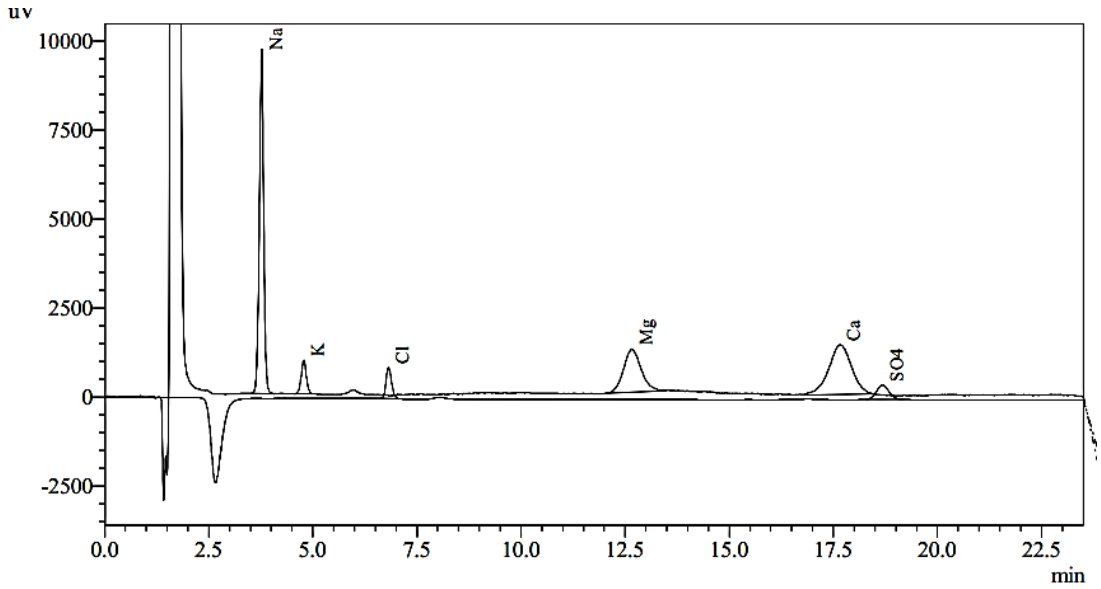

Figure 2. Quantitative result of solvents with microwave extraction by Ion Chromatography *(Water solvent; Water-ethanol solvent; Ethanol solvent; Hexane-ethanol solvent; Hexane solvent)*

The K+ content in our result was highest in the plant, the K+ participate to maintains tissue excitability and in ionic balance of the human body. And it is an essential nutrient and has an important function in the synthesis of proteins and amino acids (Zhou et al. 2005)

In spite of that, the Na+ content was not detected but Na+ plays an important function in the transport of metabolites (Zhou et al. 2005). The ratio K+ and Na+ in any food is an essential factor in the prevention of hypertension and arteriosclerosis with K depressed and No enhance blood stress (Saupi et al. 2009) , and the ion K+ is necessary for its diuretic nature (Zhou et al. 2005). Our results agreement with (Cataldi et al. 2003), but dissimilar with a species of plant.(Butnariu et al., 2012), also, agreement with species of plant by (Russo and Karmarkar, 1998), and disagreement with (Malone et al., 2002), due to the difference method and plant but same species. In the present work, an efficient, sensitive, and rapid ion chromatography technique approach was established and proven as appropriate for separating, quantifying, and identifying mineral contents of (Ca+, Mg+, K+, NH4+, Na+, Li+, SO4-, PO4-, NO3-, Br-NO2-, Cl-,BrO3-, ClO2- and F-). The high sensitivity, extraordinary linearity, precision, and rigor have been achieved. The concentration of all mineral compound had been exhibited the most inconstancy among solvent types, inside extraction technique. That is supplying a very beneficial technique for chemical analysis and biological research purposes. To the excellent of our Knowledge no preceding work has been pronounced of the mineral compound in plant of *A. pseudocotula* Boiss.

4. Conclusion

This study shows an overview analysis of the five extract solvents with the methods of microwave and conventional extraction. That shows all results of the analysis were found but as limited value. Therefore, our results were detected 8 mineral compounds from 15 ions. The microwave extraction method was a significant value of mineral compound with solvents of (distilled water and distilled water: ethanol), our study is very important and will be useful for other researchers, additionally, that suggest who interesting in our plant to use another analytical methods to get more and extensive results. That the *A. pseudocotula* Boiss, a precious plant.

Acknowledgements

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Coflict of Interest

None.

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The Efficiency of BIOAPIFIT® Wound Care Ointment in the Treatment of Venous Ulcers

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Abstract

Objective / Purpose: The objective of this study was efficacy assessment of Bioapifit® wound care ointment consisted of honey, *Cera flava,* glycerin, the oil macerates of astringent and soothing herbs combined with three essential oils for the treatment of venous ulcers. **Materials and methods**: 50 patients with total 112 venous ulcers with the total surface area of 572.5 cm^2 were treated 60 days (twice a day) with Bioapifit® wound care ointment applied on conventionally cleaned wound and covered with bandage during the whole course of the study. The healing process was assessed by Venous Clinical Severity Score (VCSS) tool twice a month. **Results:** At baseline the mean value and standard deviation of the VCSS score was 25.03 ± 4.37 and 25.53 ± 3.36 for females and males, respectively. The surface area ranged from 1.6 to 28.1 cm^2 for females and from 1.60 to 29.20 cm^2 for males. The mean value and standard deviation of the total VCSS score following the treatment decreased to 6.26 ± 4.0 and 6.47 ± 3.9 for females and males, respectively. Total number of active ulcers decreased from 112 to 17 and the total surface area of all ulcers from 572.5 cm^2 to 7.6 cm^2. No side-effects were observed during the course of the study. **Conclusion / Discussion:** Two months application of Bioapifit® wound care ointment resulted in complete closure of 84.8% of the ulcers and reduction in their surface area for 98.7% with the mean healing time of 37.2 days.

Key Words: Venous Ulcers, VCSS Tool, Honeybee's Products, Herbal Macerate

1. Introduction

Venous ulcer, the latest stage of the chronic venous disease, represents the area of discontinuity of the skin, most often located in the distal parts of the lower limbs (Marinović Kulišić, 2016). It differs in size and shape, sometimes including the entire circumference of the extremities. Possible causes of venous ulcers include inflammatory processes resulting in leukocyte migration, plasma cell and granulocyte activation, increased activity of metalloproteinase, endothelial damage, platelet aggregation, and intracellular edema. Impaired muscle activity represents another important risk factor involved in the patophysiology of venous ulcers (Collins and Seraj, 2010; Marinović Kulišić, 2016).

The important risk factors are older age (>50), body mass index (BMI) gender,

multiple childbirths, previous leg injuries, deep venous thrombosis, inadequate physical activity, smoking, static foot disorders, family history, phlebitis (Collins and Seraj, 2010; Marinović Kulišić, 2016).

Various management options were developed so far for the treatment of chronic venous disease (Collins and Seraj, 2010; Marinović Kulišić, 2016) and include: conservative treatment (compression therapy, leg elevation, dressings), mechanical treatment (vacuum- assisted closure), medications (natural venoactive drugs, pentoxifylline, glycosaminoglycans, prostaglandine E1, aspirin, iloprost, oral zinc, antibiotics/antiseptics), hyperbaric oxygen therapy, surgical intervention (debridement, human skin grafting, artificial skin, surgery for venous insufficiency). The latest was applied to the large ulcers with prolonged duration not responded to the conservative treatment. Since 1994 clinical assessment of the severity of the chronic venous disease is based on the CEAP (clinical, etiology, anatomy, and pathophysiology) classification system ranging from C0 with no disease present to C6 with the presence of active ulcers assessment of chronic venous disorders (Marinović Kulišić, 2016).

Based on the elements of CEAP classification the American Venous Forum, in 2000 developed the Venous Severity Score (VSS) grading tool as the complementary system to the CEAP classification. VSS classification is necessary for the longitudinal monitoring of the clinical condition of the patient during and after the intervention. This classification is combined with the degree of the severity of the venous disease: Venous Disability Score (VDS); Venous Segmental Disease Score (VSDS); Venous Clinical Severity Score (VCSS) (Marinović Kulišić, 2016).

The VCSS consist of ten descriptors (pain or other discomfort, varicose veins, venous edema, skin pigmentation, inflammation, induration, active ulcer number, active ulcer duration, active ulcer size, use of compression therapy) graded from 0 (no symptoms/disease) to 3 (highest degree of the symptoms/disease) (Vasquez et al., 2010).

The purpose of this work was testing of clinical performance of Bioapifit® wound care ointment composed of honey, glycerin, herbal macerates of the astringent plants, beeswax and three essential oils for the treatment of 112 active venous ulcers.

2. Patient and Method

2.1. Study Design

The study was conducted at the following locations: FINDRI GUŠTEK HEALTHCARE INSTITUTION, Ninska 5a, Sesvete, Croatia and FAMILY MEDICINE CLINIC, Vilima Korajca 19 Zagreb, Croatia. The investigator recruited the patients based on their medical history, following the predefined inclusion and exclusion criteria. The study protocol was approved by the Ethics Committee of Findri Gustek Health Care Center with EudraCT number 2019- 001379-35.

50 patients (35 females and 15 males) ranging from 57 to 77 years with total of 112 active ulcers and the total surface area of all ulcers of 572.5 cm^2 were included. All the participants signed informed consent and completed the questioner.

The patients were treated 60 days with the product. The ointment was applied on the previously cleaned wound twice a day by nurse and covered with bandage during the whole course of the study. At each changing of the bandages each wound was cleaned from the slough. Clinical evaluation of the patients before and following the therapy was done by Venous Clinical Severity Score (VCSS) tool consisting of ten descriptors each graded from 0 (no symptoms/disease) to 3 (worse possible symptoms/disease).

2.2. Description of investigational product

Bioapifit® wound care ointment is homogeneous, greasy, viscous mass of characteristic herbal odor and olive green color with pH of 4.43±0.13. It consists of the following ingredients: honey (certified organic), beeswax (*Cera flava*), glycerol, the macerates of the plant species: *Plantago major* L., *Achilea millefolium* L., *Quercus robur* L., *Salvia officinalis* L., *Olea europaea* L., *Polygonum aviculare* L., *Symphytum officinale* L., *Calendula officinalis* L., *Matricaria chamomilla* L., essential oils: Australian tea tree (*Melaleuca alternifolia* (Maiden & Betche, Cheel), thyme (*Thymus vulgaris* L. ct. thymol), oregano (*Origanum vulgare* L.).

2.3. Statistical analysis

For statistical evaluation Statistica 11.0 software package was employed. The description of the treated population was done by basic statistics and frequency tables. Statistical significance was set to $p<0.05$ in all the tests performed. The differences in the mean values of each parameter prior and after the therapy as well as different treatment periods were assessed by Newman-Keuls test. The influence of the predictor variables on the dependent variable was tested by Multiple regression method and General regression model (Oreščanin, 2016).

3. Results

3.1. Description of the population

The study included 35 females and 15 males. The number of childbirth ranged from 1 to 4 with majority of them (16 of 35) having two childbirth. 75% of the participants had previous leg injuries and 24% of them suffer from deep venous thrombosis. 82% of the participants had prevailing sedentary lifestyle or occupation with inadequate physical activity. Among the participants 68% of them are smokers. Family history of venous disease was present in 80% of the participants and phlebitis in 12% of them.

The basic statistical parameters for age and body mass index expressed separately for males and females as well as total population is presented in Table 1. The female population ranged from 57 to 77 years (67.97±4.97) and males from 61 to 77 years (67.60±4.81). Both female and male participants were overweighed with BMI ranging from 26.30 to 44.80 mg/m^2 (33.80±4.61 mg/m^2) and males from 28.70 to 42.90 mg/m^2 (36.10±4.93 mg/m^2). T-test showed no significant difference between males and females regarding age or BMI.

Table 1. The basic statistical parameters for age and body mass index separately for males and females as well as total population.

Gender	Age			Body mass index		
	$\bar{X}$±SD	Min.	Max.	$\bar{X}$±SD	Min.	Max.
Female	67.97±4.97	57.00	77.00	33.80±4.61	26.30	44.80
Male	67.60±4.81	61.00	77.00	36.10±4.93	28.70	42.90
All	67.86±4.88	57.00	77.00	34.49±4.77	26.30	44.80

X-mean value; SD-standard deviation

The results of multiple regression analysis showed very good, statistically significant correlation between VCSS score and selected predictor variables ($R=76.7$; $p<0.0000$). The variables with the highest, statistically significant contributions to the VCSS score were BMI ($p<0.0027$), family history ($p<0.043$) and age ($p<0.047$).

The results were completely in agreement with those obtained by General regression model expressed as Pareto chart of t-values (Figure 1) which identified BMI, family

history and age as statistically significant contributors to the total VCSS score.

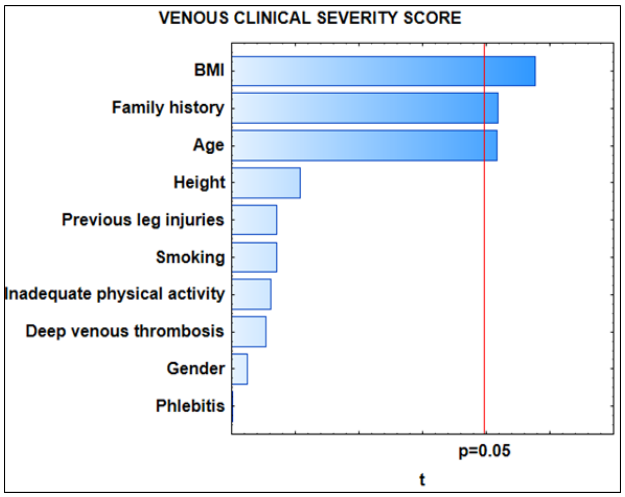

Figure 1. Pareto chart of t-values testing for the influence of predictor variable onto venous clinical severity score before the treatment

3.2. Treatment efficiency

The results of the assessment of venous ulcers according to the venous clinical severity score (VCSS) following the treatment with Bioapifit® wound care ointment were presented in Table 2 and Figure 2. Prior to the therapy the mean value and standard deviation of the total VCSS score was 25.03 ± 4.37 and 25.53 ± 3.36 for females and males, respectively (Table 2, Figure 2).

The number of active ulcers ranged from 1 to 4 and their surface area from 1.6 to 28.1 cm^2 (10.94±9.26 cm^2) for females and from 1.60 to 29.20 cm^2 (12.65 ± 10.85 cm^2) for males. There was no significant difference between males and females in all descriptors and the total VCSS score or surface area of the ulcers at baseline.

Table 2. Mean values and standard deviations for each descriptor and total value of venous clinical severity score for male (M) and female (F) population at baseline (B) and following 60 days of the treatment (F) with Bioapifit® wound care ointment

Descriptor	F-B	F-F	M-B	M-F
Pain	3.00±0.00	0.54±0.51*	3.00±0.00	0.73±0.46*
Varicose veins	2.54±0.51	1.17±0.45*	2.73±0.46	1.27±0.59*
Venous edema	2.57±0.50	0.46±0.51*	2.87±0.35	0.60±0.51*
Skin pigmentation	2.77±0.43	1.31±0.47*	3.00±0.00	1.33±0.49*
Inflammation	2.57±0.50	0.26±0.44*	2.73±0.46	0.33±0.49*
Induration	2.63±0.49	0.63±0.60*	2.67±0.49	0.87±0.35*
Active ulcer number	2.09±0.78	0.37±0.49*	2.00±0.76	0.33±0.49*
Active ulcer duration	2.20±0.76	0.74±1.15*	2.07±0.70	0.33±0.49*
Active ulcer size	2.29±0.67	0.37±0.49*	2.27±0.59	0.33±0.49*
Use of compression therapy	2.37±0.69	0.40±0.55*	2.20±0.56	0.33±0.49*
VCSS-total score	25.03±4.37	6.26±4.59	25.53±3.36	6.47±3.72*

Following the 60 days of tropical treatment with Bioapifit® wound care ointment all descriptors of the VCSS score decreased significantly. The mean value and standard deviation of the total VCSS score was 6.26 ± 4.0 and 6.47 ± 3.9 for females and males, respectively (Table 2, Figure 2). The total number of active ulcers decreased from 112 to 17 and the total surface area of all ulcers from 572.5 cm^2 to 7.6 cm^2. The mean value and standard deviation for active ulcers surface area following the therapy was 0.13 ± 0.21 cm^2 for females and 0.19 ± 0.29 cm^2 for males.

4. Discussion and Conclusion

Two months of the topical treatment with Bioapifit® wound care ointment resulted in

reduction of total VCSS score for app. 75%, complete closure of 84.8% of the venous ulcers and reduction in the ulcer's total surface area for 98.7% with the mean healing time of 37.2 days.

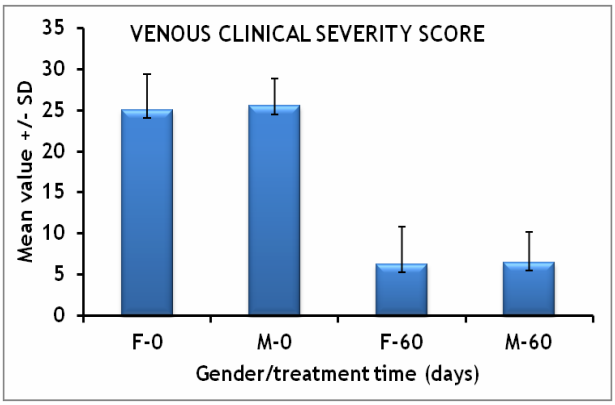

Figure 2. Mean values and standard deviations for total value of venous clinical severity score for male (M) and female (F) population prior and following 60 days of the treatment with Bioapifit® wound care ointment.

Since venous ulcers are wounds that are very difficult to heal, obtained results could be attributed to the selection of the ingredients with pH adjusting, osmotic, moisturizing, astringent and coating effect. Among them, honey was identified as most important ingredient of the ointment which thanks to its low pH value (4.16) creating an acidic wound micro-environment necessary for healing process. Debridement of slough and necrotic tissue through autolytic debridement was also present. Moreover, honey absorbed wound exudates due to high osmotic effect/high sugar content and created the environment with low water activity that all together supported wound closure and in the same time prevented pathogens growth. Previous studies connected antimicrobial activity of honey against the pathogens causing invasive wound infections including methicillin- resistant *Staphylococcus aureus* (MRSA) either to the production of hydrogen-peroxide by glucose oxidize enzyme or non-peroxide antimicrobial activity which could be connected to low pH value, osmotic effect of sugar, the presence of polyphenols and flavonoides, carbohydrate and its break-down Maillard products, aromatic acids, 10-HAD defensin-1 protein, 1,2-dicarbonyl compound methylglyoxal and bacillomycin F antibiotic like polypeptide (Lusby et al., 2002; Simon et al., 2009; Al-Waili et al., 2011).

It was confirmed that topical application of honey (directly or in the form of various types of wound dressing had very beneficial effects on wound healing. The treatment of pressure ulcers with honey alginate (Vandamme et al., 2003) resulted with rapid and complete wounds healing, reduced inflammation and deodorizing effect. Subrahmanyam et al., 2001 reported significantly faster wound healing in the patients treated with honey dressing compared to those treated with silver sulphadiazine. Moreover, completely sterile wounds were obtained in 90% of honey treated patients. It was reported that pH of the wound has critical influence on its healing potential since the wounds with pH higher than 8 showed no reduction in size (Gethin et al., 2008). Alam et al. (2014) summarized beneficial effects of honey for the treatment of diabetic wounds that were mostly connected to its antimicrobial activity, low pH value, hydrogen peroxide activity that all together stimulated wound closure. Debridement of slough and necrotic tissue through autolytic debridement and minimizing wound odor was another important mechanism (Alam et al., 2014).

A significant improvement of venous ulcer wound healing was observed following the treatment with the honey-based dressing (Alcaraz and Kelly, 2002). Mohamed et al. (2014) reported complete wound closure amputation wound after four weeks continuous treatment with natural honey. The treatment of foot ulcers with natural honey once a day resulted in complete wound closure within three weeks with no contractures or scars (Mohamed et al., 2015). The treatment of the patients with neuropathic diabetic foot ulcers with manuka

honey impregnated dressings (Kamaratos et al., 2014) resulted in complete healing after 31±4 days while in app. 78% of the patients wound became sterile following one week of the treatment which was in agreement with the results obtained in the current study.

Researchers confirmed beneficial effect of the astringent plants rich in soluble tannins in the treatment of open wounds (Abascal and Yarnell, 2005; Odukoya et al., 2007) which could be explained by surface coagulation of the proteins resulting in the shrinking of the wound as well as by forming the protective coating over damaged tissue. For that purpose oil macerates of the plants with strong astringent properties *Plantago major* L.*, Achilea millefolium* L.*, Quercus robur* L., *Salvia officinalis* L.*, Olea europaea* L., *Polygonum aviculare* L., *Symphytum officinale* L., were included in the product formulation. Moreover, the macerates of marigold flowers (*Calendula officinalis* L.) and chamomile flowers (*Matricaria chamomilla* L.) were used due to its soothing and calming effect to the wounded skin (Oreščanin, 2016).

Additionally, herbal macerate was used in the formulation due to its low pH and coating effect. Moreover, the macerate created the environment with no water activity which was unsupportive for pathogens growth and replication.

Glycerol was used in the formulation in order to provide enough moisture content of the wound necessary for the healing process. Beeswax was employed not only because of its emulsifying and thickening effect but also for wound isolation and protection from the microbial infection due to its excellent coating effect (Oreščanin, 2016). Essential oils served as natural preservatives and wound malodor correctors.

Conflict of Interest

The authors declared that they have no conflict of interest.

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Research article
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Current Perspectives on Medicinal and Aromatic Plants

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Ethnobotanical Survey of Some Plants Used in Tessala Region, Algeria

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Abstract

The area of Tessala is home to very diverse vegetation between forest and steppe species more than 1000 meters high, there are aromatic plants thus participating in the economy of the region this wealth is not exploited where the interest of our study in the framework of the valorization of the natural resources by an inventory of the flora and an ethnobotanical investigation using a standardized survey dedicated to the tradipraticiens. Subsequently, this work was complemented by the identification of field samples at the Botanical Laboratory of the faculty of medicine using flora and herbaria available to translate this traditional folk knowledge into scientific knowledge. Thus, the scientific knowledge of the medicinal flora of the region studied allowed us to gather the maximum of information concerning the therapeutic uses practiced by the local population. As a result of the floristic inventory a total of 80 medicinal plants, distributed in 50 genera, were collected and identified. Plant family with the highest medicinal plants in the study area used for various diseases treatment was Lamiaceae. The survey revealed more than 40 species used for several pathologies ranging from simple dermatological disease to hypertension and diabetes while Pistacia, *Marrubium,* and Myrtus were the most frequently utilized plant, However, to save medicinal plants from further loss, involving local communities in the cultivation of the most utilized medicinal plants is recommended.

Key Words: Aromatic plants, Ethnobotanical study, Flora, Inventory, Medicinal plants, Traditional, Valorization

1. Introduction

Nature is a huge deposit of active molecules of plant origin, and the resources of the flora are far from being fully inventoried. Around the world, we continue today to look for plants that can be used as a basis for new treatments. Today, the search for new drug molecules of natural origin continues to be an urgent necessity and is based on the quality of medicinal plants and on ethnobotanical studies that make it possible to make inventories of plants of a region, by determining their quality. by phytochemical and pharmacological studies (Guedira and Goetz, 2008). In Algeria species of spontaneous flora constitutes a significant part of local genetic resources with pastoral, forage, food, aromatic and/or medicinal value (Adrar, 2015).

Therefore, to make an appreciable contribution to the knowledge of this plant biodiversity and development of the natural

resources of Tessala, Western Algeria it has been considered useful to do a floristic and ethnobotanical study. In addition to the programs of some international organizations such as (IUCN), which aims to promote the conservation of biodiversity and the sustainable use of natural resources in North Africa and the involvement of local communities in the conservation of biodiversity, our laboratory is trying to carry out floristic, phytochemical and ethnobotanical research of medicinal plants in different regions of Sidi bel abbes, this work also aims to support the rural poor by strengthening their identity, their sources of income and their food security by promotion of the use of neglected species. (Belkhadar, 1997).

2. Material and Method

2.1. Ethnobotanical data collection and analysis

First a floristic inventory, then an ethnobotanical survey was carried out in the region of Tessala near traditional healers based on a questionnaire developed and standardized according to the pharmalex platform to draw up a list of plants with the scientific name, the family, vernacular name, usage and method of preparation.

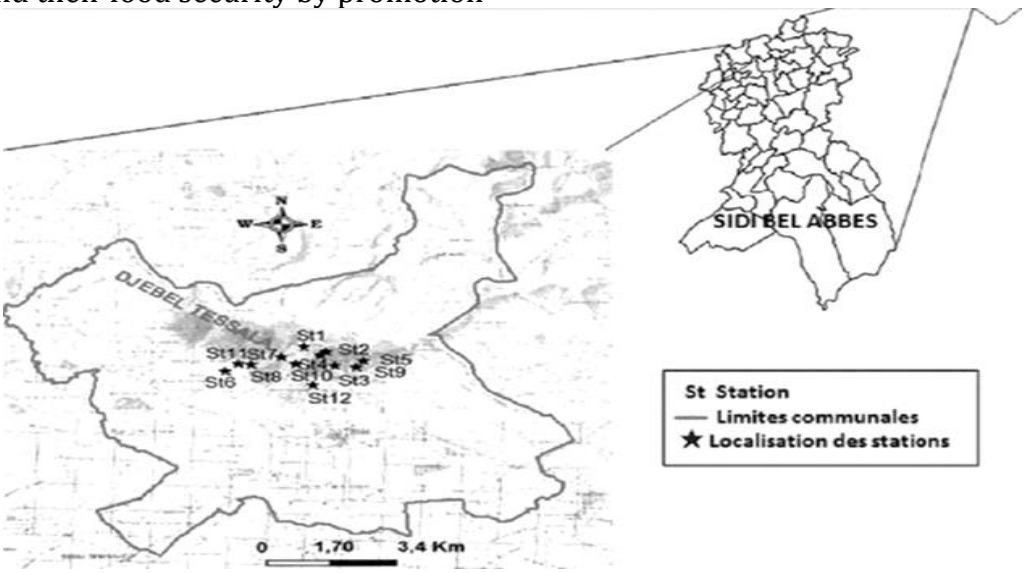

Figure 1. Presentation of the area of study

The survey carried out concerned the commune of Tessala the location of the different survey environments was identified by stratified sampling techniques. These techniques seemed appropriate for conducting ethnobotanical surveys varied from one area to another in this study. We sought to delimit and explore the maximum areas of the region. For this, two field campaigns were planned during the years 2018 and 2019. And using 40 questionnaire sheets that were prepared and rectified, we carried out ethnobotanical surveys in the region studied (Figure 1) to have as much information as possible about the use of medicinal plants by the local population. For the identification and determination of species, we used the new flora of Algeria and the southern desert regions (Paul Ozenda, 1991) and the flora of North Africa (Quezel and Santa, 1962) and the flora of the laboratory of the pharmacognosy of the faculty of medicine of Sidi bel abbes. Also, an herbarium has been made and stored in the Tlemcen pharmacognosy laboratory, an electronic version of which is available.

2.2. Presentation of the area study

The mountains of Tessala are located in the north of western Algeria, and more precisely

north of Sidi Bel Abbés (Figure 1). Composed of sedimentary rocks, they form an elongated massif, which belongs to the Tellian Atlas. They culminate at Djebel Tessala which reaches 1061 m of altitude. Most soils (Benyahia et al., 2001) belong to the class of raw mineral soils: lithosols and regosols of the French classification. There are also some rendzines Calcium brown soils are rare, the climate is the Mediterranean. Rainfall is concentrated in autumn and winter, while the drought period is 6 months, from April to September (Ferka-Zazou, 2006).

Table1. List of plants identified in the ethnobotanical survey

Scientific name	Vernacular name	Traditional use	Preparation method
Ajuga-iva	chendgoura	Diabetes	Decoction
Ampelodessma mauritanicus	Diss	Digestion	Decoction
Aristidi pungens	retam	Multiple uses	Powdre aerial part
Arthrophytum scoparium	Remeth	Hepatitis	Decoction
Artemisia herba-alba	Chih	Emmenagogue	Decoction
Asparagus acutifollus	Sekoum	Gout disease	Drop fruit
Asphodelus microcarpus	Belouz	Cold snap	Tubercule comestibl
Atractylis gummifera	Addad	Dermatological affaction	Root
Atriplex halimus	Guettaf	Emmenagogue	Decoction
Rhamnus alaternus	meliles	Ictere, jaundice	Bark decoction
Bellis annua	Hallala	Furunculosis	Cataplasm
Bourrago officinalis	Lessan elferd	Diuretic	Flower
Chamerops humilis	Gaze	Digestive	Fruit comestibl
Cistus	tanghoust	Rheumatism	Maceration
Calycotome spinosa	Guendoul	Cardiovascular	Infused flower
Coronilla valentina			
Daphne gnidium	Lazez	Sinusitis	Maceration,cataplasm
Euphorbia helioscopia	Tabera	Vomitingt, aphthae	Roots
Globularia alypum	Tesselra	Constipation	Infusion
Kundmaninia	Zeyata	Diabetes Obesity	Root
Marubium album	Merioua	Urinary infection	Decoction
Myrtus communis	Rayhan	Antiseptic	Fumigation
Lavandula stoechas	Halhal	Dyslipidemia	Infusion
Olea europea	Zitoun	Hypertension and diabetes	Dried leaves
Phillyrea angustifolia	Ktem	Hair	Tinctorial
Pinus halepensis	Zenin	Cough	Fumigation, oil
Pistachia lentiscus	Darou	Hypertension and diabetes	Oil, decoction, dried leaves
Plantago longopus	Lalema	Kidney calcul	Infusion
Quercus coccifera	Belout	Diarrhea, hernia	Decoction Bark
Rosmarinus officinalis	Iklil el jabel	Hepatoprotective Painful menstruation	Infusion
Ruta chalepensis	Fidjela	Emmenagogue	Leaves
Salvia verbenaca	keyata	Healing	Leaf application
Salvia argentea	Ferachet e neda	Healing	Fresh leaf
Saxifragas globulifera	Fetat el hejer	Calculation of the kidneys	Decoction
Scolymus hispanica	Guernina	pains	Powder
Tetraclinis articulata	Araar	Antiseptic	Fumigation
Thapsia garganica	Deryas	Rheumatism	Bulbe maceration with olive oil
Teucrium polium	Latay el khela	Dyslipidemia	Maceration
Thymus vulgaris	Zaater	Cough, flu, allergy	Infusion, decoction, cataplasm
Thymelaea hirsuta	Metnan	Pain	Decoction, infusion

3. Results and Discussion

The floristic inventory has collected more than 50 genera and 80 species distributed throughout the study area, while the survey allowed for a table with more than 40 species to better understand the relationships between species and types of diseases, we initially limited ourselves to medicinal plants with a relatively high frequency of use. In the Tell there is mainly the Aleppo Pine which is well adapted to the region, next to it there are other secondary species: The *Holm and kermes, Thuya*, *Juniperus*.

The undergrowth includes various *Cistus Pistacia , Filaria, Olea europea*, *Arbutus, Cysts, Rosemary, Dwarf Palm, Alfa, Diss* (*Ampelodesmos tenax*), next to it The *Alfa* dominates the rocky steppe, in stony soils with shallow soils. Associated with alfa, we find a range of perennial or annual plants: *Bromus squarrosus, Bromus bordaceas, Thymus ciliatus, Asphodelus, Astragalus, Rosmarinus, Cistus, Libanotus, Echinariacapitala, Avena alba, Galium, Alfa* is exploited by herds of sedentary or semi-nomadic inhabitants of the region. And a steppe to *Artemesia herba alba*: It occupies not only the previous areas but the southern area of the region. The white sagebrush, which is frequently mixed with the esparto grass (Lygeum spartum) and the whitish plantain (*Plantago albicans*), is continuous and homogeneous for tens of kilometers and only disappears in the dahyas (small closed depressions, sometimes filled with water, during heavy rains) or dominate graminaceous vegetation, including sedge bromus divided. (Quezel and Santa, 1962)

The ethnobotanical survey has made it possible to draw up a table with more than 40 species described below with several pathologies treated from a simple mycosis to kidney stones and hepatitis. The Lamiaceae family is largely predominant with more than 8 genera followed by the family. Asteraceae Apiaceae, Fabaceae, Oleaceae.

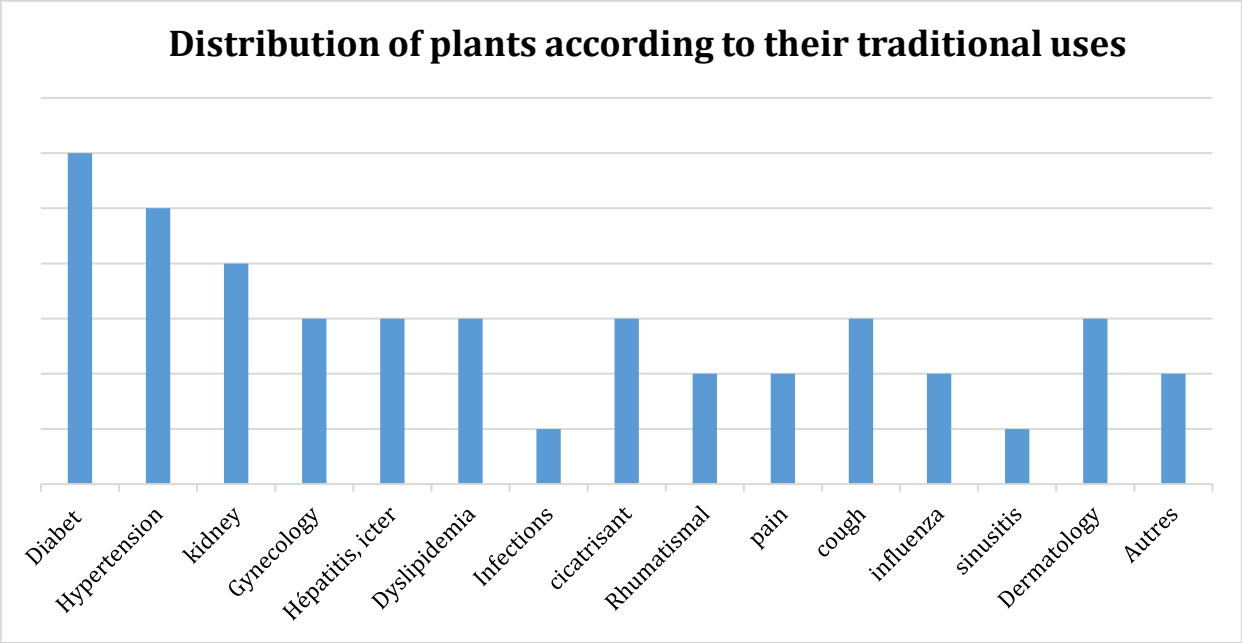

Figure 2. Distribution of plants according to their traditional uses

For the preparation method, the decoction is predominant but we also note poultices fumigations, for the traditional use diabetes is very concerned then kidney stones with 4 recommended plants, then dyslipidemia and cardiovascular diseases and finally the other

indications whose hepatitis, gastric disorders, gyneco-obstetrics, rheumatism, flu, sinusitis, dermatological disorders.

The most utilized species in the treatment of respiratory diseases (Table 1), we note the massive use of *Myrtus* for its phytotherapeutic properties particularly flu, colds, coughs, and lung diseases. It is also recognized as a plant par excellence of the diseases of winter that is to say the cooling of all kinds. We also note the use of *Slavia argentea* and its excellent qualities especially for its ability to heal wounds and burns, *Marrubium album* in the treatment of urinary infections against women and Ruta in the preparation of a meal for women suffering from amenorrhea which is accompanied by hot buffets, Daphne gnidium and Phillyrea angustifolia to prepare a tincture in cases of hair loss giving good results. These results confirm those of Belkhadar, 1997; Dif et al., 2015 and Khitri and Lardjam, 2018) ,notably in Morocco Hseini and Kahouadji and al (Tahri et al., 2012).

4. Conclusion

The region of Tessala with its abundant and varied flora is a real reserve with several species with great therapeutic potential and unveiled through this ethnobotanical survey, the species *Phillyrea angustifolia* L. is considered the preferred plant for the hair with the Daphne. It has also been noticed that Artemisia is a medicinal plant very much appreciated by women, because of its emmenagogues properties. The species Marrubium album L. is well known in the region studied for its effective action in the therapy of the urinary tract, in the end, the genus *Salvia* used for the preparation of poultice for its healing power very famous.

These species have a comparative advantage over other crops in adapting to environmental conditions, medicinal value, and resistance to adverse climatic conditions. Besides, their safeguarding is important for the protection of local identities, cultural traditions, and local know-how, for the promotion of traditional medicine by local populations of their potential. This popular knowledge, considered as an inheritance, can constitute a platform for the exchange of experiences, knowledge, and information concerning the traditional use of medicinal plants.

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Conflict of Interest

The authors declare that they have no links of interest.

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Review article
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Current Perspectives on Medicinal and Aromatic Plants

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Economically Important Sage Species from Turkey: *Salvia fruticosa* Mill. and *S. aramiensis* Rech fil.

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Abstract

Medicinal and aromatic plants have an increasing demand but this demand requires qualified material. Most of the products still obtained from nature. The mint family (Lamiaceae) contains more than 7000 species and the largest genera of the family are *Salvia*, *Scuttellaria*, *Stachys*, *Plectranthus*, *Hyptis*, *Teucrium*, *Vitex*, *Thymus* and *Nepeta*. The member of the family has a value through their secondary metabolites. Essential oils are the most important secondary metabolite in many of the species in the family. The genus *Salvia* consists of 1000 species which 107 of the taxa represented in Turkey. This review focuses on two *Salvia* species from natural flora of Turkey: *Salvia fruticosa* and *Salvia aramiensis*. *Salvia fruticosa* Mill. with wide distribution is a very important commodity with medicinal and aromatic properties. Besides that, *S. aramiensis* Rech. fil. occurs in a restricted area in Turkey but depending on low camphor and thujone content has a great potential to be part in trade. The importance of two species were estimated and future approaches were discussed. Especially the botany, distribution, domestication, spread, cultivation, previous studies on agricultural aspects, biological properties, breeding, molecular characterization of the species were discussed.

Key Words: Botany, *E*ssential oil, *Salvia*, Sage

1. Introduction

Plants have been used to cure diseases, spice in foods and bio stimulating material since ancient times. Lamiaceae family contains approximately 236 genera, and one of the most important genus is Salvia. The genus Salvia includes almost 1000 species has a great diversity and wide distributions from Far East, through Europe and across to the New World (Kintzios, 2000). Salvia genus represented by 100 species, 7 varieties in Turkey (Kusaksiz, 2019). There are many species exist in the genus that valuable in cosmetic, food, pharmaceutical industries (Carović-Stanko et al., 2016). Many of the family members contain secondary metabolites, especially essential oils that efficient as antioxidants, antimicrobial, anti-Alzheimer, anticancer, even insecticidal (Pavlidou et al., 2004; Senel et al., 2010; Exarchou et al., 2015; Sarrou et al., 2016). The amount of trade for Salvia species difficult to find. They are mostly collected from nature and sometimes being true to a species name is not possible. In 2019 ca 2400 tonnes of S. officinalis were exported from Turkey while ca 500 tonnes of S. fruticosa were exported (TUIK, 2020). S. aramiensis only collected

from the flora of Hatay and consume in the local area, there are no records for this species trade. Salvia fruticosa Mill. (Syn: S. triloba) known as “Greek sage”, “Anatolian sage” and “Dalmatian sage” is a culinary herb that used for its medicinal benefits. S. aramiensis Rech. fil., known as “Hatay sage” naturally grows in a particular region of Turkey and used as herbal tea and incense in the local area. Salvia aramiensis with low thujone and camphor levels in essential oil have a great potential to be a commodity of trade similar to S. fruticosa (Demirci et al., 2002). In developing countries traditional medicine is still utilized while these drugs only used as alternative or complementary in industrialized countries (FAO, 2005). Developed countries such as Hong Kong, the USA, and Japan are the main markets for medicinal and aromatic plants (MAPs) while developing countries are the main exporters of MAPs.

Today there is very multicultural diversity for economically important plants in the sight of conservation of genetic diversity. The knowledge of genetic diversity conservation is highly improved for industrialized plants while these information is not sufficient for MAPs. The main problem for conservating of MAPs are a leak of knowledge on population size and structure, origin, traded quantities and commodities, domestication and conservation.

In most industrialized countries, MAPs are collected from wild however with an increasing demand the collection becoming more and more problem. Many species are similar for the unconscious collectors and it is challenging to preserve these species without knowing the collection method and location (Lange, 2006). There are many organizations such as FAO, IPGRI, UPOV, ECP/GR, IUCN, WWF, EUROPAM and ISSC-MAP which are working to preserve and maintain the diversity of medicinal and aromatic plants. These organizations bring together governmental and non-governmental organizations and mostly work on in-situ conservation of endemic plants (Baydar and Telci, 2016). Because of active ingredients and new usage areas, medicinal and aromatic plants should be evaluated more carefully.

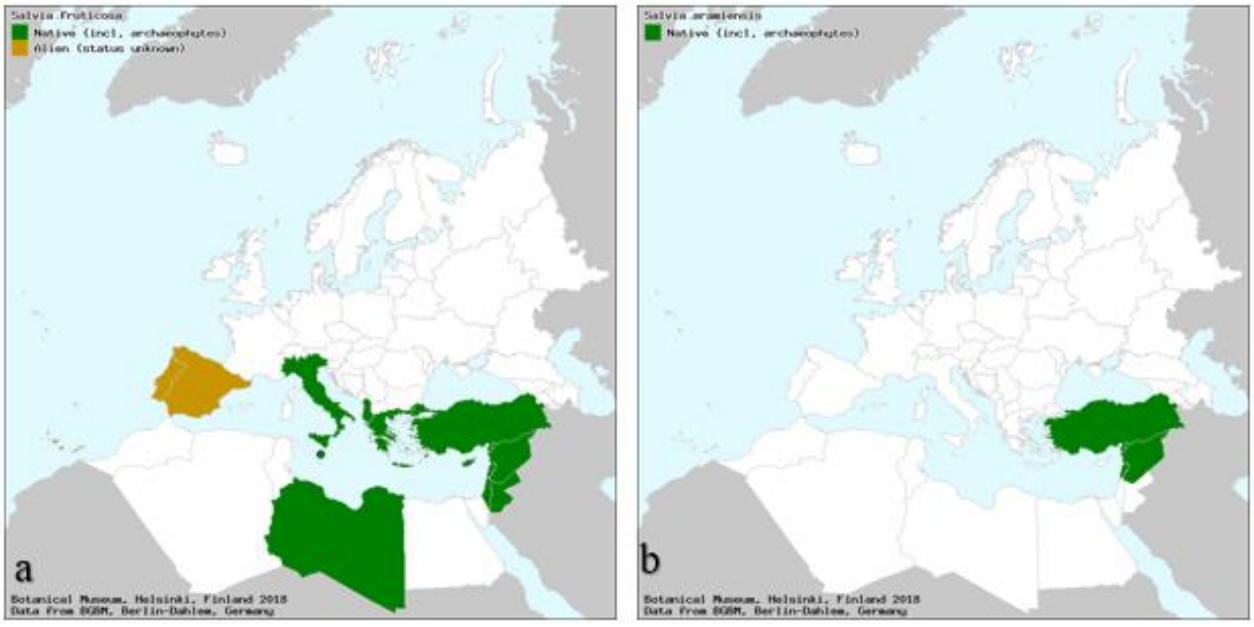

Figure 1. Distribution map of *Salvia fruticosa* (a-left) and *S. aramiensis* (b-right) (Anonymous, 2020)

2. Botany and Distribution

The genus *Salvia* with approximately 1000 species distributed in many different ecosystems (Walker et al., 2004). *Salvia fruticosa* Mill. and *Salvia aramiensis* Rech. fil. are both evergreen, perennial, semi-shrub aromatic plants. *Salvia fruticosa* native to Albania, Cyprus, East Aegean, Greece, Italy, Kriti, Lebanon-Syria, Libya, Palestine and Turkey (Hedge, 1982). *S. fruticosa* introduced into Algeria, Canary, Madeira, Morocco, Portugal and Spain (Figure 1-a). *S. aramiensis* Rech. fil. is native to Turkey, Lebanon and Syria (Davis, 1982), however, there were not any publication found from Lebanon and Syria (Figure 1-b). In Turkey *S. aramiensis* Rech. fil. is widespread only in Amanos mountains, where mostly Hatay province is located (Saroglu, 2013). Amanos Mountains, which starts from north of the Mount Cassius to southwest-northeast direction reaches to Kahramanmaraş province, limits Amik Plain and the northwest side of Asi (Orontes) River (Aytac and Semenderoglu, 2014). *S. aramiensis* Rech. fil. and *S. tomentosa* Mill. grows sympatrically in many areas however hybrid plants were not detected from any studies (Davis, 1982).

Figure 2. Leaf and flower view of *Salvia fruticosa* Mill.

Leaf and flower morphology of *S. aramiensis* Rech. fil. is very similar to *S. aucheri* var. *aucheri* Bent., however, essential oil components are very distinct (Kurkcuoglu et al., 2002). In addition, in *S. aucheri* var. *aucheri* Bent. trilobed leaf forms could be seen but in *S. aramiensis* Rech. fil. trilobed leaf could not form. Stems of *S. fruticosa* Mill. (Figure 2) are upright up to 1.6 m, *S. aramiensis* Rech. fil. (Figure 3) plants shorter up to 1 m. The feather cover in the trunk shows a lot of variation, sparse or dense below the body pubescent lanate or glandular, usually dense eglandular villous and short or long glandular-pubescent on the upper part of the trunk, sometimes glabrous in *S. fruticosa* Mill. while in *S. aramiensis* Rech. fil. dense eglandular-tomentose and stemless glandular hairs below the trunk, top at the part gently pilose or sub-glabrous. Leaves are simple in both species, in *S. fruticosa* also triple lobed could occur. *S. fruticosa* Mill. and *S. aramiensis* Rech. fil. are both entomophile and outcrossing species. *S. fruticosa* Mill. leaf

color is yellow-green and *S. aramiensis* Rech. fil. leaf color is grey-green. Gall forms named "Elma (apple)" in colloquial. Gall formation could not occur in all the *S. fruticosa* populations such as Spain and Madeiran (Rivera et al., 1994). *S. fruticosa* Mill. petals pink, lilac to violet-blue, rarely white, in *S. aramiensis* Rech. fil. mauve to pink. Habitats of the *S. fruticosa* Mill. are maquis shrub land to frigana, rocky slopes in altitudes 1-1000 m and in *S. aramiensis* Rech. fil. are red pine forest clearance and rocky slopes, altitudes 250-1000 m. Both of the species described as Mediterranean elements, to be specified *S. fruticosa* Mill. Mediterranean and *S. aramiensis* Rech. fil. East Mediterranean element. *S. fruticosa* Mill. and *S. aramiensis* placed in the same group in Flora of Turkey (Group E) (Dogan et al., 2008).

Figure 3. Leaf and flower view of *Salvia aramiensis* Rech. fil.

3. Domestication, Spread and Cultivation

S. fruticosa Mill. naturalized in Malta island, Spain, Portuguese and Croatia (Greuter et al., 1986; Radosavljevic et al., 2015). *S. fruticosa* Mill. is a culinary herb that cultivated in many different countries (Delamare et al., 2007). However, *Salvia aramiensis* Rech. fil., grows only in the Amanos Mountains and trading occurs only in close provinces (Davis, 1982; Karaman et al., 2007). In Greece dry herb of *S. fruticosa* Mill. burn in a house to cleanse it (Rivera et al., 1994). Besides, *S. aramiensis* Rech. fil. has a similar usage that dry herb of the plant burns in the house and believed to send away bad spirits. Thereat category of *S. fruticosa* Mill. is less concern (LC) at regional, national and international levels. Threat category of S. aramiensis Rech. fil. is as follows, for regional level vulnerable (VU), national-level vulnerable (VU) and international level less concern (LC) (IUCN Red List Criteria 2001 in Celep et al., 2010). The number of *Salvia fruticosa* Mill. individuals are unknown however, this species is widespread and abundant in parts of its range, but is under high collection pressure and may be declining.

Most of the *S. fruticosa* Mill. and all of the *S. aramiensis* Rech. fil. were collecting from wild populations. There are many steps until herbs become consumable, harvesters mostly uneducated people about species and collecting, intermediary buyer, exporter, international buyer and retailer that process the plants. The medicinal and aromatic plant industry needs high-quality material that also corrects in the name of the desired species. Sustainability of plant genetic

resources become more and more important. These conditions necessitated the cultivation of medicinal plants (Dudai and Yaniv, 2014). The domestication of a wild plant into similar climatic conditions is an easier otherwise acclimatizing process necessary.

Domestication is a process characterized by the occurrence of key mutations in morphological, phenological, or utility genes, which leads to the increased adaptation and use of the plant; however, this process followed by modern plant breeding practices has presumably narrowed the genetic diversity (Chaudhary, 2013). In 1980s domestication and selection of *S. fruticosa* Mill. were studied in Israel, but researchers stated that any wide-scale cultivation of this crop today does not exist (Putievsky and Ravid, 1984; Dudai and Yaniv, 2014). Domestication and characterization of *S. fruticosa* Mill. and *S. aramiensis* Rech. fil. were studied in Hatay province, 2 genotypes of *S. fruticosa* Mill. and 3 different ecotypes of *S. aramiensis* were used. In that study highest dry herb yield found for *S. fruticosa* Mill. as 1252.74 kg/da and 642.29 kg/da for *S. aramiensis* Rech. fil. In the study essential oil range was also determined, *S. fruticosa* essential oil was ranged between 1.13-3.65%, essential oil range of *S. aramiensis* was ranged between 1.13-3.06% (Ayanoglu et al., 2012). More studies should carry out both of the sage species. Cultivation for commercial purposes is rare and seeds mostly collecting from the nearest wild population for cultivation. *S. fruticosa* Mill. and *S. aramiensis* Rech. fil. utilized for their secondary metabolites. Secondary metabolites show diversity even with monomorphic and same population through age. Karasou and Kokkini (1997), point out *S. fruticosa* Mill. leaf morphology varies in the different geographical areas of Greece. The northern part of the country where the transitional climate occurs; leaves were found flat and entire, while South of Greece where real Mediterranean climate occurs; total leaf surface were decreased gradually and three-lobed, canaliculate-undulate forms appear (Karousou et. al, 2000). Murcian *S. fruticosa* Mill. populations differentiate within two main types of leaves ovate-lanceolate and ovate (Rivera et al., 1994). The higher light absorption on the leaf surface causes some morphological difference in *S. fruticosa* Mill. leaves; the number of small leaves and folding of leaf margins decreases while sparse and dense cover with hairs on the abaxial surface (Szwarcbaum, 1982).

4. Genetic Resources: Essential oil yield and content, biological properties

Considering both *S. fruticosa* Mill. and *S. aramiensis* Rech. fil. almost all the sources are wild. There are very limited number of varieties that developed by hybridization or selection of *S. fruticosa* Mill. In Southern Cyprus, *Salvia fruticosa* Mill. were recorded as a highly used shrub and some research was done to determine effective agricultural practices for cultivation in Agricultural Research Institute (Droushiotis and Della, 2002). *Salvia fruticosa* has been under protection in Israel National Parks and Reserve areas that collecting of plants are not permitted since 1956 (Putievsky, 2002). Crete is one of the most important genetic resources for *S. fruticosa* Mill. Evaluation of 37 different *S. fruticosa* populations from Crete resulted in geographic diversity occurs in the populations (Karousou and Kokkini, 1997). In the west of Crete *S. fruticosa*, branches are taller, leaves are big, dark green and the verticillasters of leaves are distinct, leaf blade flat and cornered. Through eastwards researchers found out that leaf forms were changed and in the east of island species with many small branches, dense leaf verticillasters, leaves are light green and small, leaf blade trilobed. Essential oils were also changed the highest essential oil content found in the east of the island with 3-4%. Essential oil content and composition also vary in the wild resources. The essential oil

content of 20 different *S. fruticosa* from native populations of Crete shows high diversity from 1.1 to 5.1%. In the west of the Crete 1.8 cineole content lower than in the east of the island (Karousou et al. 1998). The aromatic diversity of Turkey resulted that native *S. fruticosa* plants belong to the group CiCa which 1.8 cineole is dominant followed by camphor (Baser, 2002). *S. aramiensis* essential oil content found from different studies between 1-3% (Demirci et al., 2002; Karaman et al., 2007; Askun et al., 2010).

Many of the endemic and wild seeds of medicinal and aromatic plants have a germination problem (Abdollahi, 2012). *S. fruticosa* Mill. also have partial germination problem but with the addition of some priming applications germination in higher ranges are possible (Sonmez, 2019). However, the germination problem of *S. aramiensis* Rech. fil. seeds are still unsolved (Bahadirli and Ayanoglu, 2019). The other gaps about the genetic resources is that diseases that threatened the field collections especially *Fusarium oxysporum* and powdery mildew. In the study *Salvia fruticosa* plants were selected from natural flora of Turkey due to the high drog herb yield and essential oil content, however, during field experiments selected plants were died because of the *Fusarium oxysporum* infection (Bayram, 1999). Furthermore, *S. fruticosa Mill.* Found so susceptible to powdery mildew that causes early defoliation and senescence (Soylu et al., 2019). More detailed studies should be done to protect genetic resources and selected material from pest and diseases. There are many studies maintained in both of the species. Determining wild populations agronomic traits and essential oil (Karoussou and Kokkini, 1997; Karoussou et al., 1998; Bayram et al., 1999; Bayram, 2001; Demirci et al., 2002; Karaman et al., 2007; Mossi et al., 2011; Schmiderer et al., 2013; Cvetkovikj et al., 2015; Karik, 2015; Uysal, 2015; Sarrou et al., 2016; Kelen and Tepe, 2017; Zgheib et al., 2019); cultivated populations agronomic and essential oil features (Karik and Saglam, 2017; Ayanoglu et al., 2017); antifungal, antioxidant, antimicrobial and antimycobacterial (Sivropoulou et al., 1997; Sokovic et al., 2002; Askun et al., 2010; Giweli et al., 2013; Topcu et al., 2013; Sarrou et al., 2016; Ertas et al., 2017; Karik and Saglam, 2018); anti-alzheimer (Alim et al., 2018); optimal harvesting time from wild populations (Gul et al., 2002); hybridization in wild population (Radosavljevic et al., 2019); artificial hybridization (Putievsky et al., 1990; Bahadirli and Ayanoglu, 2019); genetic diversity with molecular markers (Skoula et al. 1999; Bahadirli et al., 2017). Universal molecular and morphologic diversity of *S. fruticosa*, pests and diseases, resistance to abiotic stress factors, different aspects of medicinal properties of both species exceedingly essential and requires more study.

Biological properties of *Salvia fruticosa* Mill. have been studied a lot compared to the *S. aramiensis*. An appropriate reason for this is the widespread distribution of *S. fruticosa* Mill. The main focus on both of the species was essential oil yield and composition (Schmiderer et al., 2013; Sarrou et al., 2016). On the other hand, ISO 9909:1997 report limits only the chemical composition of *S. officinalis* L. essential oil. In this report essential oil composition of *S. officinalis* L. should contain α-thujone (18.0-43.0%), camphor (4.5-24.5%), 1.8 cineole (5.5-13.0%), β-thujone (3.0-8.5%), α-humulene (≤12.0%), α-pinene (1.0-6.5%), camphene (1.5-7.0%), limonene (0.5-3.0%), bornyl acetate (≤2.5%), linalool and bornyl acetate (≤1.0%). The main concern of *S. officinalis* L. was about thujone content of the essential oil, however according to EMA (European Medicines Agency, 2016) exposure of 3 and 7 mg/day should not take special concerns. There is a wide variation on *S. fruticosa* essential oil composition. The major compounds mostly found as 1.8 cineoele and

camphor, however their rates are variable (Gul et al., 2002; . Cvetkovikj et al., 2015; Kavoura et al., 2019; Zgheib et al., 2019). *S. fruticosa* Mill. is an important commodity as *S. officinalis* L. within this view *S. fruticosa* essential oil also needs ISO standard limits.

S. aramiensis essential oil have been studied in a few researches. In the study where an antioxidant and antimicrobial profile of *S. aramiensis* compared with *S. aucheri* var. *aucheri* and *S. pilifera*, antioxidant and antimicrobial activity of *S. aramiensis* found stronger than the other studied species (Kelen and Tepe, 2017). In the same study, major compounds of *S. aramiensis* essential oil were found as follows 1.8 cineole (46.0%), β-pinene (10.3%) and camphor (8.7%). The anti-Alzheimer activity of *S. aramiensis* Rech fil. root extracts were studied and the highest inhibitory activity was obtained with dichloromethane extract followed by methanol extract (Alim et al., 2018). *S. fruticosa* Mil. and *S. aramiensis* Rech fil. extracts did not show antimycobacterial activity (Askun et al., 2010). Studies reveal that the essential oil content of *S. aramiensis* Rech. fil. is lower compared to the *S. fruticosa* Mill. (Demirci et al., 2002; Karaman et al., 2007). The essential oil composition of these species shows similarity yet thujone and camphor range is much lower in *S. aramiensis* Rech. fil. (Bahadirli and Ayanoglu, 2019). Karaman et al. (2007) found that major compounds of *S. aramiensis* were 1,8-sineol (%60.0), β-pinene (%9.0), myrcene (%3.70), α-pinene (%3.40) and germacrene-D (%2.90). There are many mechanisms that affect secondary metabolites of *Salvia fruticosa* Mill. altitude is one of them. The effect of different altitudes (0-200 m; 300-500 m; 600-800 m) on *S. fruticosa* Mill. essential oil yields and components were evaluated. Essential oil yield found highest in 300-500 m as 5.1% while 1.8 cineole found highest in the same altitude. Different altitudes (Kaplan and Kocabas Oguz, 2013).

5. Molecular characterization

Molecular methods have been used widely for discrimination of medicinal and aromatic plants. International studies that have samples from different countries put more general perspective while national studies observed only in limited areas. Molecular markers such as RAPD, AFLP, SSR, SNP are the main methods used for identifying wild or cultivated resources. However, most of the molecular studies associate morphological characters or locations, not secondary metabolites. The main reason for that is the variability of secondary metabolites. On the other hand, it is essential to combine both methods to describe genetic resources clearly. The advantages of molecular characterization that markers are very sensitive to any genetic differentiation and also variation could detect in the early phase of the plant growth. The disadvantages of molecular characterization are the expenses and technological requirements are not developed enough in many underdeveloped countries. Considering both sides molecular characterization is open to progress and if utilizing with breeding programs could be more possible, more and accelerate progress could obtain. Some studies revealed genetic diversity of *S. fruticosa* in the natural populations (Karaca et al., 2008; Radosavljević et al., 2011; Bahadirli, 2014; Radosavljević et al., 2015). In the study from Radosavljevic et al. (2015) hybridization between *S. fruticosa* and *S. officinalis* was detected. Skoula et al. (1999), studied genetic and essential oil profile of *S. fruticosa* in three different populations in Crete. Molecular markers (RAPD) discriminate populations in to three different group but two populations find closer, similar results were obtain also when the essential oil profile was analyzed. These results showed the importance of genetic background. Secondary metabolism pathways particularly in essential oil biosynthesis are necessary for to maintain biotechnological production in a large scale.

Chatzopoulou et al. (2010) analysed selected genes that involved in the secondary metabolite synthesis in tricomes of *S. fruticosa* with cDNA library. The results of the study indicate a series of novel genes associated with secondary metabolism.

6. Varieties and Breeding

In Turkey only one variety of *Salvia fruticosa* 'Karik'were patented. The other record was the hybrid variety 'Newe Ya'ar 4' from Israel. Alternative strategies to enhance the dissemination of varieties among researchers and farmers are crucial for the improvement and conservation of genetic resources.

Salvia fruticosa and *Salvia aramiensis* species both have been utilized as herbal tea, fragrance in pharmaceutical industry and food additives, furthermore, their essential oil constituents show antioxidant, anticancer, anti-alzheimer, antimicrobial activity. Major constraints are their essential oil and biological activity. In many of biological activity research the origin of plant is unknown, in addition, agricultural studies and conservation is disregarded. Secondary metabolites of the species are unstable, correlation between their active ingredient and biological properties are crucial.

High herb yield with active ingredient, resistant to biotic and abiotic stress factors and absence of unwanted compounds with highly desired compounds mostly are the main purposes for breeding. Survey of the natural population, characterization of the resource, selection (individual or mass), propagation of the material with vegetative or generative parts, selection, cultivation and selection for desired traits until obtaining desired traits. First artificial hybridization was done between *S. fruticosa* and *S. officinalis* by Putievsky et al. (1990) to obtain the highest adaptability to flora with high yield. The hybridization range was between *S. officinalis* × *S. fruticosa* 36% and *S. fruticosa* × *S. officinalis* 34%. Hybrids essential oils were found in the middle of parent species. Thujone content of hybrids was similar to *S. officinalis*, however, 1.8 cineole and camphor ranges were between middle of the parent species (Putievsky et al., 1990). One variety named Neve Ya'ar No:4 was developed from this hybridization study (Dudai et al., 1999). Another hybridization study was done between *S. aramiensis* and *S. fruticosa*, hybridization range between *S. aramiensis* × *S. fruticosa* found as 25.68% and *S. fruticosa* × *S. aramiensis* as 44.29%. Essential oil range between *S. aramiensis* × *S. fruticosa* 0.75-3.97%, *S. fruticosa* × *S. aramiensis* 1.04-3.84%. In the same study hybrid plants with less than 1% thujone content with higher than 60% were observed between both species (Bahadirli and Ayanoglu, 2019). Spontaneous hybrids between cultivated *S. fruticosa* and *S. officinalis* were also studied considering essential oil content and components. In the study, essential oil contents and components of the hybrids were found in the middle of the parent plants close to the *S. officinalis* parent (Karik and Saglam, 2018). Natural hybrids of *S. fruticosa* and *S. tomentosa* were propagated with cuttings and agronomic features were evaluated. In the study, essential oil contents of hybrid plants were found mostly in the middle of the parent plants however some hybrid plants essential oils were found higher than the parent plants (Evropi-Sofia, 2013). Natural hybrid plants between *S. fruticosa* and *S. officinalis* species in Croatia and Spain, in both of these study only genetic characterization, were studied (Radosavljevic et al., 2019; Rivera et al., 2019).

From the study in Hatay region artificial hybridization between *S. fruticosa* and *S. aramiensis* were done with cultivated clones from natural populations. The main purpose of that study was to obtain new plants that contain high essential oil and 1.8 cineole with

low thujone and camphor contents (Bahadirli and Ayanoglu, 2019). In other research from Israel, the study was done due to the demands of market. New variety were obtained between non-native *S. officinalis* and native *S. fruticosa* by hybridization and selection of superior genotypes. In the study the aim was, a plant with resembling *S. officinalis* in morphology and flavor additionally adaptive to intensive Israeli agricultural conditions (Dudai et al., 1999).

7. Conclusion

opens for essential oils in medicinal studies however reachable sources for public usage crucial. Biotechnological methods have very significant role in conservation, characterization and breeding of genetic resources, so their usage in the studies should increase. Furthermore functional genomic researches should be initiated to determine biotic and abiotic problems.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Conservation and characterization of *S. aramiensis* genetic resources are necessary in the first place. Increasing demand on *S. fruticosa* requires new varieties that adaptive to agrological conditions, with stable yield and diseases resistant. Therefore, it is very important to share and exchange present genetic resources. *S. aramiensis* merit more national and international recognition. Cultivation has become a key factor in sustainable flora, time and cost saving through transfer. New area of utilization

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A Study on Antioxidant and Antimicrobial Activity of *Ferulago galbanifera* Species

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Abstract

In this work, antimicrobial effects of the root, stem, leaf and flower parts extracts with ethanol and acetone of *Ferulago galbanifera* species against *Escherichia coli*, *Staphylococcus epidermidis, Enterococcus faecalis Candida albicans, Bacillus subtilis, Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella typhimurium* strains were investigated by using the disc diffusion method. In addition to that, antioxidant activity of *F. galbanifera* ethanolic extracts was measured using the DPPH method, as well as their total phenolic content using the Folin-Ciocalteu's phenol reagent technique. To our results, ethanolic extracts of leaf from *F. galbanifera* were found to have antimicrobial effect against the all microorganisms, whereas the acetonic extracts of leaf has shown antimicrobial effects to some microorganisms other than *Staphylococcus epidermidis, E. faecalis, C. albicans* species. Extracts obtained from the root and the flower parts of the plant had no antimicrobial effect on the test microorganisms. The antioxidant activity level was found to be in the following order (from the highest to the lowest): flower, leaf, stem and rood; 16.32, 215, 244, 323(µg/mL), respectively. The highest total phenolic content obtained from the parts of the plant *F. galbanifere* was for the root, while the lowest was for its leaf part.

Key Words: *Ferulago galbanifera*, Antibacterial, Antioxidant

1. Introduction

Genus of *Ferulago* W. Koch (Apiaceae) is represented by 34 species, amongst them 19 are endemic in Turkey (Saya et. al. 2012). Since the roots and fruits of the *Ferulago* species contain secretion agent out of essential oil, resin and gum, they have a distinct odour. Their usage and contents are similar to those of *Ferula* species known publicly as the same name and they (*Ferulago* species) are being used as aphrodishiac, tonic and digestive agents as well as to heal hemoroids and ascarids in folk medicine (Akalın 1999; Başer et. al. 2002). *Ferulago* is also used as a spice especially in salads for its specail smell. *Ferulago* species are named as "çaksırotu", "kisnis", "asaotu", "kuzu bası" and "kuzu kemirdi" in various regions of Turkey. The essential oils of secondary metabolits are encountered in the plants growing in the hot climatic regions of the world. Although these so called 'essence' and 'etheric oils' named owing to their smells are present in 100 families' species comprising the one third of the world flora, they are especially seen in some families of Coniferaea, Rutaceae, Apiaceae, Myrtaceae and Labiatae, mainly in their specific tissues

(Graikou et. al. 2012;Mammadov 2014; Jara – Bermeo et. al. 2016).

Essential oils (EOs) are known to exhibit antibiotic and anticeptic effects. With this special characteristics, they are called as antimicrobial agents since they are the alternatives to the food originated pathogens, chemical preservatives and antibiotics and they can destroy bacteria, fungia and yeast (Kürekçi and Sakin 2017).

Antioxidant compounds have the effect of delaying or inhibiting the oxidation of lipids and other parameters. The association of the myriad of compounds present in essential oil provides higher antioxidant activity than the summed activity of the individual components. Essential oils may also be used as food preserving agents owing to the presence of phenolic compounds as main components, which are liable to the antioxidant properties and may be an alternative to synthetic antioxidants (Marin et. al. 2016).

2. Material and Methods

2.1. Materials

For the current study, all parts (root, stem, leaf and flower) of *Ferulago galbanifera* (Mill.) W. Koch plant were collected. The collection area was Alpagut neighbourhood, Mihalgazi town (Eskişehir). After collection the samples were cleaned and dried for 7 days and Some of them were made into herbarium material (M. Sağıroğlu 6568 SAÜ Biology Herbarium).

All the chemicals and reagents (Folin-Ciocalteu, Gallic Acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Methanol, Mueller Hinton Agar (MH agar), Triptic Soy Broth, Sodium Carbonate, Ascorbic Acid) used in this study were of analytical grade and obtained from Merck Company, Germany.

2.2. Preparation of Extracts

Fifteen grams of dried parts (roots, stem,leaf and flower) of the plants were ground into a capped bottle and 150 mL of ethanol and acetone was added on top of those. These prepared extracts are kept in a cool and dark environment for 3 days and mixed in a magnetic stirrer at regular intervals. The solvents in the extracts were evaporated by using a rotary evaporator (Heidolph) under vacuum at 55°C for 15 minutes and the dried extracts were then used for all investigations. The extract concentrations were adjusted by adding own solvent (ethanol or acetone) to each extract at the doses of 6400 µg/disc for the antimicrobial activity tests. Ethanolic exracts were setting 1000µg/ml for the antioxidant activity and the total phenolics analyses. For antimicrobial activity, 15 µL of empty sterile discs with a radius of 6 mm from the raw extracts obtained were absorbed and kept in a dark sterile environment for 24 hours.

2.3. Disc diffusion method

All strains used throughout this study have been obtained from Microbiology Research Laboratory of Sakarya University. The disc diffusion method was used to determine the antimicrobial activities of the extracts. Suspensions with a density of 0.5 McFarland from previously activated microorganisms were prepared by a densitometer. Prepared microorganism suspensions to Müeller Hinton Agar were inoculated with sterile swab. Discs impregnated with extracts were slightly pressed on the inoculated plates under aseptic conditions, followed by an incubation at 37˚C for 24 h. Ethanol and acetone impregnated discs were used as negative controls and the commercial antibiotic discs (Gentamicin and Amphotericin B) were used as positive ones. If there is an inhibition zone against that pathogen around the disc as a result of the incubation, the zone diameters (mm) are

measured from the rear of the petri by using a digital caliper.

2.4. Antioxidant activity (DPPH assay)

The modified Blois method was used for determination antioxidant activity (Blois, 1958). In short, 1 ml of 0.004% solution of DPPH radical in methanol was mixed with 1 mL of extract solution in methanol (containing different concentrations of dried extract). These solutions were kept in dark place for 30 mins and the optical density was then measured at 517 nm using a spectrophotometer and methanol was used for the blank. The following equation was employed to evaluate the % DPPH radical scavenging activity: %DPPH radical scavenging = [(control absorbance- extract absorbance)/control absorbance] x 100.

2.5. Total phenolic content (TPC)

Total phenolic substance determination was determined using modified Folin-Ciocalteu method of Singleton and Rossi (1965). Taking 100 µL of the prepared extract, 200 µL of 50% Folin-Ciocalteu reagent was added and left for 2 minutes. We have then added 1 mL of 2% Na_2CO_3 solution on it and waited for 1 hour in the dark and the absorbance at 760 nm was read. The total phenolic content was determined in mg / 100g using Gallic Acid Standard.

3. Results and Discussion

The polyphenolic constituents that are present in the plant extracts produce various biologic activities together with antioxidant abilities. Nowadays, several studies are focused upon the potential healt benefits of polyphenols and their pharmacological potential as antidiabetic (Asgar, 2013), anticancerogenic (Rosa et al., 2016), antimicrobial and antioxidant (Semerci et al., 2020) agents (Unuofin et al., 2017). In the current study, the extracts obtained from the root, stem, leaf and flower parts of *F. galbanifera* has been worked out for their phenolic contents and the results were given in Table 1. The highest total phenolic content has been observed at the root part, whereas the lowest was at the leaf of the plant. The amount of the content were as follows: root 611.6, flower 311.6, stem 176.6 and leaf 43.4 mgGA/100g.

In vitro antioxidant tests are designed to imitate the oxidation-reduction reaction that are prevalently present in live biological systems and to evaluate the antioxidant potential of various chemical and biological substances (Ebrahimabadi et al., 2010). In this study a common DPPH test to measure the antioxidant activity has been employed. IC_{50} values of the extract obtained and the standard (value needed for scavenging the DPPH 50%) are given in Table1. It has been determined that the highest antioxidant activity level is for flower ethanolic extracts (16.3 µg/mL). When compared with the ascorbic acid standard, it has been found that the flower extract exhibits higher level of antioxidant activity. To our best of knowledge, there is no any study indicating that the plant *F. galbanifera* has antioxidant activity.

Table 1. Antioxidant activity and total phenolic content of the samples

Samples	Antioxidant Activity IC_{50} (µg/mL)±SD	Total Phenolic Content (mgGA/100g)±SD
Flower	16.32±0.28	311.6±2.5
Stem	244±0.8	176.7±8.7
Root	323.6±2.4	611.6±7.75
Leaf	215±1.4	43.4±1.8
Ascorbic Acid	3.2±00.1	_

Table 2. Antimicrobial activity of different parts of *Ferulago galbanifera*

Samples 6400µg/disc		Test microorganism (inhibision zone diameters, mm±SD)							
		Ec	**Se**	**Ef**	**Bs**	**Pa**	**Sa**	**St**	**Ca**
Leaf	Acetone	10.5±0.5	0	0	8.5±0.5	8±0	12.5±05	9.5±0.5	0
	Ethanol	13±0	10.5±0.5	9±0	9±0	8±0	16±0	13±0	8,5±0.5
Stem	Acetone	0	0	0	0	0	8.5±0.5	0	0
	Ethanol	0	0	0	0	0	8	0	0
Flower	Acetone	0	0	0	0	0	0	0	0
	Ethanol	0	0	0	0	0	0	0	0
Root	Acetone	0	0	0	0	0	0	0	0
	Ethanol	0	0	0	0	0	0	0	0
Antibiotic	GC	17	20	19	22	20	21	21	0
	Amp	0	0	0	0	0	0	0	16

Ec: *Escherichia coli,* ***Se:*** *Staphylococcus epidermidis,* ***Ef:*** *Enterococcus faecalis,* ***Ca:*** *Candida albicans,* ***Bs:*** *Bacillus subtilis,* ***Pa:*** *Pseudomonas aeruginosa,* ***Sa:*** *Staphylococcus aureus,* ***St:*** *Salmonella typhimurium,* ***Gc:*** *Gentamicin,* ***Amp:*** *Amphotericin B.*

Mileski et al. (2015) studied the antimicrobial adn antioxidant activities of endemic species of *F. macedonica*. They have concluded that the IC_{50} value of the extract obtained with ethanol was 1100 µg/mL. Golfakhrabadi et. al. (2016) worked out the antioxidant/antimicrobial activity of *Ferulago carduchorum* plant and found that the extract obtained for the flower part with methanol has IC_{50} value of 1 mg/mL, whereas it was 9.4 mg/mL when compared with hexan. It has been deduced that the solvent used in the extract preparation process does affect the antioxidant activity level.

In the current study it has been determined that the prepared leaf ethanolic extract has the IC_{50} value of 215 µg/mL. The difference between the works in the literature and the current one is thought to be originated from the extract preparation techniques and the plant collection areas, as well as the differences in species worked on.

The antioxidative and antimicrobial properties of essential oils obtained from several plants have taken great interest in academic studies and in food, cosmetics and pharmaceutics industries since they are thought to be the natural dopant substance candidates to substitute the synthetic antimicrobial agents. In that work, the antimicrobial activity effect of the extracts obtained from *F. galbanifera* plant *(*with acetone and ethanol) on *Escherichia coli* (ATCC 25922), *Staphylococcus epidermidis* (ATCC 12228), *Enterococcus faecalis* (ATCC 29212), *Candida albicans* (ATCC 10291), *Bacillus subtilis* (ATCC 6633), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 29213), *Salmonella typhimurium* (ATCC 14028) strains has been

summarized in Table 2. The highest antimicrobial activity has been detected for the leaf extract (with ethanol) on the bacteria *S. aureus*, producing 16 mm inhibition diameter. Ethanolic extract is found to have higher antimicrobial activity level than acetonic extract one.

Demirci et al. (2000) studied the antimicrobial effects of the essential oils of various Ferulago species and found that the highest antibacterial effect of *F. galbanifere* essential oil was detected to be on *E. coli*, followed by *S. aureus*, *S. typhimurium* bacteria. In another work, the chemical constituents and antimicrobial activities of essential oils obtained from the root, stem, leaf and flower parts of *F. trifida* were investigated and concluded that the flower part has produced 18 mm inhibition zone on *S. aureus,* whereas it was measured to be 34, 25 and 20 mm for the stem, the leaf and the root part, respectively (Tavakoli et al., 2017). In the current work, the highest antibacterial activity has been detected to be in *S. aureus* bacteria, though small differences between the parts of the plant. These antimicrobial activity differences in the various parts of the plants is thought to be originated from the fact that the chemical constituents differs in different part of the plants. Shivering the different parts of the plants in the preparation process of the extracts may create distinctive effects. In that work and the literature support this fact (Shaid Ud-Daula et al., 2016; Asraf et al., 2018).

4. Conclusion

The flower part of *Ferulago galbanifera* extracts show strong antioxidant activity due to its distinct content. From the current work together with the similar studies in the literature we conclude that different parts, i.e., flower, stem and leaf, of *F. galbanifera* are suitable to be used as a natural antioxidant and some (leaf part) as antibacterial source. The extract prepared from the leaf was determined to have a significant antibacterial effect on *S. aureus*. In this context, *F. galbanifera* leaves can contribute to natural hand and surface disinfectants and can be used as a food preservative

Conflict of Interest

The authors declare that they have no conflict of interest.

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Characterization and Optimization of Phytosome Formulation Containing Alcohol-free Umckalin from *Pelargonium sidoides*

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Abstract

This research study was designed to investigate physicochemical properties of types of phytosome containing alcohol-free umckalin from *Pelargonium sidoides*. Fourteen different phytosomal formulations were prepared by film method using *P. sidoides* in various types of phosphatidylcholine; Phospholipon 85 G (P 85 G) and phospholipon 100H (PL 100H). Physicochemical properties were measured by particle size and size distribution, zeta potential and polydispersity index of optimum 4-phytosomal formulation. Polarized light microscopy (PLM) techniques were employed for obtaining size distribution and surface appearance and lamellarity. The determination of vesicle types of phytosomes was performed by using PLM. The most suitable alcohol-free phytosome combination was chosen as PL 100H: DCP: CHOL (10: 1: 4) containing 1 % P.S (UP 4) because of its particle size and distribution (<1000 nm) and zeta potential (<-30 mV).

Key Words: Liposome, Phytosome, Umckalin, *Pelargonium sidoides*, Alcohol-free extract.

1. Introduction

Plants have been traditionally used against variety diseases since ancient times (Aslan, 2007). *Pelargonium sidoides* has an ethnobotanical and traditional plant in the treatment of some of the specific problems such as acute bronchitis, tonsillitis, pharyngitis, sinusitis and symptoms of the common flu diseases since from 20th century (Brendler and Van Wky, 2008; Lizogub et al., 2007).

The underground parts of *Pelargonium sidoides* are very well-known to include a rich of quite oxygenated coumarins and some other phenolic and polyphenolic substituents (Sharma and Yadav 2017). *Pelargonium sidoides* showed *in vitro* bactericidal, virucidal, and immune system regulating characteristics in many scientific researches (Kolodziej, 2011; Kolodziej and Kiderlen, 2007).

Umckalin is one of the important compound of the *Pelargonium sidoides (P.S.)* collected from its root via alcoholic extraction method. It has been widely used against common cold diseases in children (Lalli et al., 2008).

However, these extracts may cause problem for children and addictive patient especially in syrup formulations because of alcohol comtent (Agnich et al., 2013).

Phytosomes are submicron spheres composed of membrane similar phospholipid layers surrounding aqueous compartment including phytoactive molecules (Kidd and Head, 2005). The lipid structure is accomplished basically of phospholipid components. Phospholipids are amphiphilic; they have a hydrophilic head and a lipophilic tail. In aqueous solutions, they are arranged in bilayers, which form closed vesicles like artificial cells (Duman et al., 2014).

P.S. extract containing umckalin includes approximately 11 % ethanol in traditional and medicinal products (Wopker et al., 2020). However, ethanol containing products are reported as hazardous for infants and children (Batista et al., 2020). The extraction with the solvent mixture containing ethanol free was defined, thus obtaining a liquid preparation that provides a convenient use for children (Kohnen, 2007).

Here we present the detailed evaluation of the physicochemical properties of phytosome containing alcohol free *Pelargonium sidoides* in particle size distribution, zeta potential, polydispersity index and polarized light image parameters. The purpose of this research is to provide an optimum formulation of liposome combinations. This paper will focus mainly on the primer and seconder stability characteristics of phytosome formulations.

2. Material and Methods

2.1. Materials

Phospholipon 100 H (P100H) and Phospholipon 85 G (P85G) were provided from LIPOID GmbH (Germany). Stearylamine (SA), Dicetyl phosphate (DCP), Cholesterol (CHOL) were purchased from Sigma (UK). Pelargonium sidoides containing umckalin product (Umka®) was provided from Abdi İbrahim Pharmaceuticals. All other chemicals were of analytical grade. Polarized Light Microscope (PLM) was used in order to determine the vesicle types (NIKON).

Table 1. Phytosome formulations and their compositions.

Code	Composition	pH	Molar Ratio	Observation	Result
UPF-1	PL85G:CHOL + 1% P.S.	5.5	3:2	Phase separation	Eliminated
UPF-2	PL85G:CHOL + 1% P.S.	5.5	7:2	Phase separation	Eliminated
UPF-3	PL85G:CHOL + 1% P.S.	5.5	10:4	Phase separation	Eliminated
UPF-4	PL100H:CHOL+ 1% P.S.	5.5	3:2	Phase separation	Eliminated
UPF-5	PL100H:CHOL+ 1% P.S.	5.5	7:2	Phase separation	Eliminated
UPF-6	PL100H:CHOL+ 1% P.S.	5.5	10:4	Phase separation	Eliminated
UPF-7	PL85G:SA:CHOL+ 1% P.S.	5.5	3:1:2	Heterogenous	Eliminated
UPF-8	PL100H:SA:CHOL+ 1% P.S.	5.5	3:1:2	Heterogenous	Eliminated
UPF-9	PL85G:DCP:CHOL+ 1% P.S.	5.5	3:1:2	Heterogenous	Eliminated
UPF-10	PL100H:DCP:CHOL+ 1% P.S.	5.5	3:1:2	Heterogenous	Eliminated
UP-1	**PL85G:SA:CHOL**+ 1% P.S.	**5.5**	**7:1:2**	**Milky**	**Selected**
UP-2	**PL100H:SA:CHOL**+ 1% P.S.	**5.5**	**7:1:2**	**Milky**	**Selected**
UP-3	**PL85G:DCP:CHOL**+ 1% P.S.	**5.5**	**10:1:4**	**Milky**	**Selected**
UP-4	**PL100H:DCP:CHOL**+1%P.S.	**5.5**	**10:1:4**	**Milky**	**Selected**

2.2.Phytosome Formulations and Preparation Technique

Phytosome dispersions were prepared by film technique (Gunal et al., 2019). Briefly, phytosome was prepared by dissolving the 40 μmol mL^{-1} of phospholipids in 30 mL chloroform in a round-bottom flask. Organic solvent was removed using a rotary evaporator (Heidolph, Germany) under reduced pressure to form a thin film over the wall of the flask. In addition, ethanol was accurately evaporated using rotary evaporator from plant extract. The dried film was then hydrated over a water bath with buffer alcohol free P.S. containing umckalin. Fourteen different formulations and details are represented in Table 1.

2.3.Characterization of Phytosome Formulations

Selected four phytosome formulations were characterized by mean particle size and size distribution, zeta potential and polydispersity index. 0.1 ml of liposomes dispersions were diluted with 0.9 ml of 10 mM phosphate (pH 5.5) buffer after phytosome preparation. The size distributions of the phytosome were measured by dynamic light scattering (DLS) using Particle Sizer (Malvern, UK). The mean particle size and size distribution and polydispersity index results were obtained as the average of 6 experiments. Zeta (ζ) potential was measured by using a Zetasizer and each result was the mean of 10 measurements. All the measurements were performed at 25°C and at an angle of 90°.

The determination of vesicle types of phytosome was performed by using Polarized light microscope (PLM). Magnification of the microscope was 100 X the each phytosome dispersion. PLM techniques were employed for obtaining size distribution and surface appearance and lamellarity for 4 optimum formulation.

3. Results and Discussion

3.1. Characterization Results

Particle size is an essential factor of bioavailability for drug delivery systems such as phytosomes. According to particle size distribution results, all formulations were found below 1000 nm average diameter without UP3 as 676 nm ± 5.73, 908 nm ± 24.40, 1146 nm ± 12.30, 674nm ± 30.48, respectively.

The zeta potential is a significant property of the cumulative charge of a particle, and differences in size reflect sedimentation or fusion. The most stable phytosomes are around 30 mV. In this research paper, zeta potential results were found as 23.1 mV ± 1.04, 42.9 mV ± 1.12, -49.7 ± 1.11, -43.7 ± 1.03, respectively. The maximum stability and the minimum aggregation occur at that zeta potential value (30-50 mV) so that expected from DLVO theory. When the zeta potential goes below the critical value (<30 mV), the attractive forces supersede the repulsive forces and flocculation occurs. These loosely packed particles or flocs settle faster than the deflocculated particles because of their larger sizes (Awad et al., 2005; Kumar et al., 2007).

Table 2. Results of particle size, zeta potential and polydispersity index of phytosomes (n=3).

Code	Composition	Molar Ratio	Average diameter (nm)	PDI	Zeta potential (mV)
UP-1	PL85G:SA:CHOL+ 1% P.S.	7:1:2	676±5.73	0.461±0.02	+23.1±1.04
UP-2	PL100H:SA:CHOL+ 1% P.S.	7:1:2	908±24.40	0.670±0.03	+42.9±1.12
UP-3	PL85G:DCP:CHOL+ 1% P.S.	10:1:4	1146±12.30	0.568±0.02	-49.7±1.11
UP-4	PL100H:DCP:CHOL+1%P.S.	10:1:4	674±30.48	0.323±0.01	-43.7±1.03

When the results are evaluated from PDI (poly dispersity index) viewpoint, it was determined PDI as 0.461±0.02, 0.670±0.03, 0.568±0.02, 0.323±0.01, respectively. In addition, it was reported that uniform and stable phytosome dispersions have<0.5 PDI value (Colas et al., 2007). In this research, PDI value was found as 0.323±0.01 nm optimum for UP4. All data were given Table 2.

3.2. Microscopic Observation Results

Microscopic observations of the liposomes were imaged with Polarized Light Microscope. According to PLM results, multilamellar vesicle type liposomes were mainly observed in all formulations in Figure 1.

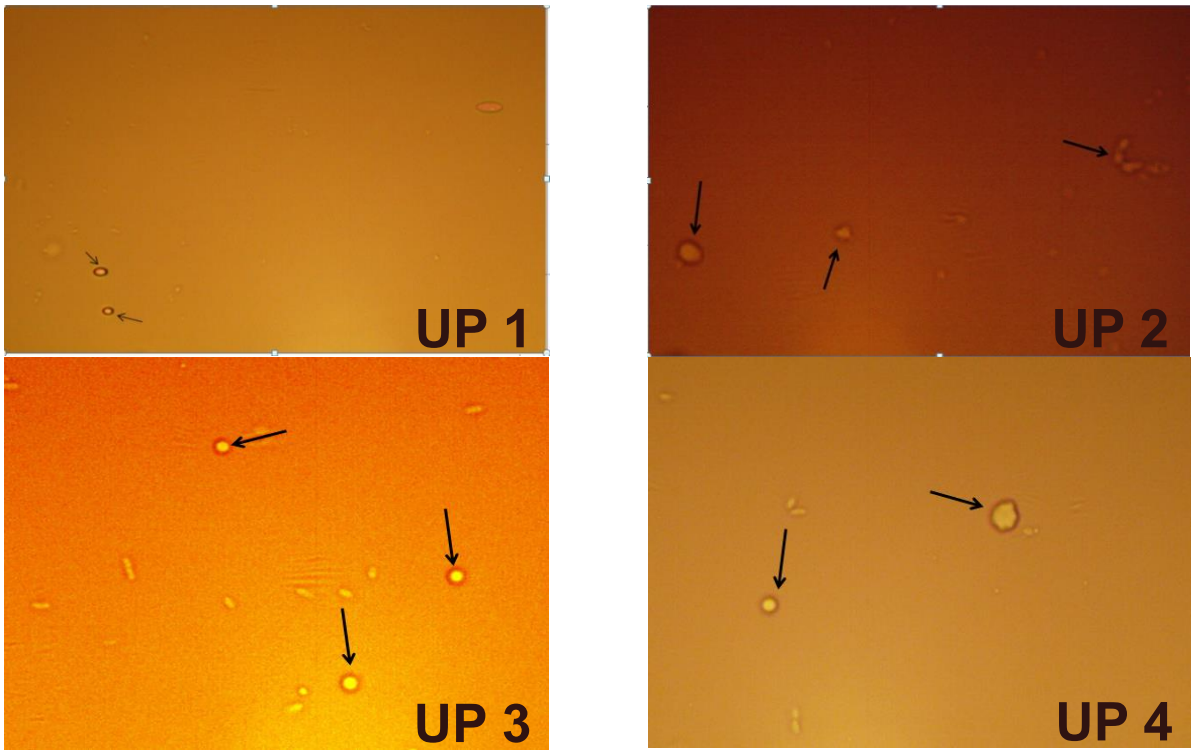

Figure 1. PLM images of phytosome formulations (UP1, UP2, UP3, UP4).

4. Conclusion

In this study. appearance, mean of particle size and size distribution, polydispersity index, and zeta potential values were considered to select the optimum phytosome formulation. So, the optimum formulation was selected depending on the formulations from UP1 to UP4 (Table1). Two types of phytosome formulations were designed (gel and liquid). Phase transition temperature of gel state liposomes (PL100H) was higher than liquid state liposomes (P85G). Stearylamine and dicetylphosphate were employed as charge inducer negative and positive, respectively.

The charge inducer was incorporated into the liposome bilayer in order to stabilize liposome dispersions physically. This effect depends on electrostatic interaction between vesicles and it provides electrostatic charge to the vesicle surface (Ethemoglu et al., 2017). The objective of this study is to develop a delivery system, which is containing alcohol free P.S. for children, with novel carrier system and stable phytosome formulation. The optimum liposome formulation (UP4) composed of (PL 100H: SA: CHOL – 1% P.S.) was selected in all formulations.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Free Radical Scavenging Activity and Chemical Constituents of the Unripe Fruits of *Spondias pinnata* (L.f.) Kurz. from Nepal

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Abstract

Spondias pinnata (L.f.) Kurz.(Anacardiaceae) is widely used as food and for medicinal properties. This study aims to disclose the free radical scavenging potential, total phenolic and flavonoid contents and phytochemical constituents of 70% methanol extract of unripe fruits of *S. pinnata* collected from Kaski district, Nepal. The free radical scavenging activity was evaluated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay method. The total phenolic content (TPC) and total flavonoid content (TFC) were estimated by using Folin-Ciocalteu's phenol reagent and aluminium chloride methods, respectively. *S. pinnata* fruits extract showed potent free radical scavenging activity with IC_{50} value 2.75±0.23 µg/ml. TPC and TFC values were found to be 229.24±0.46 mg GAE/g and 192.58±3.81 mg QE/g, respectively. Detailed chemical isolation of the extract afforded caffeic acid methyl ester (**1**) and rhamnetin 3-*O*-sophoroside (**2**). In conclusion, *S. pinnata* fruits were found to be rich source of phenolic and flavonoid compounds and possessed strong free radical scavenging property. However, further study is needed to explore its potential health benefits and bioassay guided chemical analysis should be performed to isolate and identify the bioactive compounds.

Key Words: *Spondias pinnata*, DPPH, Total phenolic content, Total flavonoid content, Isolation

1. Introduction

Medicinal plants, both in crude and refined form, are considered as an important therapeutic aid for treatment of diseases in humans and animals. More than 70% of world's population depend on traditional medicinal plants to maintain their health and cure their illness (Joseph and Jini, 2013). The chemical constituents obtained from different parts of medicinal plants serve as lead molecules in the development of modern medicines and also as potent components of various functional foods and nutraceuticals (Atanasov et al., 2015; Ayaz et al., 2019; Yeung et al., 2019). Therefore, medicinal plants need to be investigated for biological activities and phytochemical composition to provide safe and effective remedies.

Spondias pinnata (L. f.) Kurz. (Synonyms: *Mangifera pinnata* L. f., *Spondias acuminata* Roxb., *Spondias mangifera* Willd.) (Figure 1) is a deciduous tree belonging to family Anacardiaceae. It is mostly found in lowlands and hill forests of Nepal, Bhutan, China (southern), India and Myanmar. In Nepal, it is locally known as "Amara" and fruits are eaten fresh or pickled (Manandhar, 2002) and bark juice is given for stomach ache, diarrhea, dysentery and rheumatism (Bora et al., 2014). *S. pinnata* leaves are used in the formulation of an herbal beverage which is consumed to treat heart burn, urolithiasis and diabetes and to improve immunity in Indonesia (Sujarwo and Keim, 2019). Previous studies have reported the hypoglycemic activity of methanol extract (Dash and Mondal, 2009), anti-hyperlipidaemic and insulinotropic effects of aqueous extract (Attanayake et al., 2014) and cytotoxic activity of the methanol extract (Ghate et al., 2014) of the bark. Likewise, antioxidant activities of methanol extract of bark (Hazra et al., 2008) and leaves (Sai et al., 2019) are also reported.

In addition, the phytochemical and nutritional characterization of raw fruits has demonstrated various constituents such as phenolic compounds, flavonoids, amino acids and minerals (Satpathy et al., 2011) and triterpenoids such as β-amyrin and oleanolic acid (Sameh et al., 2018) . Recently, we reported the total phenolic content (TPC), total flavonoid content (TFC), antioxidative and α-amylase inhibitory activity of the methanol extract of the leaves of *S. pinnata* (Sai et al., 2019). In continuation, this this paper, we aimed for the evaluation of the free radical scavenging activity, estimation of TPC and TFC and isolation of secondary metabolites from the unripe fruits of *S. pinnata*.

(a) (b)

Figure 1. The unripe fruits of *S. pinnata* (a) and dried peels and pulp (b) used for experiments

2. Materials and Methods

2.1. Instruments and chemicals

^{1}H- and ^{13}C-NMR spectra were measured on BRUKER AVANCE 600 NMR Spectrometer (Bruker, Billerica, MA, USA). Column chromatography (CC) was carried out with MCI gel CHP20P (75~150 μm, Mitsubishi Chemical Industries Co. Ltd., Tokyo, Japan), Sephadex LH-20 (Amersham Pharmacia Biotech, Tokyo, Japan), and silica gel 60 (0.040–0.063 mm, Merck KGaA, Darmstadt, Germany). Thin-layer chromatography (TLC) was performed on a pre-coated silica gel 60

F254 (Aluminum sheet, Merck KGaA, Darmstadt, Germany). DPPH, gallic acid and quercetin were purchased from Wako Pure Chemicals, (Tokyo, Japan). Ascorbic acid and aluminium chloride were procured from Qualigens Fine Chemicals, India. Folin-Ciocalteu's phenol reagent was from Sigma Aldrich, USA.

2.2. Plant collection and extraction

The unripe fruits of *S. pinnata* were collected from Kaski district, Gandaki province, Nepal in the month of August, 2017 and were identified by Dr. Radheshyam Kayastha, former Professor, Tribhuvan University, Nepal. A voucher specimen of this plant (PUCD-2018-07/08) was deposited at the Laboratory of Pharmacognosy, Pokhara University, Nepal. The fruits were thoroughly washed with tap water and peeled along with pulp to remove seeds. Air dried peel and pulp (1 kg) of *S. pinnata* was extracted twice with 70% methanol at room temperature by maceration. The filtered extract was evaporated using rotary evaporator to obtain 240 g of dried extract. The dried extract was stored in refrigerator and used for further experiments.

2.3. Evaluation of DPPH free radical scavenging activity and TPC and TFC

Evaluation of DPPH free radical scavenging activity and estimation of TPC and TFC values were performed according to the method described in previous paper (Sai et al., 2019).

2.4. Chemical isolation

S. pinnata fruits extract (236.0 g) was suspended in water and applied to MCI gel CHP-20P column. The column was then eluted with water, by 40% methanol, 70% methanol and methanol to obtain 45 fractions each of 100 mL. TLC pattern of each fractions were observed in suitable solvent system and fractions with similar spots were combined to obtain 10 major fractions (SPFW-1 to SPFW-10).

Fraction SPFW-8 (1.23 g) was then applied into Sephadex LH-20 column and eluted with 50% methanol followed by methanol to obtain 6 sub-fractions (SPFW-8-1 to SPFW-8-5). SPFW-8-5 was obtained as pure compound **1** (100 mg). Subfraction SPFW-8-2 (180 mg) was further purified by silica gel column chromatography and eluted with $CHCl_3$: MeOH: H_2O (8:2:0.2) to obtain compound **2** (70 mg).

2.5. Caffeic acid methyl ester (1)

White amorphous power. ^{1}H-NMR (600 MHz, CD_3OD) δ_H: 7.53 (1H, d, J = 15.9 Hz, H-7), 7.03 (1H, d. J = 2.0 Hz, H-2), 6.93 (1H, dd, J = 2.0 Hz, 8.2 Hz, H-6), 6.77 (1H, d, J = 8.2 Hz, H-5), 6.25 (1H, d, J = 15.9 Hz, H-8), 3.75 (3H, s, OCH_3). ^{13}C-NMR (150 MHz, CD_3OD)□□$_C$: 166.9 (C-9), 148.3 (C-4), 145.5 (C-8), 145.1 (C-3), 129.3 (C-1), 121.3 (C-6), 115.7 (C-5), 114.7 (C-2), 111.3 (C-7), 51.1 (OCH_3) (Fujioka et al., 1999).

2.6. Rhamnetin 3-*O*-sophoroside (2)

Pale amorphous powder. ^{1}H-NMR (600 MHz, CD_3OD) δ_H: 7.70 (1H, d. J = 2.1 Hz, H-2'), 7.57 (1H,dd. J = 2.1, 8.5 Hz, H-6'), 6.90 (1H, d. J = 8.5 Hz, H-5'), 6.58 (1H, d. J = 2.1 Hz, H-8), 6.33 (1H, d. J = 2.1 Hz, H-6), 5.37 (1H, d. J = 7.6 Hz, H-1"), 4.77 (1H, d. J = 7.6 Hz, H-1"'), 3.88 (3H, s, OCH_3), 3.20-3.80 (remaining sugar protons). ^{13}C-NMR (150 MHz, CD_3OD) δ_C :179.8 (C-4), 167.2 (C-7), 162.7 (C-5), 159.3 (C-2), 158.3 (C-9), 149.9 (C-4'), 145.9 (C-3'), 135.3 (C-3), 123.2 (C-6'), 122.8 (C-1'), 117.9 (C-2'), 116.2 (C-5'), 106.6 (C-10), 104.9 (C-1"'), 101.2 (C-1"), 99.1 (C-6), 93.1 (C-8), 82.8 (C-2"), 78.2 (C-5"), 78.1 (C-5"'), 77.9 (C-3", C-3"'), 75.6 (C-2"), 71.4 (C-4"'), 71.1 (C-4"), 62.4 (C-6", C-6"'), 56.6 (OCH_3) (Goda et al., 1999).

2.7. Statistical analysis

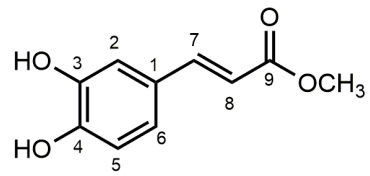
Results are expressed as mean ± SD (n=3). All the data analysis was carried out using Microsoft Excel 2007.

3. Results and Discussion

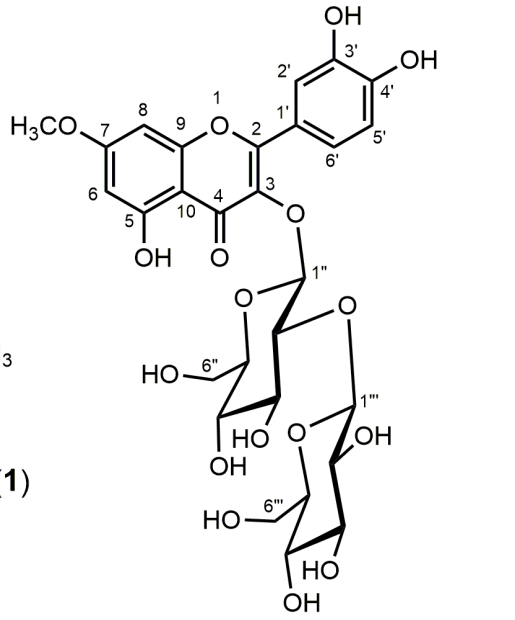
Various plants belonging to genus *Spondias* are used in traditional medicine systems and are studied for their chemical constituents and pharmacological activities (Sameh et al., 2018). In this study, we evaluated the free radical scavenging activity of the 70% methanol extract of dried peels and pulp of unripe fruits of *S. pinnata* collected from western Nepal. Additionally, estimation of TPC and TFC and isolation of secondary metabolites was also performed. Antioxidant activity of *S. pinnata* fruits extract was evaluated by using DPPH free radical scavenging assay method and expressed in terms of IC_{50} values (µg/ml) (Table 1). *S. pinnata* fruits extract showed strong antioxidant activity with IC_{50} value of 2.75 ± 0.23 µg/ml compared to positive control, ascorbic acid (IC_{50} = 3.16 ± 0.02 µg/ml). The total phenolic (TPC) and flavonoid content (TFC) values of *S. pinnata* fruits extract were found to be 229.24±0.46 mg gallic acid equivalent (GAE)/g and 192.58±3.81 mg quercetin equivalent (QE)/g, respectively (Table 1). The strong antioxidant activity of *S. pinnata* fruits can be attributed to the presence of phenolics and flavonoids which are already established as potent free radical scavengers by many studies (Satpathy et al., 2011; Dirar et al., 2019) and it is further supported by the results of total phenolic and flavonoid contents in this study.

Table 1. IC_{50} values for DPPH assay, and TPC and TFC values of extract of *S. pinnata* fruits

Sample	IC_{50} values for DPPH assay (µg/ml)	TPC (mg GAE/g)	TFC (mg QE/g)
S. pinnata extract	2.75 ± 0.23	229.24 ± 0.46	192.58 ± 3.81
Control(ascorbic acid)	3.16 ± 0.02	-	-



caffeic acid methyl ester (**1**)



rhamnetin 3-*O*-sophoroside (**2**)

Figure 2. Structures of isolated compounds

The detailed chemical analysis of the extract afforded two compounds. The structures of these compounds were elucidated on the basis of NMR spectroscopic data and comparison with literature values as caffeic acid methyl ester (**1**) (Fujioka et al., 1999). and rhamnetin 3-*O*-sophoroside (**2**) (Goda et al., 1999) (Figure 2.). Caffeic acid methyl ester (methyl caffeate) is a hydroxycinnamic acid derivative and rhamnetin 3-*O*-sophoroside (7-*O*-methylquercetin 3-*O*-sophoroside, **2**) is a flavonoid glycoside. Previous study on raw fruits of *S. pinnata* has reported the presence of other hydroxy cinnamic acid derivatives i.e. chlorogenic acid and *p*-coumaric acid and hydroxybenzoic acid derivatives i.e. gallic acid, salicylic acid and ellagic acid. This is the first report on isolation of caffeic acid methyl ester (**2**) from *S. pinnata.* However, it should be noted that it can be an artifact generated during isolation procedures. It has been also reported from various plant sources such as *Angelica japonica* A.Gray (Apiaceae) (Fujioka et al., 1999), *Heynea trijuga* Roxb. ex Sims (Meliaceae) (Devkota et al., 2014), *Phegopteris decursivepinnata* Fée (Thelypteridaceae) (Watanabe et al., 2018) among others. Rhamnetin 3-*O*-sophoroside (**2**) has been reported from only two sources previously i.e. *Nasturtium officinale* R.Br. (Brassicaceae) (Goda et al., 1999) and *Ranzania japonica* T. Ito (Berberidaceae) (Iwashina and Kitajima, 2009). Goda et al. (1991) also reported the strong inhibitory activity of compound **2** on histamine release from RBL-2H3 cells induced by antigen stimulation.

There are more than 8000 naturally occurring phenolic compounds including flavonoids reported from the medicinal plants, fruits and vegetables. Flavonoids are the largest group of polyphenolic compounds which are reported to have several pharmacological activities such as anti-inflammatory, antimicrobial, antidiabetic, antithrombogenic, hepatoprotective and antitumor activities (John et al., 2014; Khan et al., 2019). For example, quercetin exhibited anti-inflammatory activity by inhibiting cyclooxygenase and lipooxygenase pathways and thus reducing the formation of inflammatory mediators (Kim et al., 1998). Similarly, quercetin is reported to possess antidiabetic activity by regeneration of pancreatic cells in streptozotocin-induced diabetic rats (Vessal et al., 2003). Flavonoids and polyphenolic compounds are primarily known for their free radical scavenging activities. Their polyphenolic nature enables them to scavenge injurious free radicals such as superoxide anion and hydroxyl radicals (Adhikari-Devkota et al., 2018). The possible mechanism of antioxidant action can be suppressing the formation of reactive oxygen species by inhibition of enzymes or chelating trace elements (Pietta, 2000). Flavonoids have been found to inhibit enzymes such as xanthine oxidase and protein kinase C as well as chelate some trace elements which are responsible for reactive oxygen species generation (Ursini et al., 1994).

4. Conclusion

In conclusion, the unripe fruits of *S. pinnata* possessed strong free radical scavenging activity along with high content of total phenols and flavonoids. Two compounds, caffeic acid methyl ester (**1**) and rhamnetin 3-*O*-sophoroside (**2**) were isolated from 70% methanolic extract of the fruits. Further research should focus on the *in vivo* bioactivity evaluation of the extract and compounds and isolation and structure elucidation of other bioactive compounds.

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Conflicts of Interest

The authors declare no conflict of interest.

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