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CONTENTS **PAGE**

International Journal of Minor Fruits, Medicinal and Aromatic Plants. Vol. 8 (2) : 01- 11, December 2022

Review article

Taxonomic diversity of spice crops available in Bangladesh Agricultural University Botanical Garden and their medicinal properties

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ABSTRACT

Spice crops have versatile economic uses and medicinal properties. People commonly use spices in the cooking of foods. Many spice crops were collected from home and abroad and are being conserved in Bangladesh Agricultural University Botanical Garden (BAUBG). A survey was carried out to update the list of spice crops available in BAUBG and to record their diversity, and economic and therapeutic uses.Thirty spice species identified in BAUBG belong to 16 families. A brief description of the therapeutic/medicinal uses of each species has been presented in the text.

Keywords: Bangladesh, Culinary Herbs, Medicinal Values, Spice, Therapeutic Properties

INTRODUCTION

Spices, the culinary agents, are used for seasoning or flavoring a food or curry either in dried, powdered, or fresh form. Bangladeshi people use and cultivate a taxonomically diverse group of spices and condiments in their homes as well as in the crop fields commercially to satisfy their daily needs. They also collect some of them from the wild vegetation (the natural forest). Most of the spices were found to be multi-purpose and were reported as they are used in medicine by local peoples for a long time. There is a significant demand for herbs and spices in the area because locals use a lot of them in their everyday culinary preparations.Spices are enriched with bioactive compounds that have high antioxidant properties and provide the potential to safeguard the human body (Shan *et al*., 2005). Spice crops produce secondary metabolites that may not be essential for their normal growth or function, but they are known to have anti-disease potential (Craig, 1999).

Often the fresh leaves of *Anethum graveolens, Coriandrum sativum*, *Cymbopogon citratus, Eryngium foetidum*, *Foeniculum vulgare, Mentha spicata*, and *Piper betle* are used as condiments. The seeds and roots of the spices and condiments are used both in dried and ground forms. The bulbs of Onion and Garlic are used in both fresh and dry conditions. *Capsicum annum* and *Capsicum frutescens* both are used as fresh vegetables or in dried and ground form (usually mixed with other spices and condiments). *Curcuma longa* L. and *Zingiber officinale* are used for their spicy rhizome and used either in dried or powdered forms.

The Bangladesh Agricultural University Botanical Garden (BAUBG)is the second-largest botanical garden in Bangladesh in terms of area, but the largest in terms of species. Presently, BAUBG conserves a total of 1800 species belonging to 287genera and 168 families. There are some species of spices that are also conserved in it. The majority of the spices are annual herbaceous plants, whereas just a small percentage are trees and some are a climber. This study attempts to catalog all the accessible spice plants at the BAUBG and describe their therapeutic/medicinal uses.

MATERIALS AND METHODS

Bangladesh Agricultural University Botanical Garden (BAUBG) is situated on the west bank of the old Brahmaputra River (Fig.1). The University is geographically located at 90°26’29.6’’E and 24°43’26.8’’N.It is dominated bya tropical monsoon climate having relative humidity between 80-84% and an average rainfall of about 2000 mm (Jone *et al*., 2022).

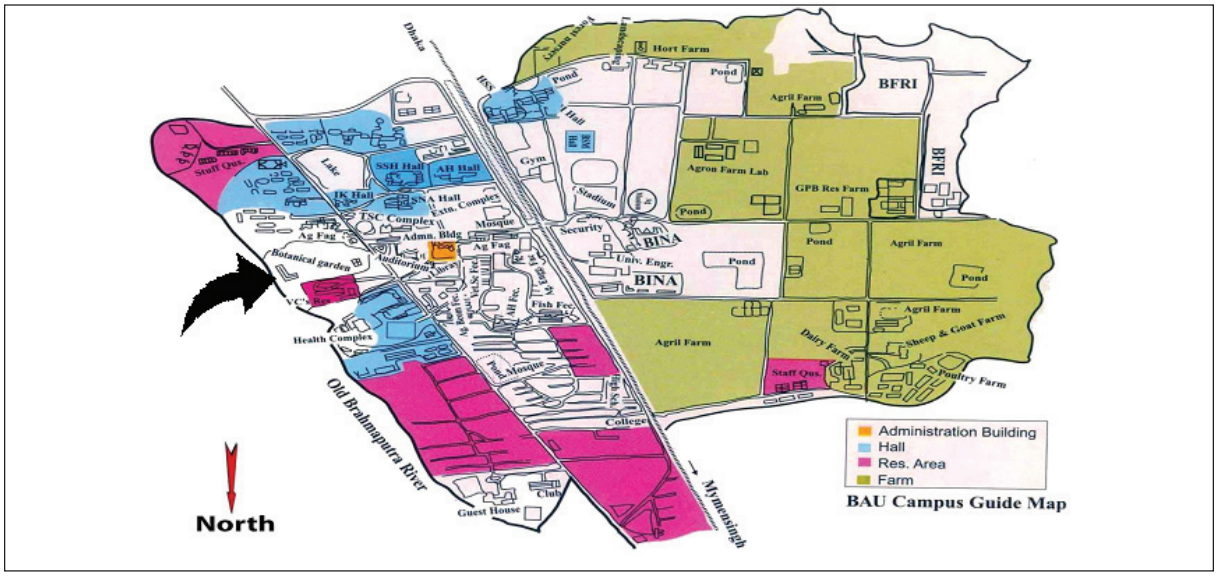

Fig. 1: Bangladesh Agricultural University map with the research area (BAUBG) marked by an arrow sign

The study has been planned to document the spice crops available in the BAUBG along with their taxonomic diversity, medicinal properties, and other uses. All the data presented in the text were formulated by visiting the garden and were re-checked with two websites named The Plant List (http://www.theplantlist.org/) and The Plants of World Online (https://powo.science.kew.org/). The genera and species under each family along with their medicinal uses have been described in alphabetical order. The valid name(s) of each species, its habit, and conservation status have been stated in tabular form.

RESULTS AND DISCUSSION

Thirty species under 23 genera belonging to 16 families of spice crops were found in BAUBG. About 54 per cent of the spices found in the garden are herbs, whereas 30% are trees, 13% are climbers, and 3% are shrubs. Apiaceae has the highest number of genera (4) followed byZingiberaceae(3), Myrtaceae(2), and Rutaceae (2). The rest 12 families have a single genus in each. Piperaceae has the maximum number of plants (5species) whereas nine families have single species (Fig. 2). Spices can be used as seeds, fruit, bark, or any other plant part for culinary or medicinal purposes. Seeds of almost all the 30 spice crops are used for culinary or medicinal purposes whereas single spices are used as bark and flower bud. In this section, we described the medicinal uses of 30 spice crops with graphs to classify them according to various classes.

For each taxon, the latest nomenclature, synonym, and uses have been included below.

***Allium cepa* L.:** Synonym : *Allium angolense* Baker, *Porrum cepa* (L.) Rchb.

Onion expressed antibacterial activity against pathogenic microorganisms. Aqueous and alcoholic extract of the onion bulb has powerful hepatoprotective properties. Eating onion regularly can reduce liver diseases. The unutilized outer layers of the red variety of onion are an important source of natural antioxidants (Miri and Roughani, 2018). Fresh onion juiceis used to reduce pain and inflammation (Upadhyay, 2016).(Fig: 5-A)

***Allium sativum* L.:** Synonym: *Allium longicuspis* Rege, *Porrum sativum* (L.) Rchb.

Garlic is extensively used in treating some issues such as cardiovascular diseases, arthritis, pulmonary complaints, headache, bites, abdominal growths, respiratory infections, skin disease, wounds, ulcers, tumors, aging symptoms, diarrhea, and worms (Rahman, 2007). Allicin is the compound of garlic that has antibiotic, and antibacterial properties (Mikaili *et al*., 2013). It has hypocholesterolemic, antithrombotic, antihypertensive, and antioxidative properties (Petrovska and Cekovska, 2010). (Fig: 5-B)

***Alpinia calcarata* (Haw.) Roscoe :** Synonym: *Alpinia alata* A. Dietr.

It is used to treat indigestion, vomiting, and nausea. It is also beneficial to the lungs or other pulmonary diseases. It can be used as a laxative

Table 1: List of Spices with family and scientific names, habits, and conservation status available at BAUBG

SL	Common name (Local name)	**Family** and **Scientific name**	Habit	CS
		Amaryllidaceae		
1	Onion (Peyaj)	*Allium cepa* L.	Herb	LC
2	Garlic (Rosun)	*Allium sativum* L.	Herb	LC
		Apiaceae		
3	Dill (Radhuni)	*Anethum graveolens* L.	Herb	LC
4	Coriander (Dhania)	*Coriandrum sativum* L.	Herb	LC
5	Culantro (Bilatidhonia)	*Eryngium foetidum* L.	Herb	LC
6	Fennel (Mouri)	*Foeniculum vulgare* Mill	Herb	Rare
		Bixaceae		
7	Lipstick tree (Doirang)	*Bixa orellana* L.	Herb	LC
		Lamiaceae		
8	Spearmint (Pudina)	*Mentha spicata* L.	Tree	LC
		Lauraceae		
9	Ram-tejpata	*Cinnamomum bejolghota* (Buch-Ham.) Sweet	Herb	LC
10	Indian Bay Leaf (Tejpata)	*Cinnamomum tamala* (Buch-Ham.) Nees & Eberm.	Tree	DD
11	Cinnamon (Daruchini)	*Cinnamomum verum* J. S. Presl	Tree	NE
		Leguminosae		
12	Fenugreek (Methi)	*Trigonella foenum-graecum* L.	Herb	LC
		Myristicaceae		
13	Nutmeg (Joitri & Jaifal)	*Myristica fragrans* Houtt.	Tree	LC
		Myrtaceae		
14	Allspice	*Pimenta dioica* (L.) Merr.	Tree	Rare
15	Clove (Lobongo)	*Syzygium aromaticum* (L.) Merr. & L. M. Perry	Tree	LC
		Pandanaceae		
16	Pandan (Polao Pata)	*Pandanus amaryllifolius* Roxb.	Tree	LC
		Piperaceae		
17	Betel leaf (Pan)	*Piper betle* L.	Climber	LC
18	Indian Long Pepper (Pipla)	*Piper longum* L.	Herb	Rare
19	Black Pepper (Golmorich)	*Piper nigrum* L.	Herb	LC
20	Javanese Long Pepper (Choi)	*Piper retrofractum* Vahl	Climber	LC
21	Mountain Long Pepper (Bon Pan)	*Piper sylvaticum* Roxb.	Climber	LC
		Poaceae		
22	Lemon grass	*Cymbopogon citratus*(DC.) Stapf	Climber	CD
		Ranunculaceae		
23	Black Cumin (Kalo Jira)	*Nigella sativa* L.	Herb	LC
		Rosaceae		
24	European Plum (Alu Bukhara)	*Prunus domestica* L.	Herb	LC
		Rutaceae		
25	Pan Bilash	*Clausena heptaphylla* (Roxb.) Wight &Arn.	Tree	LC
26	Curry leaf	*Murraya koenigii* (L.) Spreng.	Shrub	LC
		Solanaceae		
27	Chilli (Morich)	*Capsicum annuum* L.	Tree	LC
		Zingiberaceae		
28	Snap Ginger (ChotoAlachi)	*Alpinia calcarata*(L) Maton	Herb	LC
29	Turmeric (Holud)	*Curcuma longa* L.	Herb	LC
30	Zinger (Aada)	*Zingiber officinale* Roscoe	Herb	LC

CD= Conservation Dependent, DD= Data Deficient, LC= Least Concerned, NE= Not Evaluated

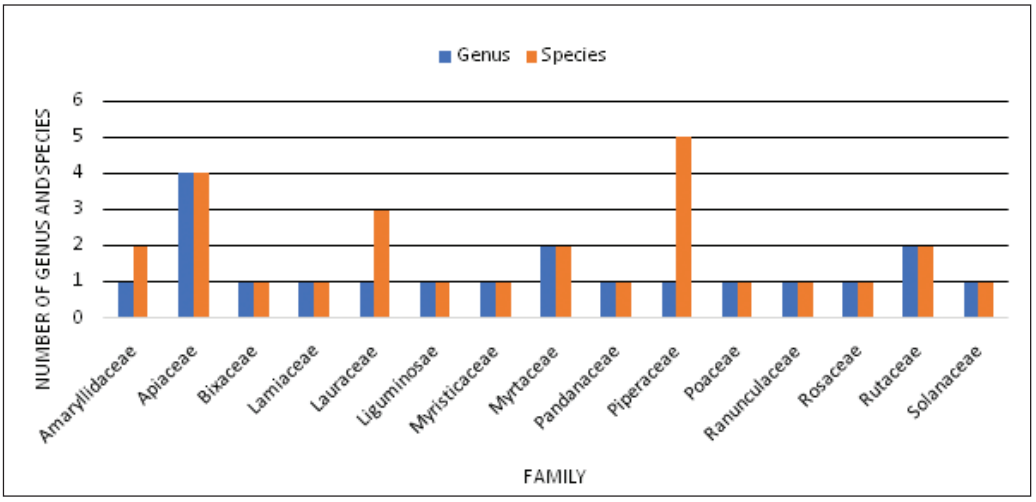

Fig. 2: Genera and species distribution of the spice crops available in BAUBG under different families.

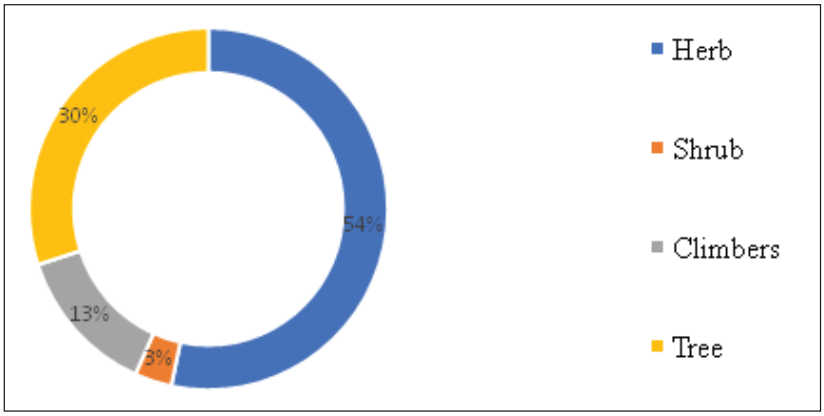

Fig. 3: Habit-wise diversity percentages of the spice cropsavailable in BAUBG.

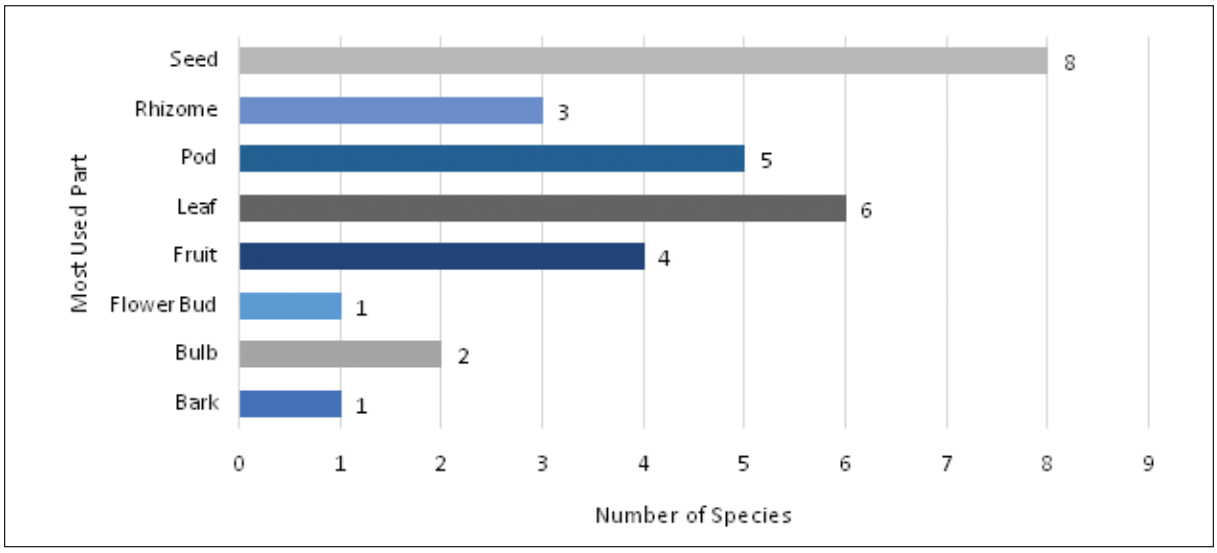

Fig. 4: Distribution of species based on the plant parts commonly used for culinary and/or medicinal purposes.

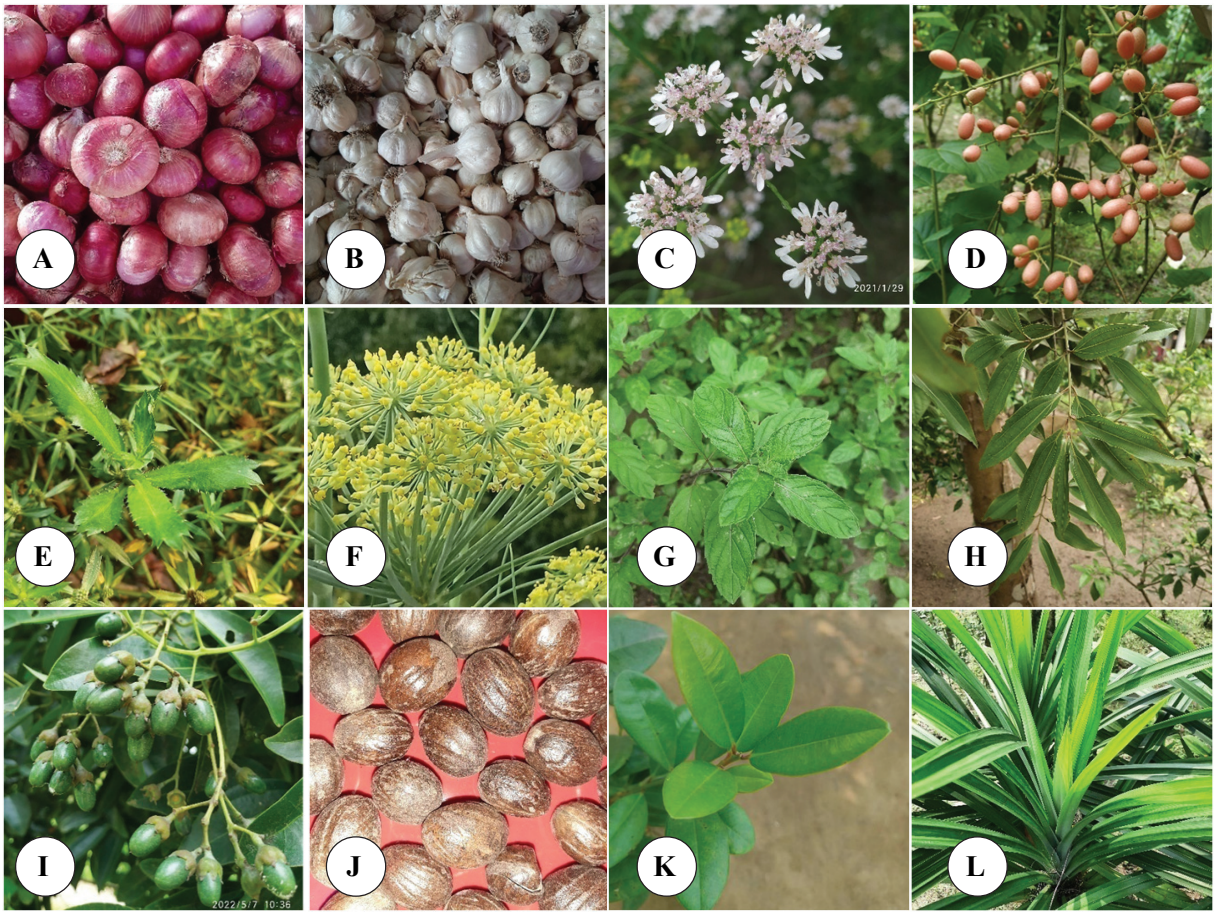

Fig. 5: A. *Allium cepa* **B.** *Allium sativum* **C.** *Coriandrum sativum* **D.** *Clausena heptaphylla* **E.** *Eryngium foetidum* **F.** *Foeniculum vulgare* **G.** *Mentha spicata* **H.** *Cinnamomum tamala* **I.** *Cinnamomum verum* **J.** *Myristica fragrans* **K.** *Pimenta dioica* **L.** *Pandanus amaryllifolius*

and it prevents stomach pain, flatulence, and gripping. (Fig. 6-K)

***Anethum graveolens*L.:** Synonym : *Anethum arvense* Salisb.

It is commonly used in Ayurvedic medicine to treat abdominal discomfort, digestion, and constipation. It also cures ulcers, abdominal pain, eye diseases, and urinal pain.

***Bixa orellana* L. :** Synonym: *Bixa americana* Poir.

The seeds have been used as laxative, cardiotonic, hypotensive, and antibiotic. It is also used as an anti-inflammatory agent for bruises and wounds as well as for the treatment of bronchitis and wound healing. Leaves are effective against bronchitis, and eye inflammation (Vilar *et al*., 2014).

***Capsicum annuum* L. :** Synonym : *Capsicum conoide* Mill.

Chilli is widely used in beverage industries, pharmaceuticals, cosmetics, and as a flavoring and coloring agent in meat processing (Rymbai *et al*., 2011). Traditionally, Chilli fruits are used for the healing of wounds and to treat cough, toothache, rheumatoid arthritis, infections, and sore throat. It has some other major properties, like anticancer, antibacterial effects, counterirritant, and antiseptic as well. Dyspepsia and flatulence can be protected by chilis (Singletary, 2011; Pawar *et al*., 2011).(Fig: 6-I).

***Cinnamomum bejolghota* (Buch-Ham.) Sweet:** Synonym : *Cinnamomum sikkimense*

Lukman.The plant extracts possess warming stimulant, astringent, digestive, carminative, blood purifier, antiseptic, antioxidant, antifungal, antiviral, antibacterial, anti-inflammatory, and immunomodulatory properties. The Plant also helps lowering cholesterol and blood sugar levels (Kumar *et al*., 2019).

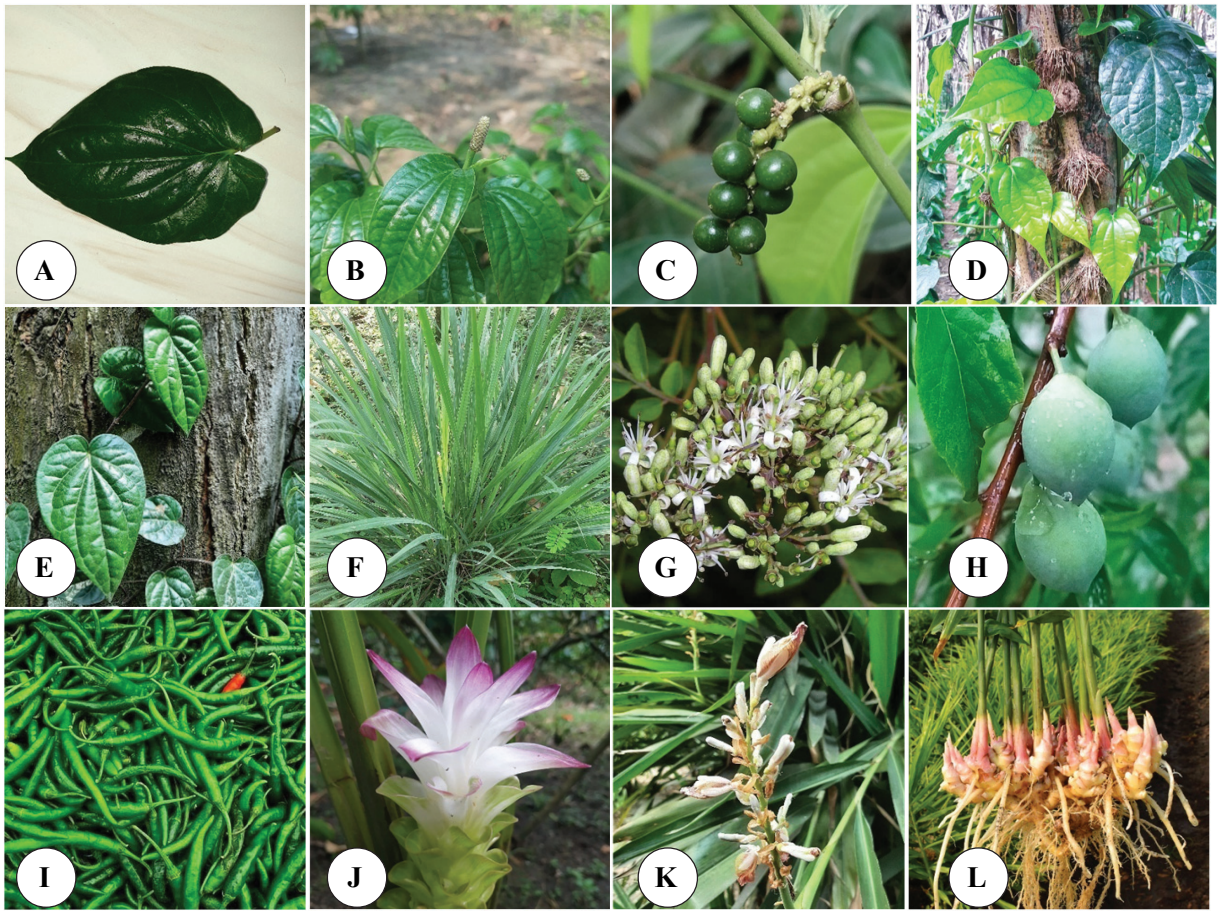

Fig. 6: A. *Piper betle* **B.***Piper longum* **C.** *Piper nigrum* **D.** *Piper retrofractum* **E.** *Piper sylvaticum* **F.** *Cymbopogon citrates* **G.** *Murraya koenigii* **H.** *Prunus domestica* **I.** *Capsicum annuum* **J.***Curcuma longa* **K.***Alpinia calcarata* **L.** *Zingiber officinale*

***Cinnamomum tamala* (Buch-Ham.) Nees & Eberm. :** Synonym: *Cinnamomum albiflorum*

Nees.Bay leaves are one of the most essential spices used in cooking and have been used as a traditional medicine to treat several diseases such as indigestion, earaches, rheumatism, and sprains, and to enhance perspiration (Fang *et al*., 2005). It has very powerful antioxidant, analgesic, and anti-inflammatory properties (Sayyah *et al*., 2003)(Fig. 5-H).

***Cinnamomum verum* J. S. Presl :** Synonym: *Camphorina cinnamomum* (L.) Farw.

Cinnamon contains a large number of essential oils and resins, mainly cinnamaldehyde, cinnamate, eugenol, and cinnamic acid. These chemical constituents of cinnamon possess anti-inflammatory, anticancer, antimicrobial, antidiabetic, and antioxidant properties. It is also used against cardiovascular diseases and neurological disorders (Manojlovic *et al*., 2012). Cinnamon is also beenused to cure dental problems in traditional medicine (Aneja *et al*., 2009; Azad *et al*., 2016)).(Fig. 5-I)

***Clausenaheptaphylla* (Roxb.) Wight & Arn. :** Synonym: *Amyris anisata* Roxb. ex Steud.

It is known to be used in paralysis, headache, muscle pain, ulcerated nose, and malarial fever. The leaf extract of this plant has great anti-bacterial properties. The bark can be used to treat cattle wounds and sprains (Fakruddin *et al*., 2012). (Fig: 5-D)

***Coriandrum sativum* L. :** Synonym: *Coriandrum globosum* Salisb.:

The primary ingredients found in Coriander are geranyl acetate, geraniol, camphor, ápinene, linalool, and ã-terpinene (Nadeem *et al.,* 2013). Traditionally, coriander is used to treat anxiety, loss of appetite, convulsion, dyspeptic complaints, and insomnia. It is used as an anti-hyperglycemic agent

and it controls blood glucose as well (Gallagher *et al.,* 2003). (Fig. 5-C)

***Curcuma longa* L. :** Synonym : *Curcuma brog* Valeton:

In traditional medicine, turmeric is used for treating rheumatism, body ache, skin problems, intestinal worms, amenorrhea, diarrhea, constipation, intermittent fevers, ulcers, arthritis, hepatic diseases, urinary discharges, dyspepsia, inflammation, leukoderma, dental diseases, dyspepsia, acidity, indigestion, flatulence, colitis and hepatitis (Ammon and Wahl, 1997).(Fig. 6-J).

***Cymbopogon citratus* (DC.) Stapf :** Synonym: *Andropogon ceriferus*Hack.

The plant is used in folk medicine as an analgesic, antiemetic, antitussive, antispasmodic, hypotensive, anticonvulsant, antirheumatic, and antiseptic, and for treating gastrointestinal and nervous disorders as well as fevers (Shah *et al.,* 2011; Premathilake *et al*., 2018). (Fig. 6-F).

***Eryngium foetidum* L. :** Synonym : *Eryngium antihystericum* Rottler.

The plant is used to treat burns, earaches, fevers, constipation, asthma, worms, snakebites, malaria, diarrhea, hypertension, infertility complication, etc. The areal parts have anthelminthic activity as well as antibacterial, and anti-inflammatory properties (Paul *et al*., 2011). (Fig. 5-E).

***Foeniculum vulgare* Mill. :** Synonym: *Anethum dulce* DC.

Fennels are widely used in treating different respiratory ailments. It can also be used to treat a wide range of digestive, reproductive, and endocrine systems and as a galactagogue agent for lactating mothers (Badgujar *et al*., 2014).(Fig. 5-F).

***Mentha spicata* L. :** Synonym : *Mentha aquatica var. crispa* (L.) Benth.

Spearmints are traditionally used to treat colds, fever, cough, asthma, jaundice, obesity, and digestive problems. Volatile oils from spearmint have efficient inhibiting properties against fungi, bacteria, and other parasites (El Menyiy, 2022; Amel *et al*., 2022). (Fig. 5-G).

***Murraya koenigii* (L.) Spreng. :** Synonym : *Bergera koenigii* L.

Curry leaves have stimulating properties and are used to treat common body aches. It is also used in treating edema, fresh cuts, inflammation, itching, bruises, dysentery, and piles (Balakrishnan, 2020).(Fig. 6-G)

***Myristica fragrans* Houtt. :** Synonym : *Aruana silvestris* Burm.f.

Nutmeg possesses antioxidant, immunomodulatory, and radio-protective activities due to the presence of â caryophyllene, eugenol, and lignans (Naeem *et al*., 2016). It shows a strong antibiotic property against bacteria and fungi as well (Dorman and Deans, 2004).(Fig. 5-J).

***Nigella sativa* L. :** Synonym : *Nigella cretica* Mill.

Black cumin is effectively used to treat jaundice, coughs, tumors, rhinitis, cataracts, alopecia, tertian fever, migraine, rheumatism, headache, paralysis, abdominal disorders, hydrophobia, orchitis, ulcers, vitiligo. (Kunnumakkara *et al.,* 2009).

***Pandanus amaryllifolius*Roxb. :** Synonym: *Pandanus hasskarlii* Merr.

Pandan is high in vitamins, minerals, and antioxidants, which it is widely used to treat health issues like headaches, earaches, and arthritis. It also reduces atherosclerosis formation, heals burns, and controls blood sugar (Keim *et al.,* 2020). (Fig: 5-L)

***Pimenta dioica* (L.) Merr. :** Synonym: *Pimenta officinalis* Lindl.

The plant has hypotensive, antibacterial, anti-neuralgic, antiproliferative, anti-tumor and analgesic properties. Crushed allspices are used to treat sore joints and muscle aches (Myalgia). It is also used to relieve respiratory congestion and toothache (Zhang &Lokeshwar, 2012).

***Piper betle* L. :** Synonym: *Betela mastica* Raf.

Betel leaves are widely used for chewing purposes but they possess various health benefits. It has analgesic properties that instant relief from pain, ease constipation, improve digestion, reduce respiratory issues, maintain oral health, relieve joint pain, manage diabetes, and so on (Rai *et al*., 2019)(Fig. 6-A).

***Piper longum* L. :** Synonym : *Chavica longa* H. Karst.

It is commonly used to treat asthma, bronchitis, respiratory infections, constipation, cholera, diarrhea, chronic malaria, and stomachache. It can be used to treat gonorrhea, paralysis of the tongue, viral hepatitis, and so on (Kumar *et al*., 2011). (Fig. 6-B)

***Piper nigrum* L. :** Synonym : *Piper aromaticum* Lam.

Black pepper is widely applied as spice and seasoning due to having beneficial health effects. This plant has been used as a chemo-preventive, anti-thyroid, thermogenic action, andanti-inflammatory as well as growth stimulator (Panda and Kar, 2003). The plant has been used for the treatment of large intestinal toxins indigestion, gastric acidity, and diarrhea. It is effective against disorders like asthma, fever, and cold (Parganiha *et al.,* 2011).(Fig. 6-C)

***Piper retrofractum* Vahl :** Synonym : *Piper chaba* Hunter

It can be used as a stimulant and also used to treat fever, asthma, hemorrhoids, bronchitis, liver ailments, jaundice, edema, and abdominal pain. Its root and fruits are useful in treating ingestion, poisoning, and anorexia (Salleh *et al*., 2020). (Fig. 6-D)

***Piper sylvaticum* Roxb. :** Synonym : *Piper malmoris* Wall.

The plant is used to treat the common cold, asthma, cough, headaches, wounds, indigestion, rheumatic pain, tuberculosis, etc. Mashed leaves can be used as an anti-inflammatory agent (Adnan *et al*., 2020)(Fig. 6-E).

***Prunus domestica* L. :** Synonym : *Druparia prunus* Clairv.

Plum fruits are used as febrifuge, stomachic, and laxative. It is also used to treat constipation. Prussic acid produced by this plant substance is poisonous which stimulates respiration and improves digestion (Zhebentyayeva *et al*., 2019)(Fig. 6-H).

***Syzygium aromaticum* (L.) Merr. & L. M. Perry :** Synonym : *Caryophyllus aromaticus* L.

Clove is used to treat sore throats, headaches, asthma as well as the digestive system, respiratory, and dental disorders (Lee *et al*., 2009) and it has a huge application in diarrhea, dyspepsia, and gastritis (Singh *et al*., 2012). (Fig. 5-K)

***Trigonella foenum-graecum* L. :** Synonym : *Trigonella tibetana* (Alef.) Vassilcz.

Fenugreek has been used to treat respiratory infections like bronchitis and pneumonia. It has hypoglycemic, hypolipidemic, and anti-hypertensive activities. Fenugreeks are used to treat reproductive disorders, treat hormonal disorders, and reduce menstrual pain (Mandal & Mandal, 2016).

***Zingiber officinale* Roscoe :** Synonym: *Amomum zingiber* L.

Ginger has been widely used to treat ailments like arthritis, cramps, fever, and helminthiasis, rheumatism, muscular aches, pains, sore throats, sprains (Dissanayake *et al*., 2020). It is also used to treat nausea, morning vomiting, colic, heartburn, flatulence, diarrhea, loss of appetite, sore stomach, gas, bloating, and dyspepsia (Prasad and Tyagi, 2015). (Fig. 6-L)

CONCLUSION

In the Indian subcontinent including Bangladesh people commonly use different parts of spices crops for culinary purposes to enhance the palatability or storage quality of foods and feeds. The spice crops possess some bioactive compounds to impart therapeutic/medicinal propertieslike antimicrobial, antioxidant, anti-inflammatory, anti-carcinogenic, cardio-tonic, and so on narrated in the text. This is a baseline work on spice crop diversity at BAUBG and its therapeutic uses for the scientific community.

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Evaluation of the antibacterial activity of essential oils of *Rosmarinus officinalis* L and *Rosmarinus eriocalyx* from the region of Sidi Bel Abbes (Algeria).

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ABSTRACT

Antibiotic resistance is currently a major public health problem facing hospitals around the world, another concern and that of the increased risks of additive synthetic food, added to foods that objective is to preserve foodstuffs. All these parameters are behind the urgent need for a natural antimicrobial agent. Rosmarinus officinalis L. is used in traditional medicine as an antispasmodic, ingredients in beauty products and so good in the preservation of food products. In this context, the objective of this study is to evaluate the antimicrobial activity of essential oils extracted from two species of the same genus of rosemary of wild origin "Rosmarinus eriocalyx" and "Rosmarinus officinalis" of a home garden in the Sidi Ali Benyoub region, against three pathogenic bacteria (Staphylococcus aureus, Escherichia coli, and Pseudomonas aeruginosa). Our work is divided into two parts, the first consists of extracting a volume of essential oil from 100g of each plant species by the hydrodistillation method which is the most effective and the most useful one, the second part is based on the evaluation of the effect of these oil extracts on the growth of the three pathogenic bacteria "Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa" taken from a biological sample "the urine" in the presence of a urinary tract infection at the level of the Central Laboratory, bacteriology department of the SBA center, which is highlighted by the aromatogram tests and the monitoring of bacterial growth in the absence and in the presence of essential oils. The diffusion method on the agar medium made it possible to determine the diameters of the zones of inhibition. The results show that the quantitative analysis of the essential oils of Rosmarinus eriocalyx and Rosmarinus officinalis L gave good yields of 1% and 0.63%. Regarding the antibacterial activity of both oils against pathogenic bacteria, the results show us that Rosmarinus eriocalyx (wild) has a higher activity compared to "Rosmarinus officinalis L". We can see that these oils have antibacterial activity only against Pseudomonas aeruginosa but the other two bacteria "Staphylococcus aureus and Escherichia coli" can be resistant for these oil extracts.

***Keywords*:** Antibacterial activity, aromatogram, essential oils, pathogenic bacteria, *Rosmarinus eriocalyx*, *Rosmarinus officinalis* L, yield

INTRODUCTION

Through the years, interest of using plant compounds has been growing faster in worldwide due to their benefits on health (Nandi and Ghosh, 2016). Many researchers have reported essential oil (EO) as potential alternative antimicrobials (Solórzano-Santos and Miranda-Novales, 2012). Rosemary *Rosmarinus officinalis L.* is a spontaneous aromatic plant widely distributed in Algeria, belonging to the Labiatae family (Lamiaceae) commonly called by the local population "Eklil". Rosemary likes limestone soils and adapts very well to arid and rocky regions. It is easily recognized, all year round availability. Its leaves, the flowering tops, which we will have taken care to dry or the essential oil which are used in

herbal medicine. Rosemary has been the subject of recent research in the pharmaceutical and food industries. It has anti-inflammatory and antispasmodic properties (Gianmario *et al.*, 2007) and action on the nervous system (Gonzalez *et al.*, 2007; Suzana *et al.*, 2007). Rosemary has excellent antioxidant and antimicrobial properties (Thoresen and Hildebrand, 2003). Recent research has shown that it has a variety of pharmacological activities, such, as cancer chemoprevention, anti-diabetic, hepatoprotective. Thus, rosemary is considered one of the most effective herb for treating headaches, poor circulation (Rocha *et al.*, 2015). Rosemary, like all aromatic and medicinal plants, contains chemical compounds with antibacterial properties. The use of these plant-based molecules can have many advantages over current synthetic products. Whose essential oils have antibacterial activity against some pathogens responsible for several human diseases such as Escherichia coli, staphylococcus aureus, pseudomonas aeroginosa, etc. (Bennadja *et al*., 2013). With this in mind, our choice fell on these two plants answered in Algeria (*Rosmarinus officinalis* L and *Rosmarinus eriocalyx*), widely used in culinary flavoring. In our region of Sidi-bel-abbés *Rosmarinus officinalis* L and *Rosmarinus eriocalyx* are important and more used as a condiment and/or food uses, found in the kitchen; it is also a bee plant, medicinal plants traditionally used against inflammation, bacterial and viral infections, and antispasmodics.

The aim of this work is to evaluate the antibacterial activity of the essential oil of *Rosmarinus officinalis* L, *Rosmarinus eriocalyx* from the ecotype of the region of Sidi Ali Benyoub and Mazaourou of Sidi-Bel-Abbés (Algeria) against pathogenic bacteria such as *staphylococcus aureus, pseudomonas aeroginosa, Escherichia coli.*

MATERIALS AND METHODS

Place of internship

Our experimental study is programmed at the level of the laboratory of Physiology and Nutrition and General Microbiology within the Department of Biology at the Faculty of Natural and Life Sciences of the University of Djilali Liabes of Sidi-Bel-Abbés (SBA).

Plant material

This work is focused on the two plants of "*Rosmarinus officinalis L*" and "*Rosmarinus eriocalyx"*, the choice of these plants is linked on the one hand to the importance of this species "Rosemary" as a medicinal and aromatic plant and among the most used throughout the world and on the other hand to the strong use of their extract of essential oils in traditional medicine. The species of "*Rosmarinus eriocalyx"* was provided at the level of the forest of ''Sidi Ali Benyoub" which is located in Sidi-Bel-Abbés province in the north-west of Algeria, it is located at 34°56'44 "N, 0°43'10"W. Latitude: 34.9456, Longitude: -0.719433 between Tabia and Mezaourou.), and for the second plant *'Rosmarinus officinalis L'* was provided at a small home garden at ''MEZAOUROU'' which is located in the province of Sidi-Bel-Abbés in the north-west of Algeria Latitude: 34.8173, Longitude:-0.623319, 34° 492 23 North, 0° 372 243 West). Both plants were dried to extract these oils

Extraction of essential oils (EO_s)

The extraction of EO from the dry leaves of the two plants *Rosmarinus officinalis L* and *Rosmarinus eriocalyx* is carried out using the hydrodistillation method at the level of the laboratory of Physiology and Nutrition of the department of biology, of the Faculty of Sciences of the Nature and Life at SBA.

Extraction process

This is the simplest and the oldest method of steam distillation. The process consists of immersing (50g) of the vegetable raw material (Rosemary) in a flask filled (100ml) with distilled water on a heat source. Everything is then brought to boil. The heat allows the plant cells to burst and then release odorous molecules. These aromatic molecules form an azeotropic mixture with water vapor. The vapors are condensed in a cooler and the essential oils separate from the water by density difference (the same steps for both species). The duration of a hydrodistillation can vary considerably, up to several hours depending on the equipment used and the plant material to be treated. The duration of the distillation influences not only the yield but also the composition of the extract.

EO analysis

Quantitative analysis

Determination of extraction yield

According to the standard (AFNOR, 1982), the yield of essential oil (Y_{EO}) is defined as the ratio

between the mass of essential oil obtained after extraction (m) and the mass of the plant material used (m_0). The yield is expressed as a percentage, it is expressed by the following formula. $Y_{EO}(\%)=m/m_0 X 100$ (Chama *et al.*, 2020).

Y_{EO}: Essential oil yield in %

m : Mass of essential oil in grams.

m_0: Mass of fresh plant material used in grams

Qualitative analysis

Organoleptic control

It consists of determining the organoleptic characteristics of the EOs obtained: smell, color, appearance, and flavor.

Study of Physico-chemical properties

In order to determine the quality of the EOs, we determined a certain number of physico-chemical characteristics.

Determination of relative density

The density of an oil is the ratio of the mass of a certain volume of oil at 20°C (Chama *et al.*, 2020).

$$D\frac{20}{20}=\frac{m}{m_0}$$

Where: m: the mass in grams of the oil, m_0: the mass in grams of distilled water

Determination of the refractive index

The refractive index is used for the identification and as a criterion of purity of EOs and liquid compounds, and to check the quality of the extraction.

pH

The pH or "hydrogen potential" measures the chemical activity of hydrogen H+ ions in solution. pH measures the acidity or basicity of a solution. This method describes the ionic acidity of the product to be analyzed; its principle consists in introducing the electrode of the pH meter into the product after adjusting the calibration temperature. The reading is done directly on the pH meter.

Research method and identification of the three pathogenic bacteria

The tested bacterial strains of *Staphylococcus aureus*, *Escherichia coli,* and *Pseudomonas aeruginosa* are brought from a biological sample (urine) of a patient suffering from a urinary tract infection; this experiment of isolation of the strains of interest is carried out at the central laboratory level CHU Abdelkader Hassani SBA in the department of bacteriology. Once, the samples are subjected to a direct examination including microscopic observations in the fresh state and a Gram stain. The identification of the strains was carried out at the level of the General Microbiology laboratory of the Faculties of Nature and Life Sciences, Department of Biology, University Djilali Liabés SBA.

Sampling: The collection of urine is carried out during the morning in a sterile pot, throwing away the first jet and then collecting the other urine.

Storage: The urine is stored at 4°C to prevent the proliferation of germs.

Sowing Seeding is carried out using a sterile platinum loop in streaks in three Petri dishes, each dish containing a selective culture medium for the isolation of the strains of interest. The media used are: Chapman for *Staphylococcus aureus*, Mac Conkey for *Escherichia coli*, Hektoen for *Pseudomonas aeruginosa*

Incubation: the inoculated Petri dishes are incubated at 37° C. for 18-24 hours.

Identification of isolated strains

Identification is based on the determination of morphological (macroscopic and microscopic) and biochemical characters

Macroscopic observation makes it possible to distinguish the shape, color, and morphology of bacterial colonies.

Microscopic observation makes it possible to study the morphological aspect of the cells of a microbial species. It includes the examination:

Fresh Microscopic

Observation in the fresh state consists of depositing a drop of bacterial suspension between slide and sterile cover slip and observed at magnification (X40). This method makes it possible to observe: The morphology of bacteria, Mobility, Grouping mode.

In the colored state Microscopic observation in the stained state is carried out on a bacterial smear, fixed and stained by the Gram method, this method makes it possible to observe: Color: Gram-positive or Gram-negative

Biochemical tests

The study of biochemical bacteria is essentially based on the search for catalase, oxidase to confirm that it is a bacterium of interest.

Oxidase test

Principle

The purpose of this test is to determine the presence of an active cytochrome oxidase using a determined substrate: N-dimethyl-para phenylene diamine, which will react in its presence with a color change (formation of a purple-colored compound).

Technical

The search for oxidase consists of placing a disk of it on a sterile slide and soaking it with a drop of distilled water. Then, pick up a colony using a Pasteur pipette and place it on the disc. A rapid color change to purple indicates a positive reaction.

Catalase test

Principle

In the presence of molecular oxygen, certain metabolic reactions lead to the formation of hydrogen peroxide. Catalase is an enzyme that breaks down hydrogen peroxide H_2O_2 into water H_2O and oxygen O_2.

Technical

It consists of depositing a drop of hydrogen peroxide H_2O_2 on a slide. Using a sterile Pasteur pipette, pick up a few colonies of the strain to be tested and place them in the drop of hydrogen peroxide. The appearance of many gas bubbles indicates a positive reaction.

Conservation

The conservation was carried out in the short term (about a week); the boxes are maintained at more than 4°C in anaerobic atmosphere.

Evaluation of the antibacterial activity of essential oils of *Rosmarinus officinalis* and *Rosmarinus eriocalyx* against pathogenic bacteria

Aromatogram technique on solid medium

Principle

The aromatogram is based on a technique used in medical bacteriology and which is carried out in vitro, called antibiogram or method by diffusion in agar medium or even disc method. This method has the advantage of being very flexible in the choice of essential oils tested, of applying to a very large number of bacterial species, and of having been widely evaluated for more than 50 years of worldwide use (Wilkinson, 2006). It is the technique we used to initially assess the antibacterial activity of EO (Chama *et al.*, 2020).

The antibacterial activity is evaluated by the aromatogram method, which makes it possible to determine the sensitivity of the different bacterial species to the essential oils used.

Technical

This method consists of depositing sterile cellulose discs 6mm in diameter on the surface of Petri dishes containing agar which is already solidified and seeded with the microorganisms of interest, these discs will be soaked in a quantity of essential oil to test; Petri dishes were incubated at 37°C for 24 hours. After incubation, the reading of the results is done by measuring the diameter in millimeters of the clear zone around the discs; this zone is called the zone of inhibition (formation of a translucent halo). The larger the diameter of the zone of inhibition, the more sensitive the strain is to the substance tested, the smaller it is, the more resistant the microbial strain.

Revivification and transplanting of stumps

Revivication of microbial strains are carried out by the streak method. The latter is then revived from storage boxes of selective media for each bacterium on nutrient agar. The cultures are incubated in an oven at 37°C for 24 hours.

Preparation of the inoculum

It was prepared from a young 24-hour culture. For this, bacterial suspensions were made by taking 3 to 5 well-isolated colonies, which were deposited in 10 ml of distilled water. Then, we ensured a good agitation. Then incubated at 37°C for 24 hours. After incubation, centrifugation of this bacterial suspension was performed.

Preparation of culture media

The culture medium used for the aromatogram test is Mueller Hinton (MH) medium, the process consists of pouring this agar into sterile petri dishes with a diameter of 90 mm to a thickness of 4 mm

and then allowing them to cool and solidify at room temperature in the sterile area.

Preparation of different EO concentrations

The preparation of EO concentrations consists of making a stock solution of tween 80 for preparing the different dilutions of essential oil. The process consists of diluting 2.5 ml of pure Tween 80 in 90 ml of distilled water, this solution is sterilized at 120°C for 15 minutes. In a volume of 9ml of this stock solution, we added 1ml of essential oil, in order to obtain a well-homogenized solution, we stirred the contents with a vortex, and then we carried out the successive dilutions of 10^{-1} to 10^{-6}.

Preparation of EO discs

The discs are prepared from Whatman paper 6mm in diameter, then they are put in a test tube, sterilized in an autoclave at 120°C for 20 minutes, then soaked in the extracts of essential oils of *Rosmarinus officinalis* and *Rosmarinus eriocalyx* at room temperature.

Seeding

The seeding of the bacterial suspension is carried out by the calibration technique using a Pasteur rake pipette on the Mueller Hinton medium prepared beforehand. In order to better appreciate the reading of the inhibition zones, two tests are carried out for each oil.

Disc repositories

Using sterile forceps; Whatman paper discs previously immersed in essential oils were placed on the surface of the inoculated Petri dishes.

Incubation

In order to inoculate the bacterial strains and deposit the discs, the boxes are incubated at 37°C for 24 to 48 hours.

Expression of results

The reading of the results is done after the incubation time by measuring the diameter of the zone of inhibition around each disc using a caliper or a ruler graduated in millimeters. The larger the diameter of this zone, the more the strain is sensitive to the essential oil (Djenane *et al*., 2012). To interpret the results, we used the scale of estimation of antimicrobial activity, which is given in the following table (Table 1).

Table 1: Reading of antimicrobial activity results (Djenane *et al*., 2012)

Diameter (∅) (mm)	Strain sensitivity
∅ < 7	**Not susceptible or resistance (-)**
8< ∅ <14	**Sensitive (+)**
15< ∅ <19	**Very sensitive(++)**
∅ >20	**Extremely sensitive(+++)**

RESULTS AND DISCUSSION

EO analysis

Rosmarinus officinalis L

Y_{EO}: Essential oil yield in %

m: Mass of essential oil in grams 0.63g.

m_0: Mass of fresh plant material used in grams 100g.

$$Y=\frac{0.63}{100}\times 100 \text{ , } Y = 0.63$$

Rosmarinus eriocalyx

YEO: Essential oil yield in %

m: Mass of essential oil in grams 1g.

m_0: Mass of fresh plant material used in grams.

$$Y=\frac{1}{100}\times 100 \text{ , } Y = 1$$

The yield obtained during the extraction is 0.63% for *Rosmarinus officinalis* and 1% for *Rosmarinus eriocalyx*, the yield may vary from one harvest to another this may be due to the various factors that come into play, among them, we cite the nature of the soil, the harvest period, the drying time, the extraction method, but this value remains within the standards between 0.5-2 according to (AFNOR, 1982).

The results obtained on the yield of essential oils extracted from Rosemary of the two populations studied in the region of Sidi Ali Beyoub and Mezaourou of the wilaya of Sidi-Bel- Abbés

comply with Afnor standards (0.5-2%). However, they show a significant difference between the sample from sidi ali benyoub (1%) of the *Rosmarinus eriocalyx* species and the sample from Mezaourou (0.63%) of the *Rosmarinus officinalis* species, his difference is due to the different factors such as; soil type, harvest time, drying time, etc. Regarding *Rosmarinus eriocalyx* is obtained from the mountain of Sidi Ali Benyoub (soil type) which is an upper region in the month of April, and the drying time is 12 days. Moreover, *Rosmarinus officinalis* is obtained from a garden in Mezaourou which is a lower region in the month of April and the drying time is 7 days; these conditions are influenced on the yield results.

Few are the works on Rosmarinus essential oil, and comparing to the yield of essential oil, our recorded value is close to that mentioned by Bendif *et al.* (2017) which was 0.73% for the oil extracted from leaves 0,6% and 0.82%, respectively. On the other hand, it is higher than that found in Annaba by (Ouibrahim, 2014) which was estimated at 0.36% and that obtained in Morocco (0.54%) (Derwich *et al.*, 2011). Low compared to that reported by Bendif *et al*. (2018) but this is obtained after supercritical CO_2extraction, which was 3.7%. The yield of essential oil from plants is often minimal, typically 1% or less (Carson and Hammer, 2011). The difference in yield is quite normal because it varies according to several parameters including climate, plant nutrition, stress (Croteau, 1986) and the collection period (Akrout *et al*., 2003).

Qualitative analysis

Organoleptic control

The essential oil of *Rosmarinus officinalis* and *Rosmarinus eriocalyx* are extracted by a simple hydrodistillation technique, this extraction method is standardized for the extraction of essential oils (Marie *et al*., 2004). The organoleptic parameters of this essential oil are in agreement with those listed in the standards (AFNOR, 1999). It is in liquid form with a light yellow color and it has a camphor odor, these results with the AFNOR standard are presented in the table (Table 2)

Table 2: Organoleptic characteristics of essential oils of *Rosmarinus officinalis L* and *Rosmarinus eriocalyx*.

	Appearance	Color	Odor	Flavor
AFNOR (fresh form)	Mobile, clear liquid	Almost colorless to pale yellow	Characteristic fresh, more or less camphor depending on the origin	Bitter and very slightly spicy
Essential Oil	Liquid	Pale yellow	Camphor	Spicy and fragrant

Study of Physico-chemical properties

Determination of relative density

The density of an oil is the ratio of the mass of a certain volume of oil to20°C, it varies between 0.900 and 0.905 (AFNOR, 1994). Our result showed that the essential oil density of *Rosmarinus eriocalyx* is 0.895g/cm.

The determination of the refractive index of *Rosmarinus eriocalyx* oil at 20°C is 1.464

pH

The hydrogen potential "pH" of *Rosmarinus eriocalyx* is 6.28

Identification of isolated strains

After the incubation of the dishes for the three strains, we identified:

Macroscopic identification

Staphylococcus aureus: fairly large colonies of about 1mm in diameter, round, regular, domed, smooth and shiny of the Smooth type. They are also cream, pigmented with a yellow color.

Escherichia coli: they are rounded, smooth colonies of a red or colorless color with regular edges 2 to 3mm in diameter.

Pseudomonas aeruginosa: these colonies are smooth, of average size of 1 to 2mm, with a domed central part and an irregular edge, shiny and mucous or even runny.

Microscopic identification

In the fresh state

Table 3: microscopic characteristics in the fresh state of the isolated strains

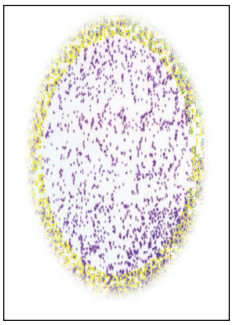
This table represents the microscopic characteristics of the different bacterial strains isolated

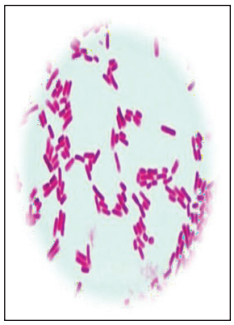
	Staphylococcus aureus	*Escherichia coli*	*Pseudomonas aeruginosa*
Form	Shell	Rod bacillus	Bacillus
Mobility	Motionless	Mobile	Very mobile
Mode of grouping	Motionless Bunch	Mono/diplobacillus	Isolated of grapes or diplobacillus

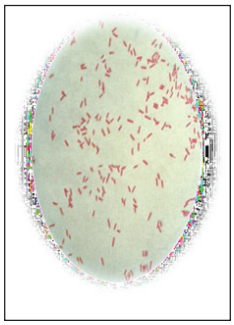
Table 4: Gram type of strains isolated

This table represents the gram type of each bacterial strain

Staphylococcus aureus	*Escherichia coli*	*Pseudomonas aeruginosa*
Gram typeGram-positive	Gram-negative	Gram-negative







Biochemical identification

Oxidase test

Staphylococcus aureus: absence of color change on the oxidase disc: oxidase negative

Escherichia coli: the absence of color on the oxidase disc: oxidase negative

Pseudomonas aeruginosa: presence of dark color on the disc: positive oxidase

Catalase test

Staphylococcus aureus: the appearance of gas bubbles on the drop of H_2O_2: positive catalase

Escherichia coli: presence of gas bubbles in the H_2O_2: positive catalase

Pseudomonas aeroginosa: the appearance of gas bubbles: positive catalase

Table 5: Catalase and Oxidase Biochemical test results

This table represents the biochemical characteristics

	Staphylococcus aureus	*Escherichia coli*	*Pseudomonas aeruginosa*
Oxidase test	-	-	+
Catalase test	+	+	+

Results of the antibacterial activity of essential oils against pathogenic bacteria "aromatogram"

These results showed us the zones of inhibition around the discs soaked in essential oils for the purpose of evaluating the activity of these oils vis-à-vis the strains tested.

Rosmarinus eriocalyx

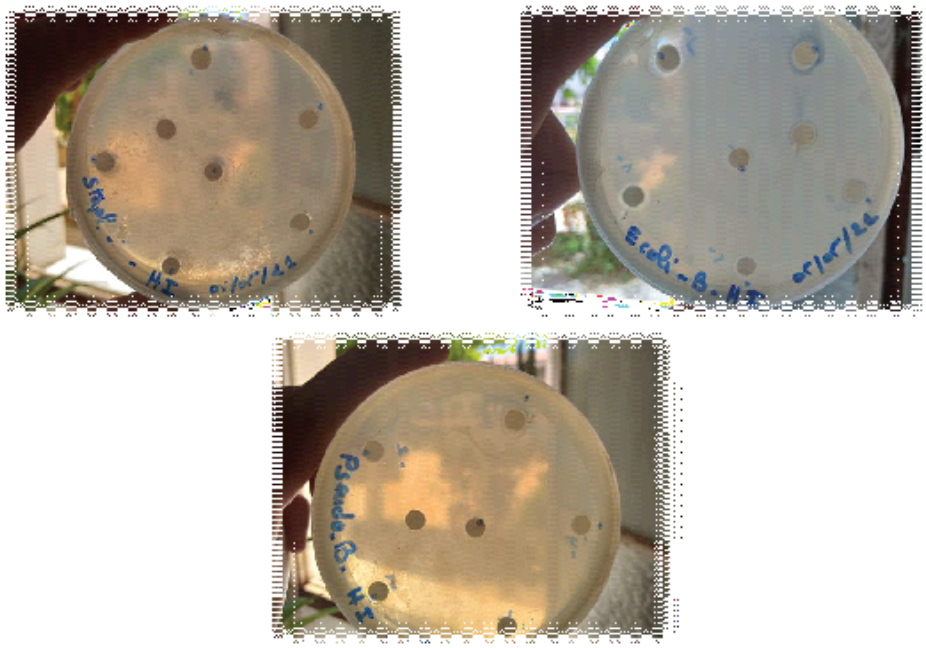


Fig. 1: Results of the effect of essential oil of *Rosmarinus eriocalyx* against pathogenic strains inoculated on MH medium at 37°C for 24 to 48 hours.

Rosmarinus officinalis

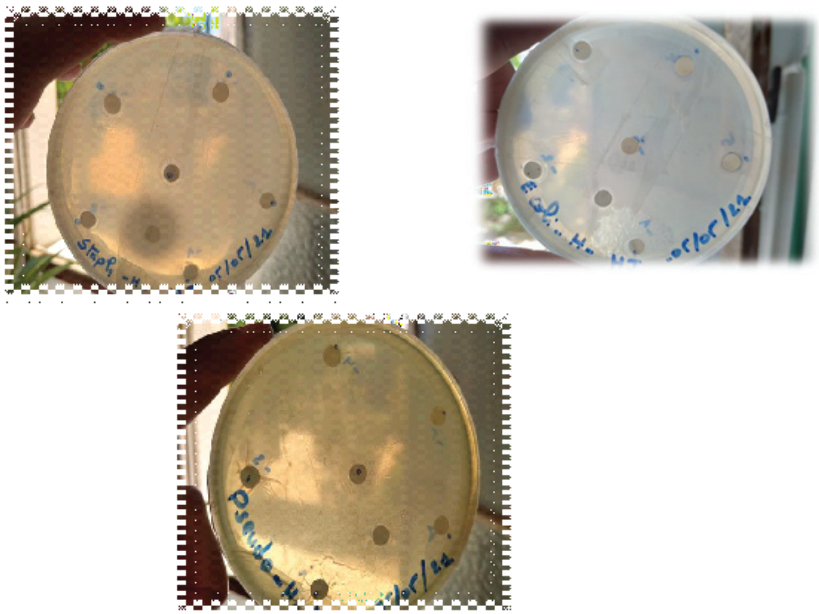


Fig. 2: Results of the effect of essential oil of *Rosmarinus officinalis* against pathogenic strains inoculated on MH medium at 37°C for 24 to 48 hours.

The results obtained from the aromatogram showed that *Staphylococcus aureus* and *Escherichia coli* were the most resistant bacteria to extracts of *Rosmarinus officinalis L* and *Rosmarinus eriocalyx* from both sources. Knowing that, *E.coli* has a strong resistance against the extract of *Rosmarinus eriocalyx* with a diameter of 2mm compared to the other extract of *Rosmarinus officinalis* with a diameter of 6 mm, on the other hand *Staphylococcus aureus* has a weak resistance to the extract of *Rosmarinus eriocalyx* with a diameter of 6mm compared to the other essential oil of *Rosmarinus officinalis* which represents a diameter of 3mm. On the other hand, the species of *Pseudomonas aeruginosa* marked a significant zone of inhibition of 19mm; therefore it has a high sensitivity compared to the essential oil of *Rosmarinus eriocalyx* on the other hand it has a lesser sensitivity vis-à-vis the extract of *Rosmarinus officinalis* by a diameter of 13 mm.

Previous studies on the essential oil of *Rosmarinus officinalis* reveal antimicrobial activity and indicate a similarity with the results obtained in the present work (Boutabia *et al*., 2016) compared to the strains studied; they noticed that bacteria (*Pseudomonas aeruginosa, E.coli*) represent inhibition diameters of (20, 2 mm) respectively while the results are not similar by the work presented by Bertella, (2019) they found that the *Staphylococcus aureus* bacteria is very sensitive vis-à-vis the two extracts tested by a diameter of 16.5 mm. Benbelaid *et al*. (2016) found that *Rosmarinus tournefortii* essential oil exhibited significant antibacterial activity against *Staphylococcus aureus* with an inhibition zone of 40 mm, so the results reported by Benbelaïd *et al.* (2016) and Bendeddouche *et al*. (2011) found that there is no antimicrobial activity of the essential oil of *Rosmarinus tournefortii* on *Pseudomonas aeruginosa* with an inhibition zone which was zero. While the results exhibited by Baratta *et al.*(1998) showed that rosemary essential oils destroyed *Pseudomonas aeruginosa*. Our results on the antibacterial activity of rosemary essential oils are consistent with those obtained by Lograda, (2014). The diameters of the zones of inhibition obtained do not exceed 20 mm, they are close to our results and reflect the modest or even weak antibacterial potential of the EO of *R. officinalis*. On the other hand, Djeddi and his colleagues (2007) reported the strong antibacterial activity of the essential oil of *R. officinalis* from the National Park of El Hamma (Algiers). On the other hand, according to Caillet and Lacroix, (2007*) S. aureus* and *E. coli* were moderately sensitive. Several studies have highlighted the high sensitivity of Gram (+) bacteria compared to Gram (-) bacteria (Falleh *et al*., 2008; Hayouni *et al*., 2007; Turkmen *et al*., 2007; Shan *et al*., 2007; Koné *et al*., 2004), This can be attributed to the difference in the outer layers of Gram (-) and Gram (+) bacteria. Gram (-) bacteria, apart from the cell membrane, have an additional layer the outer membrane, which consists of phospholipids, proteins and lipopolysaccharides, this membrane is impermeable to most molecules. Nevertheless, the presence of porins in this layer will allow the free diffusion of molecules with a molecular mass below 600 Da. However, inhibition of the growth of Gram (-) bacteria has been reported, particularly in combination with factors that may disturb cell integrity and/or membrane permeability, such as low pH values and increased NaCl concentrations (Georgantelis *et al*., 2007). The hypersensitivity of the *Staphylococcus aureus* strain ATCC can be explained by the probability of sensitivity of Gram (+) bacteria to external environmental changes, such as temperature, pH and natural extracts due to the absence of the outer membrane (Balentine *et al.*, 2006). Some studies reveal no selective antimicrobial activity against Gram (+) or Gram (–) bacteria (Guesmi and Boudabous, 2006). The resistance of the *Sterptocoque* sp strain can be attributed to the ability of the antibacterial agent to diffuse uniformly in the agar (Hayouni *et al*., 2007). The zone of inhibition increases considerably with the concentration of the extracts, which was also observed by Dordevic and his collaborators, (2007). Several classes of polyphenols such as phenolic acids, flavonoids and tannins serve as a defense mechanism in plants. against pathogenic micro-organisms, insects and herbivores (Falleh *et al*., 2008). Polyphenols, such as tannins and flavonoids like epigallocatechin, catechin, myricetin, quercetin, (Shan *et al*., 2007) and luteolin (Askun *et al*., 2009) are important antibacterial substances. Generally, all plants of the *Lamiaceae* family known for their phenolic compounds, have been

proven active against a variety of microorganisms (Gortzi *et al*., 2007). The antimicrobial activity of rosemary has also been attributed to rosmarinic acid, chlorogenic acid and caffeic acid (Tsai *et al*., 2007), carnosic acid and a few essential oil compounds, mainly borneol and camphor (Ramirez *et al*., 2007). The differences found can be attributed to several factors such as inherent factors (variety, ambient conditions, ecological factors, seasonal variations), extraction methods (Moreira *et al*., 2005; Sagdic and Ozcan 2003; Celiktas *et al*., 2007a, Turkmen *et al*., 2007). The antimicrobial action of HES takes place in three phases: Phase 1- The destruction of the bacterial wall by EO, causing an increase in permeability and then the loss of cell constituents. Phase 2- Acidification of the interior of the cell, blocking the production of cellular energy, and synthesis of structural components. Phase 3- Destruction of the genetic material, leading to the death of the bacteria. Several studies have shown that $Gram^-$ bacteria are supposed to be more resistant than $Gram^+$, this is due to structural differences in their outer membranes (Inouye *et al*., 2001; Lopez *et al*., 2005; Bozin *et al*., 2006), the penetration of active compounds present in HES is therefore different (Kheyer *et al*., 2014). In $Gram^-$ bacteria, the outer membrane constitutes an effective permeability barrier, rich in lipopolysaccharide whose negative surface charges prevent the diffusion of hydrophobic molecules (Nikaido, 2003), however, some low molecular weight phenolic compounds can adhere to these bacteria by attachment to membrane proteins and lipopolysaccharides using their functional groups and sneak up to the more vulnerable inner membrane (Dorman *et al*., 2000). In other words, hydrophobic compounds are capable of disrupting the plasma membrane and the outer membrane of $Gram^-$ bacteria by inducing its permeability and cell death (Wang *et al*., 2012).

CONCLUSION

In this work, we undertook a study on the antimicrobial activity of essential oils of *Rosmarinus officinalis* from a garden in Mezaourou and *Rosmarinus eriocalyx* is harvested at the level of a drill in Sidi Ali Benyoub of Sidi-Bel-Abbés. It can be seen that these two essential oils have a weak antibacterial activity against pathogenic strains (*Staphylococcus aureus* and *Escherichia coli*), on the other hand Pseudomonas aeruginosa was sensitive to the essential oil of *Rosmarinus eriocalyx* and *Rosmarinus officinalis*. This is why we can conclude that Rosemary has an antibacterial activity against pathogenic bacteria especially *Pseudomonas aeruginosa*, the results vary according to the type of plant material, its region and the dilutions of which they can present another effect, against these tested bacteria . This leads us to say that the essential oil of Rosemary could constitute a good basis of treatment for various problems due to the bacterial strain of *Pseudomonas aeruginosa*. All of these results obtained constitute only a first step in the search for biologically active substances of natural origin. A chemical analysis is desirable to obtain a more in-depth view of the qualitative and quantitative composition of these oils studied in order to highlight the therapeutic effect of this medicinal plant *Rosmarinus officinalis L* Finally, the results obtained on the antimicrobial activity made it possible to justify the traditional use of plant extracts from two plants chosen at the level of our region.

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Study on antimicrobial activity of the essential oil of *Achillea maritima* L collected from Oued Righa beach, the North East of Algeria

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ABSTRACT

In this work we tested the antibacterial and the anti yeast activity of the essential oil extracted from the plant Achillea maritima L., harvested from Oued Righa, Filfla, the state of Skikda, Algeria, towards seven bacterial strains of Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, and a yeast strain of Candida albicans. The content of total polyphenols in the plant methanolic exctract is 27.75 mg EGA / g methanolic extract whereas the rate of total flavonoids is 11.33 mg EQ/g methanolic extract. The essential oil exerted a weak antibacterial activity on Staphylococcus aureus showing an inhibition diameter zone of (9±0.27 mm), the rest of microbial strains was totally resistant to the tested essential oil. The minimal inhibitory concentration showed that the tested microbial strains could grow on all the dilutions. Our Achillea maritima L. essential oil has a weak antimicrobial activity, thereby it is recommended to do the extraction of essential oil using methods other than the hydrodistillation.

***Keywords*:** *Achillea maritima* L.*;* Antibacterial activity; Aromatogram; Essential oil; Minimal inhibitory concentration.

INTRODUCTION

Many bacterial strains have developed mechanisms of antibio-resistance. Therefore it is very necessary to search new antimicrobial agents from different sources (Chebaibi *et al*., 2016). Plants are a potential source of antimicrobials in many parts of the world (Alviano *et al*., 2009). Medicinal plants have been recognized for their use in traditional medicine practices since prehistoric times (Mirihagalla and Fernando, 2021). At the end of the XIXe century and the beginning of the XXe century many scientific research works have been related with the action of antiseptic actions of many essential oils (Chebaibi et *al*., 2016; Karaalp *et al*., 2009). People around the world, wildly use the medicinal plant *Achillea* L. in the treatment of various diseases (Yener *et al*., 2020). *Achillea maritima* L. is a species that lives in the maritime sands of the North of Algeria. It is an aromatic perennial herb, densely white lanate, with stems up to 50 cm, ascending, stout and woody. Leaves are 5–17 mm, oblong to oblong- lanceolate, entire or crenulate, fleshy and sessile. Inflorescence corymbose, with several medium capitula. It was reported to exhibit many biological ethnopharmacological activities such as the treatment of wound healing, bladder inflammation, wound healing, dysentery and digestive disorders like diarrhea and gastric gas (Lee *et al*., 2019; Muselli *et al*., 2007). It is also used as a decorating plant which has an effect against flying insects (Christodoulopoulou *et al*., 2005)

Our research aims to test the possibility of using the essential oil extracted from the plant *Achillea maritima* L. collected from Oued Righa beach, the state of Skikda, the North East of Algeria, in the treatment of some microbial infections caused by multiresistant bacteria and yeasts. It is the first time that the antimicrobial activity of the plant variant growing in Algeria was revealed.

MATERIALS AND METHODS

Vegetal material

The aerial parts of the plant (Fig.1) were collected in January 2021 from Oued Righa beach,

the town of Skikda, the North East of Algeria whose geographical coordinates are 36.9025, 6.8779. The plant was identified by Dr. Sakhraoui N., a botanist in the university of August 20th, 1955-Skikda. The collected parts were then dried and stocked in a dry and dark place

Extraction of essential oil

It was done by the hydrodistillation method, using a clvenger apparatus. 20 grams of the plant were cut into small pieces and placed in a ball of 500 ml impregnated with distilled water. The whole was boiled during 2 or 3 hours. Vapors charged with essential oil, traversing a refrigerant column, condenses in a clean beaker. The distillate was then decanted to separate the organic phase charged with essential oil from the aqueous phase. Water particles in the extracted essential oil were eliminated by adding magnesium sulfate. Pure essential oil was then preserved at 4°C, away from light, in small dark glass bottles (Makhloufi *et al*., 2012). The extraction yield was calculated using the following formula of Harbone (1998):

Extraction of oil yield= weight of the extract/ weight of the vegetal material) x 100.

Preparation of the methanolic extract

The aerial parts of the plant washed and dried at room temperature were grinded. Ten grams of the powder were macerated in methanol 70% and continuously stirred for 24 h. The obtained solution was applied onto a filter paper and poured into Petri dishes to be dried at 30°C for 48h. The obtained methanolic extract was used for the determination of total polyphenols and total flavonoids.

Dosage of total polyphenols

The concentration of polyphenolic content was determined using the method of Folin-Ciocalteu (Singleton, 1999). 1ml of Folin-Ciocalteu diluted 10 times, was added to 0.2 ml of the methanolic extract solution previously diluted in dimethyl sulfoxide (DMSO) at a concentration of 1mg/ml. 0.8 ml of Na_2Co_3 (7.5%) were then added to the mixture. The whole was incubated at room temperature during 2 hours. The absorbance of the mixture was measured using a spectrophotometer at 765nm, against a blanc without methanolic extract. The concentration of polyphenols was calculated from a linear calibration curve (y = ax + b) previously created with different concentrations of Gallic acid (0-1000mg/ml). The concentration of polyphenols was expressed in milligram of equivalent Gallic acid per gram of methanolic extract (mg EGA/g methanolic extract) (Boizot and Charpentier, 2006).

Dosage of total flavonoids

The concentration of total flavonoids was measured using the colometric method of aluminum chloride with some modifications (Brighente *et al*., 2007). 1 ml of the methanolic extract solution diluted in DMSO at a concentration of 1mg/ml as well as the dilutions of quercetin standard solution (20, 40, 60, 80 and 100 ug/ml) were added to 1 ml of $AlCl_3$ (2 % w/v). After an incubation period of 40 minutes at room temperature, the absorbance was measured at 430 nm against a blanc without methanolic extract. The concentration of total flavonoids was determined from a linear calibration curve of quercetin (y = ax + b). It was expressed in milligramg equivalent quercetin per gram of methanolic extract (mg EQ/g methanolic extract) (Bahorun *et al*., 1996).

Bacterial strains

Antimicrobial activity of *Achillea maritima* L. essential oil was tested against seven bacterial strains and a yeast strain. The pathogenic strains (02 strains of *Escherichia coli*, 01 strain of *Klebsiella pneumoniae*, 01 strain of *Pseudomonas aeruginosa* and 01 strain of *Staphylococcus aureus*) were provided by the laboratory of RENAD clinic, Skikda and the microbiological laboratory of Abderrazak Bouhara hospital, Skikda. The type strains *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were obtained from the hospitalian center of Louis Giorgi, Orange-France. *Candida albicans* ATCC 10231 was provided by the microbiological laboratory of the faculty of sciences, Skikda University.

Re-isolation of the pathogenic bacterial strains

To confirm the purity of the microbial strains, they were re-cultured in Hektoen medium for *Escherichia coli* strains and *Klebsiella pneumoniae*, nutrient agar for *Pseudomonas aeruginosa*, Chapman medium for *Staphylococcus aureus* strains and Sabouaud agar for *Candida albicans* ATCC 10231.

Evaluation of essential oil antimicrobial activity (Aromatogram)

The sensitivity of the bacterial strains towards *Achilllea maritima* L. essential oil was tested using the agar medium direct contact technique (Aromatogram) according to the following steps.

Preparation of mother solution

Essential oil was added to distilled water to obtain a mother solution concentration of 2560 ug/ml (Khadri , 2009). Tween 80 (0.05%) and ethylic alcohol 95% (2% v/v) were added to the mother solution to get a homogenate solution.

Preparation of standard inoculums

Re-isolated bacterial strains were firstly transplanted and incubated at 37° C for 18-24h. The yeast strain was incubated at 28 °C for 18-24h. The bacterial inoculums was prepared by diluting 2 or 3 separated colonies in physiological water to get a suspension of 0.08-0.1 absorbance read at 625 nm (0.5Mc Farland) (CA-SFM, 2012). The yeast suspension was standardized at an absorbance of 0.08-0.1 read at 449 nm.

Seeding

Muller Hinton medium was firstly poured in sterile Petri dishes. After solidification, the medium was seeded with the bacterial inoculums using a sterile swab. For the yeast strain Muller Hinton medium was replaced by Sabouraud agar. 6 mm sterile Wattman filter disks loaded with 10 ul of the essential oil mother solution were deposited aseptically. A control disc loaded of distilled water, tween 80 and ethylic alcohol was added as control disc. The plates were then incubated at 37° for 24h. The trial was performed in triplicates and the antimicrobial activity of the essential oil was evaluated after calculating the mean of the inhibition zones diameters as:

not sensitive (≤ 8 mm), sensitive (9-14 mm), very sensitive (15-19 mm) and extremely sensitive (≥ 20mm) (Moreira *et al*., 2005).

Preparation of mother solution and determination of minimal inhibitory concentration (MIC)

It was determined by the agar dilution method. Serial dilutions of the microial mother solution from 2560-10 ul/ml were prepared according to the method of dilution in geometric progression at a rate of 2 (1/2-1/256) (Khadri, 2009). In Petri dishes, 1 ml of each dilution was mixed with 9 ml of Muller Hinton or Sabouraud melted agar. After homogenizing the mixture, Petri dishes were inoculated with the same suspension prepared for the Aromatogram. The bacterial plates were incubated at 37°C for 24-48 h and the yeast plate at 28°C for 18-24h. The MIC was determined by the first concentration that inhibits the microbial growth.

Antibiogram of the tested strains

The sensitivity of the tested bacterial strains to *Achillea maritima* L. essential oil was compared with their sensitivity to the used antibiotics. Antibacterial susceptibility (Antibiorgam) of the bacterial strains to the tested antibiotics was determined according to the Comity of the Antibiogram-French Society CA-SFM (2010). Bacterial inoculums of 0.5 Mac Farland, an equivalent of 10^6 cells/ml, was prepared. Muller-Hinton medium was then seeded by the bacterial inoculums using the swabbing technique to get tight streaks. The antibiotic disks were deposited o the surface of the medium and the plates were incubated at 37 °C for 24 h. The bacterial strains were classified as resistant or sensitive depending on the inhibition zones obtained around the antibiotics disks (Comity of the Antibiogram-French Society CA-SFM, 2012).

Statistical analysis

The diameter of the inhibition zones was calculated in triplicates. The obtained values were presented as mean± Standard Deviation (SD) (Steel *et al*., 1997).

RESULTS AND DISCUSSION

Yield of essential oil extraction

The obtained essential oil was characterized by a blue color and a characteristic smell. The yield of extraction was 0.08%. It was superior than that of *Achillea maritima* L. collected from Corsica (0.02-0.06%) (Muselli *et al*., 2007). It was however weaker than the yield obtained from the essential oil of the same species collected from Portugal (0.3–0.4%) (Cabral *et al*., 2013). Comparing our results with those of El-Kalamouni *et al*. (2017) who worked on *Achillea millefolium*, we found that our

yield is very close to theirs (0.07%). Our results are inferiors than those of Lamamra (2018) who reported that the essential oil extracted from *Achillea santolinoides* harvested from Djelfa, Algeria was 2.19%. Hatem and his collaborators (2018) showed that the essential oil of *Achillea fragrantissima* gathered from the North of Bekaa-Lebanon, had a yield of 1.25%. The yields of essential oils were different despite the use of the same technique of extraction (Hydrodistillation). This is explained by the geographic origin of the plant, the environmental factors of the plant chemotype (Bruneton, 1993). The temperature, the rate of humidity, the soil composition and the sunshine duration are the main factors that affect the chemical composition of essential oils. Fadil *et al*. (2015) proved the effect of time of hydrodistillation and the drying duration on the maximization of essential oil yield.

Rate of total polyphenols

The polyphenols content was calculated from the linear calibration curve of Gallic acid whose equation is $y = 0.004x + 0.024$ with $R^2 = 0.996$ (Table1). The obtained results indicate that the rate of polyphenols in *Achillea maritima* L. was 27.75 mg EGA/g methanolic extract. This rate is inferior than that recorded by Haliloglu and his collaborators (2017), who reported that the methanolic extracts of flowers and leaves of *Achilleae sivasica* are characterized by polyphenolic rates of 54.7 and 51.1 mg EGA/g extract respectively. They are however superiors of the polyphenolic contents of flowers and leaves aqueous extracts of the same plant (22.7 and 17.9 mg EGA/g extract respectively) (Haliloglu *et al*., 2017).

Rate of total flavonoids

The total flavonoids content was calculated from the linear calibration curve whose the equation is $y = 0.009x + 0.048$ with $R^2 = 0.998$ (Table 1). We obtained a rate of 11.33 mg EQ/g methanolic extract. The recorded rate is less than the rate recorded by Haliloglu and his collaborators (2017) in the methanolic extract of leaves and flowers of *Achilleae sivasica* (31.4 and 27.1 mg EQ/g extract respectively). Our result was however superior than the total flavonoids concentrations found in the aqueous extract of leaves and flowers of *Achilleae sivasica* (4.4 and 3.7 mg EQ/ g extract respectively) (Haliloglu *et al*., 2017). It was noted that the method of extraction and of quantification may influence the rate of total polyphenols (Lee *et al*., 2003) as well as the genotypic and geographic differences (Khadri, 2019). The temperature applied during the hydrodistillation has a negative effect on the rate of polyphenols. The alcoolic extract however increases the extraction of polyphenols by increasing permeability of the cell wall and facilitating the extraction of polyphenols (Seidel, 2006). The plant physiological state during the harvest also affects the rate of polyphenols (Khadri, 2019).

The sensitivity of bacterial strains to the tested antibiotics

The obtained results (Table 2A & Table 2B) reveal that the strain of *Staphylococcus aureus* was resistant to 50% of antibiotics. *Escherichia coli* isolated from urine was resistant to 30% of antibiotics. The strain of *Escherichia coli* isolated from diabetic foot was however resistant to 56% of the tested antibiotics. *Pseudomonas aeruginosa* was characterized by a high rate of resistance (67%). The type strains were sensitive to all the applied antibiotics (100%). Most of the clinical bacterial strains are resistant to the applied antibiotics (Silver, 2011). Chromosomal mutations, transmission of resistance genes by conjugative plasmids, phages or transposons are the main causes of the bacterial antibio-resistance. This resistance leads to the research of new antimicrobial substances more efficient than the synthetic drugs and more tolerated by the human organism (Garcia-Ruiz *et al*., 2008; Kempf and Zeitouni, 2009).

Evaluation of antimicrobial activity of essential oil of *Achillea maritima* L.

The obtained results (Table 3 & Fig. 2) indicate that the extracted essential oil was active only towards *Staphylococcus aureus* which showed an inhibition zone of (9±0.27 mm) (Moreira *et al*., 2005). On the other hand, the clinical strains of *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and the type strains of *Escherichia coli ATCC* 25922, *Staphylococcus aureus* ATCC 29213 and *Candida albicans* ATCC

Table 1: Rate of total polyphenols and total flavonoids in the methanolic extract of *Achillea maritima* L.

	Concentration of total polyphenols (mg EGA/g methanolic extract)	Equation of the curve	R^2
Methanolic extract	27.75	y = 0.004x + 0.024	0.996
	Concentration of total flavonoids (mg EQ/g methanolic extract)	**Equation of the curve**	**R²**
	11.33	y = 0.009x + 0.048	0.998

Table 2A: Sensitivity of the tested Enterobacterial strains and *Pseudomonas aeruginosa* to the tested antibiotics

Bacteria **Antibiotics**	*Eshcerichia coli* isolated from urine	*Escherichia coli* isolated from diabetic foot (pus)	*Escherichia coli* ATCC 25922	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*
COT	R	R	S	R	/
AMX	R	R	S	R	R
CZ	S	R	S	R	S
FO	S	S	S	S	R
CIP	S	R	S	S	S
AMC	S	R	S	R	R
NIT	S	S	S	S	S
FOX	/	/	/	/	R
P	/	/	/	/	R
TE	/	S	S	/	/
AMP	/	S	S	/	R

Table 2B: Sensitivity of the tested staphylococci strains to the tested antibiotics

Bacteria **Antibiotics**	***Staphylococcus aureus***	***Staphylococcus aureus*** **ATCC 29213**
COT	/	/
AMX	/	/
CZ	/	/
FO	/	/
CIP	/	/
AMC	/	/
NIT	/	/
FOX	R	S
P	S	S
TE	S	S
AMP	R	S

S:sensitive, R: resistant, / not tested

COT: Cotrimoxazole, AMX: Amoxicilline, CZ: Cefazolin, FO: Fosfomycine, CIP: Ciprofloxacine, AMC: Amoxicilline + acide clavulanique, NIT: Nitrofurantoin, FOX: Cefoxitine, p: Pénicilline, TE: Tetracycline, AMP: Ampicilline

Table 3: Sensitivity of the microbial strains to the essential oil of *Achillea maritima* L. (Aromatogram)

	Aromatogram	
Microbial strain	**Inhibition zone (mm)**	**Sensitivity**
***E. coli* (pus)**	6 ± 0.43	-
***E. coli* (urine)**	6 ±0.5	-
***E. coli ATCC* 25922**	6 ±0.3	-
S. aureus	**9 ±0.27**	+
***S. aureus* ATCC 29213**	6 ±0.1	-
K. pneumonia	7 ±0.5	-
P. aeruginosa	6 ±0.35	-
***C. albicans* ATCC 10231**	6 ±0.35	-
Control disc	**6 ±0.5**	-

- not sensitive (≤ 8mm), + sensitive (9-14 mm)

10231 grew without any inhibition power around the essential oil discs. The obtained inhibition zones were 6± 0.1-7±0.5mm. Cabral and his collaborators (2013) in turn showed weak antimicrobial activity of *Achillea maritima* L. against the same tested species of *Candida* (*C. albicans* ATCC 10231). The results of El-kalamouni *et al*. (2017) on *Achillea millefolium* collected from France are also in agreement with ours. They recorded an inhibion zone of 9.1 mm against *Staphylococcus aureus* and no antimicrobial activity was found against *Escherichia coli.* Daniel *et al*. (2020) also reported a weak antimicrobial activity of *Achillea millefolium* L. essential oil against *Escherichia coli, Staphylococcus epidermidis Candida. albicans* and *Klebsiella pneumoniae. Achillea fragrantissima* was however characterized by a considerable antimicrobial activity against *Staphylococcus aureus, Escherichia coli, Enterobacter faecalis*, *Salmonella enteritidis, Pseudomonas aeruginosa* and *Candida albicans* with inhibition zones of 45 mm, 40mm, 38mm, 31mm, 30mm and 15mm respectively (Hatem *et al*., 2018). Previous studies confirmed that essential oils have pronounced antimicrobial activities against Gram positive bacteria in comparison with Gram negative bacteria. This is awarded to the external hydrophilic membrane of Gram negative bacteria which prevents the penetration of hydrophobic compounds to the targeted cell (Wan, 1998).

Minimal inhibitory concentration of essential oil of *Achillea maritima* L.

The obtained results were confirmed quantitatively by the Minimal Inhibitory Concentration (MIC) in solid medium (Fig. 3 & Fig. 4). The tested strains grew at all the concentrations (1/2-1/256). Our results are in contradiction with those of El-Kalamouni et *al.* (2017) which revealed that essential oil of *Achillea millefolium* L. was characterized by a minimal inhibitory concentration of 120ug/ml against *Staphylococcus aureus*. Yener *et al*. (2020) confirmed that *Achillea vermicularis* of Turkey exerts a strong antimicrobial activity against *Escherichia coli* with a minimal inhibitory concentration of 30ug/ml. Essential oil of *Achillea millefolium* collected from Bresil showed a minimal inhibitory concentration of 30 mg/ml against *Escherichia coli, Klebsiella pneumoniae, Staphylococcus epidermidis* and *Candida albicans* (Daniel *et al*., 2020).

The essential oil of *Achillea maritima* L. collected from Oued Righa the state of Skikda, Algeria did not show a significant antimicrobial activity. This is due to many factors among which we cite the low concentration of polyphenols known for their antimicrobial effects like sabinene, 1,8-cineole and the camphor (Pattnaik, 1995), the loss of essential oil volatile compounds during the storage and the extraction (Chemloul, 2014), the low diffusion of the extracted essential oil into the agar and the period of collection.

Fig.1:Aerial parts of *Achillea maritima* L. collected from Oued Righa, Skikda

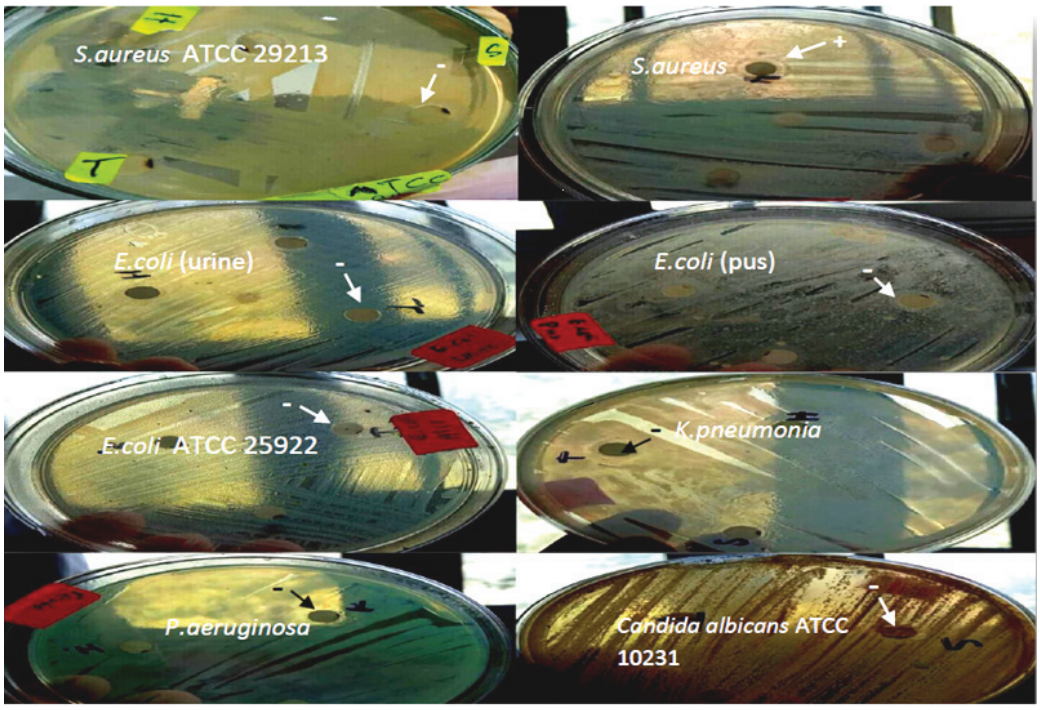

Fig. 2: Antimicrobial activity of *Achillea maritima* L. essential oil using the method of Aromatogram
- not sensitive (inhibition zone ≤ 8mm), + sensitive (inhibition zone 9-14 mm)

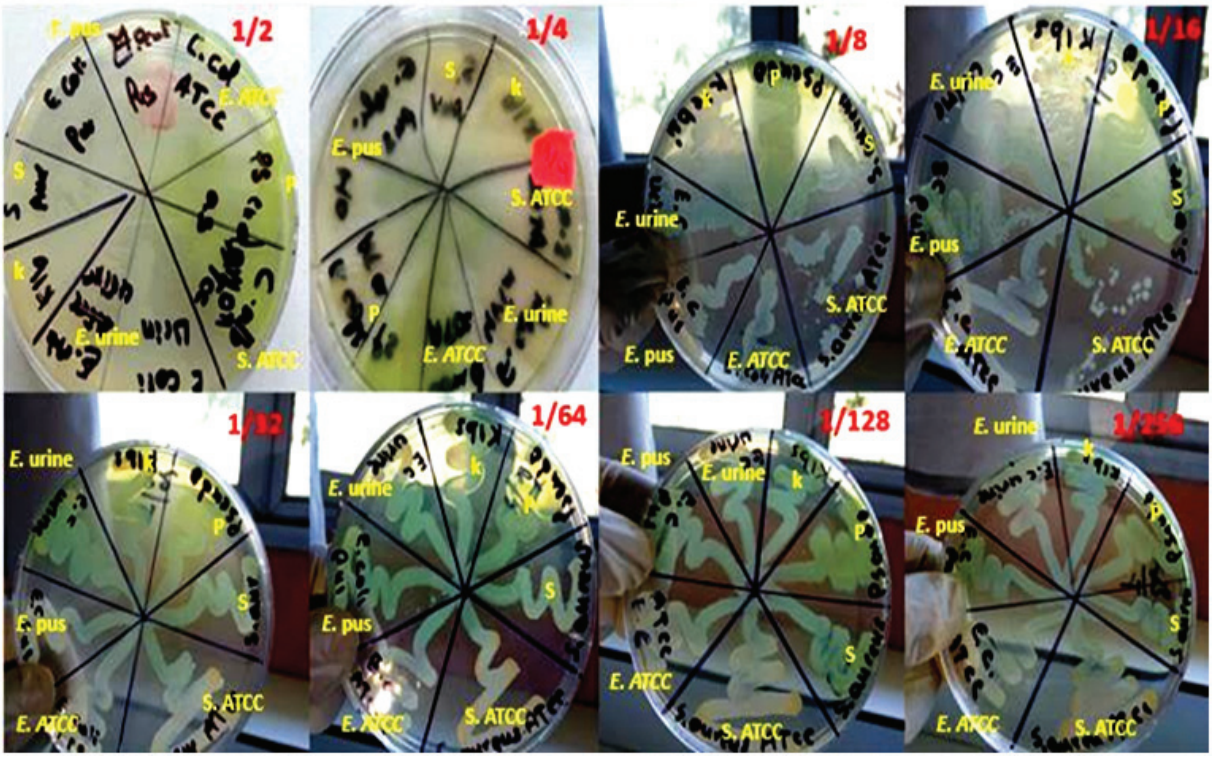

Fig. 3: Minimal inhibitory concentration of *Achillea maritima* L essential oil towards the tested bacterial strains.
E.pus: *Escherichia coli* isolated from pus, E.urine: *Escherichia coli* isolated from urine, E. ATCC: *Escherichia coli* ATCC 25922, K: *Klebsiella pneumonia*, P: *Pseudomonas aeruginosa*, S: *Staphylococcus aureus*, S. ATCC: *Staphylococcus aureus* ATCC 29213

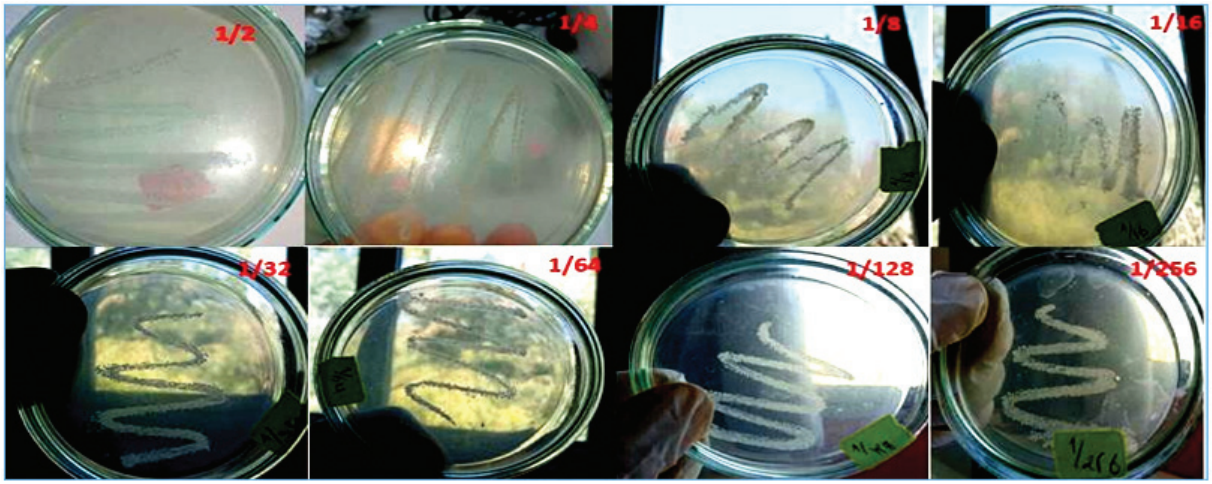

Fig. 4: Minimal inhibitory concentration (MIC) of *Achillea maritima* L. essential oil towards the tested *Candida albicans* ATCC 10231

Gas chromatography analysis showed that the essential oils of *Achillea maritima* L. aerial parts collected from different beaches in Portugal are mainly composed of chrysanthenone (40.4–57.2%), filifolone (12.2–15.5%), cischrysantenyl acetate (10.1–12.2%) and alpha-pinene (6.7–7.2%) (Cabral *et al*., 2013). Romeo *et al*. (2007) revealed that the major volatile components extracted from *Achillea maritima* L. aerial headspace, collected from Messina (Italy) and analyzed by gas chromatography-Mass spectrometry, are santolina triene (24.35%), camphor (17.91%) and artemisyl acetate (13.19%). Gas chromatography and gaz chromatography-mass spectrometry analysis of the oils obtained by hydrodistillation of the headspace parts of *Achillea maritima* L. growing in Greece, showed that the major components of the oils are chrysanthenyl acetate (30.4%); camphor (12.9%) and artemisia alcohol (12.6%) (Tsoukatou *et al*., 2000). In fact, an essential oil chemical composition varies according to the plant vegetative cycle (Khadri, 2019) in addition to the plant chemotype.

CONCLUSION

The sensitivity of some microbial strains towards the essential oil extracted from *Achillea maritima* L. collected from Oued Righa the state of Skikda Algeria was tested for the first time. The studied essential oil exerts a weak antimicrobial activity. *Staphylococcus aureus* is the only sensitive bacterial strain to the action of *Achillea maritima* L. essential oil. The obtained results of the aromatogram were confirmed by the minimal inhibitory concentration where the strains could grow at all the concentrations (1/2-256). Thereby it could be interesting to take in consideration other aspects among which:

- The extraction of essential oil using methods other than the hydrodistillation
- The extraction, the purification, the identification and the dosage of active components
- Testing the antimicrobial activity towards other pathogenic microbial strains.

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Phytotherapy of urinary calculi: the mass reduction of calcium oxalate stones in vitro by the aqueous extract of *Urtica dioica* l.

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ABSTRACT

The purpose of this study is to evaluate in vitro the effect of an aqueous extract of Urtica dioica on the dissolution of oxalocalcic type kidney stones at the mesoscopic scale. The weight of the stones used in our experiment varied from: 0.0625g to 1.1049g. Type identification of kidney stone samples is performed by Infrared spectroscopic analysis. The presence of carboxylate ion of calcium oxalate is highlighted by absorption bands in the 1312.41 cm^{-1} and 1606.36 cm^{-1} areas. The aqueous extract of the aerial part of the plant Urtica dioica was prepared by infusion for 30 min of 5g of powder in 100 ml of saline solution (9 g/L of NaCl), previously brought to the boiling point, and there was then filtered. The stones were left in contact with the extract for 6 weeks, under constant magnetic stirring in 50 ml of aqueous extract. Kinetic evolution of the pH and the evaluation of the dissolution capacity of the extracts were carried out every week. The results obtained are very satisfactory where we observe a loss of mass which increases with time in order to reach a rate of 63%. This confirms the dissolution of stones and the increase of pH by the effect of the presence of the base of calcium oxalate in the aqueous medium. According to this study, we emphasize the need to suggest Urtica dioica as a means to reduce the occurrence of this urological disease and to establish less expensive tests and treatments.

***Keywords*:** Calcium oxalate, dissolution rate, kidney stones, urticadioica

INTRODUCTION

Renal lithiasis is a disease that affects from 4 to 20% of the population in different countries, characterized by the formation of crystals in the kidneys (El Habbani *et al.,* 2021). It is a multifactorial disease due to a sequence of physicochemical steps, in particular supersaturation, nucleation, growth, aggregation, and retention in the renal tubules (Ammork *et al.,* 2020).The recurrence rate of this pathology is 70-81% in men compared to 47-60% in women (Annand *et al.,* 2021). Extra corporeal shock wave lithotripsy (ESWL) is the treatment of choice for this disease, but this technique causes acute kidney damage and small fragments of unremoved crystals (Parveen *et al.,* 2021). In Algeria, alternative medicine is still widely used by the population with the use of medicinal plants based on recommendations made here and there (Dif *et al.,* 2022). Therefore, we proposed to evaluate in vitro the effect of an aqueous extract of *Urtica dioica* on the dissolution of oxalocalcic type kidney stones.

MATERIALS AND METHODS

Urinary stones

Samples of kidney stones were eliminated spontaneously in patients suffering from urinary lithiasis, after shock wave treatment at the urology

department of the University Hospital of Sidi-Bel-Abbes. These stone samples were washed with distilled water, air-dried on filter paper, and stored in sterile containers. The weight of the stones used in our experiment varied from: 0.0625g to 1.1049g. Type identification of kidney stone samples is performed by Infrared spectroscopic analysis on a Mattson Genesis II FTIR instrument. The samples were processed as KBr pellets (Singh, 2008).

Plant material

The plant studied belongs to the Urticaceae family under the scientific name: *Urtica dioica*. We are interested in this study in the aerial part of the plant. The plant is harvested at the level of the forest of the Ex-ITMA, wilaya of Sidi-Bel-Abbes, during the flowering period: February-April 2019. The aerial part (leaves) was dried at room temperature and protected from light for a period of 4 months; then reduced to powder and sieved. The powder is stored in an airtight glass bottle protected from light. The identification of the plant *Urtica dioica* was done with the help of a specialist in Botany of the laboratory of Water Resources and Environment at the University of SAIDA.

Extraction process

Our experimental work was carried out at the laboratory of Agronomic Sciences, Faculty of Natural Sciences and Life, and laboratory organic physical chemistry and Macromolecular LCPOM of the University Djillali Liabès of Sidi-Bel-Abbes. The extract of *Urtica dioica* leaves was prepared by infusion for 30 min of 5g of powder in 100 ml of a physiological aqueous solution of sodium chloride (NaCl) at 9g /L, previously brought to boiling point (Hannache *et al.,* 2012). The extract was then filtered.

Experimental device

The aqueous extract of the plant (*Urtica dioica*) is used to demonstrate, in vitro, the dissolving power of urinary stones at the mesoscopic scale, by putting the stones in the presence of 50 ml of each sample at room temperature under constant magnetic stirring (130 rpm). The stones were left in contact with the extract for 6 weeks, which is the recommended treatment period in traditional medicine (Meiouet *et al.,* 2011). Each week, the stones are removed from the different solutions, washed with distilled water, dried at 40°C for 18 h in an oven, and then weighed with a 10^{-4} g precision balance to evaluate the mass loss. The sample is put back into the extract with the same conditions to count the second week, until the sixth week of the experiment. The study is performed on three samples of the same type of kidney stones. In parallel, the same experiment was performed, where physiological saline water (NaCl 9g/l) was used instead of *Urtica dioica* extracts. Furthermore, the dissolution of the urinary calculi in the extract is monitored by evaluating the pH values during six weeks of immersion with agitation and at room temperature. The pH is measured weekly using a pH meter with a 10^{-4}g precision scale. The Ca^{+2} and Mg^{+2} ions were determined according to the method described by Rodier *et al*. (2009), before and after the six weeks contact of calcium oxalate stones with the aqueous extract, by an EDTA solution containing ethylene diamine tetra acetate ion in the basic medium of ph=10.

RESULTS AND DISCUSSION

Analysis of kidney stones

The presence of oxalate is highlighted by absorption bands in the 1600 cm^{-1} and 1300 cm^{-1} areas (Fig. 1). The oxalocalcic dependent species is hyperoxaluria represents the major cause of urinary lithiasis, among its causes, is the excessive consumption of food rich in oxalate as tea. Urinary lithiasis ranks third among the most common urologic diseases (Wigner *et al*., 2022). With a wide range of sizes, kidney stones can have a diameter ranging from a few millimeters to several centimeters (Idm'hand *et al*., 2019). These require other treatments and techniques such as ESWL (Kim *et al*., 2020). On the other hand**,** Ahmed *et al.* (2018) stated that ESWL is less effective for calcium oxalate monohydrate (COM) stones.

The spectrum below shows two characteristic bounds, the first one around 1312.41 cm^{-1} of the C-O-C bond due to the presence of the carboxylate ions and the second one around 1606.36cm^{-1} of the carbonyl function C=O which confirms the presence of the carboxylate ion of calcium oxalate. The large bung presented between 3000 and 3500cm^{-1} is due to the presence of humidity from 50 to 75%. The IR spectrum data confirms that our urinary stone samples used in this study are of the oxalocalcic type.

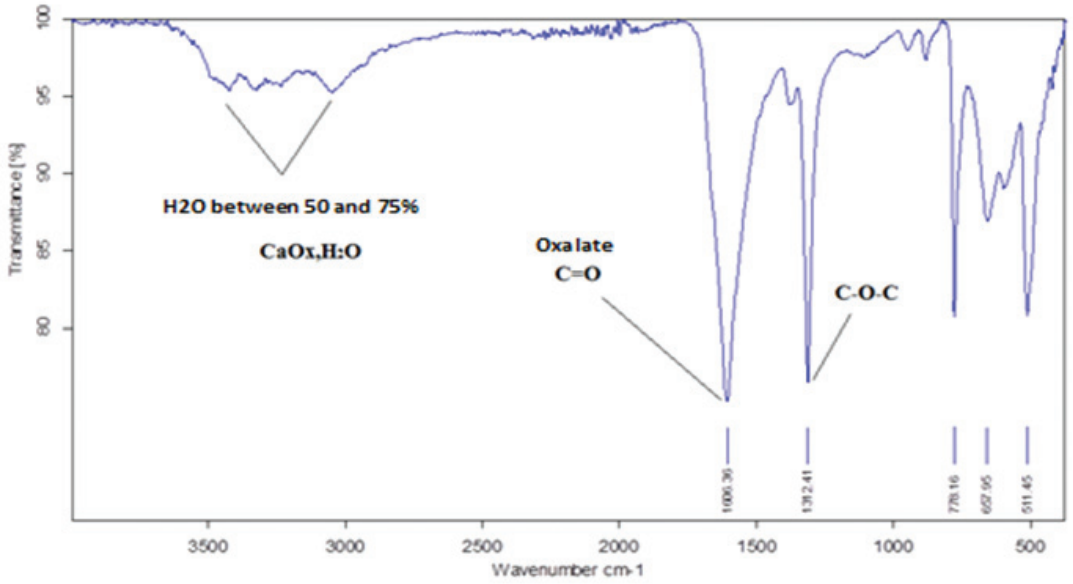


Fig. 1: FT-IR analysis of urinary stones

Kinetic evolution of ph and evaluation of the dissolution capacity of extracts

Herbal medicine is an integral part of the cultural practices of our local communities, which constitute an important heritage for the management of urological diseases (Bencheikh *et al.,* 2021; Guerrouj *et al.,* 2021). In recent years, various plants and traditional medicines have been proposed to reduce calcium oxalate kidney stones, which can be useful in its prevention and treatment (Afkari *et al.,* 2019). According to recent ethnobotanical studies in Algeria, the infusion of leaves is the most used practice (Belhouala *et al*., 2021; Zatout *et al.,* 2021). As well, pharmacological research on medicinal plants used in anti-lithiasis therapy has revealed their therapeutic potential in vitro models (Manasa Reddy *et al.,* 2018). The complete mechanisms of actions of medicinal plants are not well known, but herbal medicine is more effective in the treatment of urolithiasis (Chandel *et al.,* 2019).

The pH values are obtained at the beginning and at the end of the experiment for each week during the whole duration of our study (Figure 2).The urinary stone samples used in this study are of the calcium oxalate type.

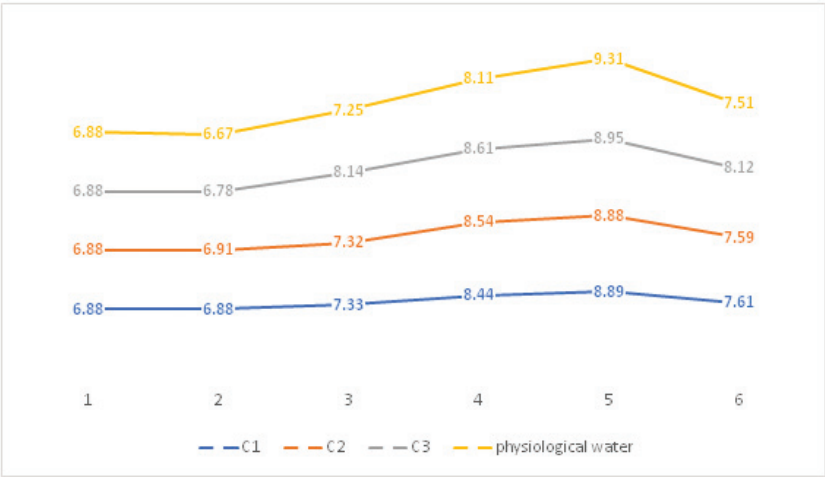


Fig. 2: pH evaluation

We observed a very important increase in the pH values and the highest value of 9, 31 where we find the greatest efficiency. The pH in physiological water remains almost constant where we can say that the properties do not change during the experiment (no dissolution of kidney stones in physiological water).

The dissolution capacity of the extracts (A%) was evaluated by the mass lost from the samples of the kidney stones by the effect of the tested extracts in a period of one week. The formula used to calculate the dissolution capacity in our extract (A%) is:

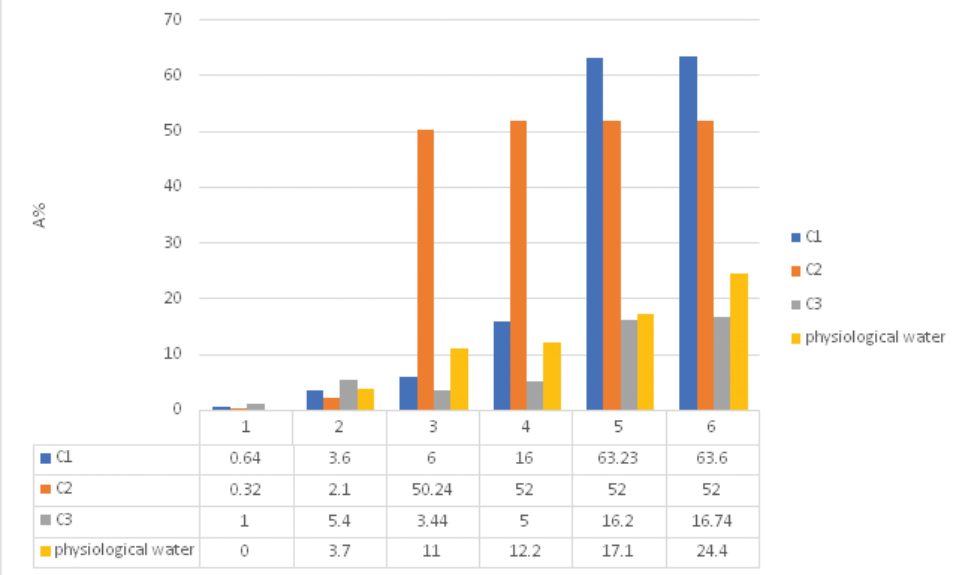
$$A\% = \frac{\Delta m}{m_{initial}} \times 100$$

A%: The dissolution rate of the kidney stones

Δm : The lost in a week

$m_{initial}$:The mass of the initial sample0

The different results of the dissolution rates in the experimental medium with the sample masses of the kidney stonesare shown in the Fig. 3.



	1	2	3	4	5	6
C1	0.64	3.6	6	16	63.23	63.6
C2	0.32	2.1	50.24	52	52	52
C3	1	5.4	3.44	5	16.2	16.74
physiological water	0	3.7	11	12.2	17.1	24.4

Fig. 3: Dissolution capacity of extracts

The results show a rapid evaluation for the C2 stone sample, a rate of 50% is obtained in the third week and the maximum in the fourth week. The sample C1 has a good dissociation but the dissolution takes five weeks with a rate of 63%. Sample C3 does not give good results because it is very bulky.

The results of the measurements of the concentration of calcium Ca^{2+} and magnesium Mg^{2+} ions, in the extract solution before and after the emersion of the stones obtained are presented in the Table 1:

Table 1: Concentration of Ca^{2+} and Mg^{2+} ions

	$[Ca^{2+}] + [Mg^{2+}]$ mmol/l	
Aqueous extract of Urtica dioica	Before	After
	15	900

The possible mechanism by which *Urtica dioica* affects calcium oxalate could be the result of several valuable chemical compounds, such as phenolic compounds (such as chlorogenic acid, ferulic acid, ellagic acid, naringin, myricetin, rutin), flavonoids (such as kaempferol), tannins, coumarins (e.g., scopoletin), lignans (secoisolariciresinol, 9,9-bisacetyl-neo-olivil and their glucosides), phytosterols (e.g., β-sitosterol), fatty acids, polysaccharides, isolectins, triterpenic acids and

monoterpendiols Taibi *et al.,* 2021). Studies have shown that the main bioactive of *Urtica dioica* are flavonoids, anthocyanins, and saponins (Nirumand *et al.,* 2018), which could inhibit calcium and oxalate deposition and crystal growth by disintegrating mucoproteins (Al-Assaf *et al*., 2020). In our study, the dissolution or degradation of the stones in the medium leads to the release of calcium oxalate ions of basic character. The presence of oxalate will increase the pH of the medium. The experimental data show a positive evaluation of pH with the loss of mass of the studied urinary stones. Tannins and polyphenols inhibit CaO_x crystal formation and dissolve preformed CaO_x crystals by promoting calcium complexation (Bawari *et al.,* 2018). The results obtained justify well the evaluation of the dissolution of the stones in the extract of our plant. We observe a very important increase in the concentration of Ca^{2+} and Mg^{2+} ions in the solution of the extract after emersion of the stones during the six weeks. Flavonoids could effectively inhibit CaO_x stone formation in vitro, correlating with their diuretic, antioxidant, and anti-inflammatory properties (Zeng *et al.,* 2018). According to the study of Taïbi *et al.* (2021) in the Tiaret region of Algeria, the medicinal benefits of *Urtica dioica* are related to its nutritive, depurative, diuretic, antioxidant, anti-inflammatory, and stimulating effects. Antioxidant therapy could be one of the effective methods to prevent the nucleation and fixation and growth of calcium oxalate crystals (Keles *et al.,* 2020). The process of calcium oxalate crystallization is of great interest in medicine, it is the main component of 75-80% of kidney stones (Khan *et al.,* 2021). A more negative crystallite surface inhibits the growth and agglomeration of urinary crystals (Sun *et al.,* 2017). The study of Polat (2019) shows that *Urtica dioica* extracts can reduce the size of calcium oxalate crystals leading to the formation of COD (calcium oxalate dihydrate: $Ca\text{-}C_2O_4\text{-}2H_2O$, weddellite) and inhibiting the growth and aggregation of COM (calcium oxalate monohydrate: $CaC_2O_4\text{-}H_2O$, whewellite) crystals. Furthermore, Polat, (2019) indicates that the surface of the crystals becomes negative due to the coverage of the crystal surface by the negatively charged *Urtica dioica* ions, which induces the increase of the zeta potential over time.

CONCLUSION

The present study shows the efficacy of the aqueous extract of *Urtica dioica* in dissolving oxalocalcic type urinary stones in vitro. Therefore, we emphasize the need to suggest *Urtica dioica* as a means to reduce the occurrence of this disease and to establish less expensive tests and treatments. However, further studies are needed to determine the mechanism of action of this plant in preventing kidney stone formation in vivo.

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Bio-chemical study of essential oils of *Thymus vulgaris* on pathogenic strains responsible for urinary tract infections

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ABSTRACT

The present work investigates the chemical composition; the essential oils antioxidant and antibacterial activities of Thymus vulgaris on the pathogenic bacteria strains responsible of the urinary tract infections. The essential oils obtained by the hydrodistilation of studied plants areal parts; were analyzed by GC/MS. The essential oil'antioxidant activity capacity was measured using: the DPPHand the FRAP methods, while the antibacterial activity was determined by the agar well diffusion method against twelve pathogenic strains, responsible of the urinary tract infections. For the DPPH and the FRAP tests, The Thymus vulgaris from the two Algerian regions presented a very interested activity. Moreover, it was found that the studied plants essential oils exhibited an important resistance against the most of the pathogenic bacteria tested. The relation study between the essential oils chemical composition, the antibacterial and the antioxidant activities, reveal the presence of different strong correlation with some major identified compounds. As a conclusion, the tested essential oils in this study showed an interesting strong antioxidant, and antibacterial activities. These effective activities are due to the presence of several chemical compounds.

***Keywords*:** Antibacterial activity, antioxidant activity, essentials oils, *Thymus vulgaris,* urinary tract infections.

INTRODUCTION

Urinary tract infections are a common reason for consultation and prescribing in general medicine. They are considered as the second site of bacterial infection after lung infections (Barrier Letertre, 2014). They are also, one of the most common infections in both outside and in hospital. Several studies showed that these infections affect about 40% to 50% of women (Dauquanand Abdullah, 2017; Giordani *et al.,* 2008). A portion of the world's population uses traditional medicine to heal. This widespread use is explained by, its accessibility and availability of this medicine in developing countries on the one side, and the harmful effects of synthetic drugs on the other side (Kanoun *et al.,*2020).The natural resources uses, particularly medicinal plants becomes very important and interesting for the new antibacterial products search; more effective in view of the resistant strains emergence to antibiotics (Lavigne, 2007; Prasanth *et al.,* 2014). Medicinal plants becomes important for pharmalogical research and drug development or production, not only when plant components are directly used as therapy agent, but also as materials for drug synthesis or as models for pharmacologically compounds (Burt, 2004).

Algeria, by its climatic diversity and its geographical situation, possesses a considerable set of natural species that represents a great importance phylogenetic patrimony, considering their spatial distribution and their role in ecological equilibrium (Ruberto and Barrata, 2000). The studies indicated that the essential oils of *Thymus vulgaris* have an antioxidant proprieties by protecting cells against the free radicals damaging impact (Hussain *et al.,* 2011). The aim of this study was to investigate the chemical composition of essential oils of *Thymus*

vulgaris and its antimicrobial activity collected from two regions (Chlef and Sidi-Bel-Abbes), against a pathogenic strains responsible of urinary tract infections. The antioxidant capacity tests such as FRAP and DPPH scavenging activity are also demonstrated. Finally, the correlation between the *Thymus vulgaris* essential oils chemical composition and its antibacterial and antioxidant activities was explored by a statistical analysis, which was carried out, in order to determine the natural chemical compounds responsible for the different essential oils activities.

MATERIALS AND METHODS

Plant material

This study was performed on the *Thymus vulgaris* leaves harvested from Medajaja region (Chlef) and Tessala region (Sidi-Bel-Abbes) (Algerian west), during the flowering stage (late May and early June 2019). Two samples from two different regions were collected in order to compare their essential oils composition differences, as well as their antioxidant and antibacterial activities. The plant samples were identified by the regional flora; as well as by floristic and taxonomic references. Once harvested, The *Thymus vulgaris* fresh leaves were air-dried for 20 days. After drying, the leaves are ground with an electric grinder until a fine powder was obtained, which was kept in bottles and stored, away from light and humidity until use.

Used bacteria

All clinical strains were obtained from the Chlef Hospital Microbiology Laboratory, which are involved in urinary tract infections. These microorganisms were identified using conventional, morphological and biochemical tests. The stored bacterial cultures were maintained in PBS (Phosphate Buffered Saline), with 20% glycerol at -70°C. The essential oils used in this research were extracted by hydrodistillation using a Clevenger apparatus. For this, a 50g sample of each plant powder was mixed with 500 ml of distilled water, and then placed in the hydrodistillation instrument for 3 hours. The extracted essential oils were then dried on anhydrous sodium sulfate and then stored in dark glass bottles at +4°C until use (Mansour *et al.,* 2021).

Essential oils analysis by GC–MS

The essential oils chemical composition was determined, by a gas chromatography coupled with Mass Spectrometry GC/MS type Perkin Elmer 500, equipped with a capillary column Elite Series 5 % Phenyl Dimethyl polysiloxane (30 m x 0, 25 mm), with a film thickness of 0.25 mm, and the a split injector was calibrated at 250°C. The injection mode is split (leak ratio: 1/50, flow rate: 66 ml / min). Samples are diluted in methanol (1/20 v/ v), 2 ml constituting a manual injection (Hussain *et al.,* 2011). The gas used is the helium with 1 ml/ min of flow rate/min. The column temperature was programmed from 60 to 275°C (Burits and Bucar, 2000), the fragments are carried out by electronic impact under 70 EV field, with abalayage of 80 to 600 Uma, a quadruple analyzer and a solvent's delay: 5.90 mn. The component identification was based on Kovats indices (KI) and the equipment was connected to a computer system that manages a library of NIST 98 mass spectra.

Antimicrobial activity determination

The antibacterial activity of essential oils was identified by the agar well diffusion, approach as reported by Rather *et al*. (2012). Firstly, the clinical strains were cultured in Brain Heart Infusion (BHI) broth at 37°C for 24h. After incubation, each bacterial suspensions was adjusted to *turbidity* standard of *0.5 McFarland units* in the same medium .0.1ml from the bacterial suspension was uniformly spread on the agar Mueller Hinton surface, the Petridishes were then dried. Moreover, 5 mm diameter wells were formed on the petridishes surfaces under aseptic conditions, 50 µl of each essential oils were applied in the well formed on the inoculated Petridishes; which were incubated at 37°C for 24h. After incubation, The inhibition zones diameters were measured in mm, including the well diameter (5mm) (Burits and Bucar, 2000; Oyaizu,1986). All tests carried out in triplicate, and the results are expressed as the zone diameter mean values.

The Minimum Inhibitory and Bactericidal Concentrations were determined using a dilution test. The Minimum Inhibitory Concentrations (MICs) are defined as the lowest concentration of an antibacterial agent, that inhibits the visible growth of a microorganism after an overnight incubation, for the Minimum Bactericidal Concentrations (MBCs), are defined as the lowest concentration of an antibacterial, that kill the

microorganism after sub-culturing on the antibacterial agent-free medium. These methods are based on those described by Ericsonand Sherris (1971). In the dilution test, the microorganisms are tested for their capacity to generate a visible growth on a series of agar plate (agar dilution) or in a broth (broth dilution) containing the antibacterial agent dilutions (in mg/l) which, under defined *in vitro* conditions, inhibits the appearance of the microorganism visible growth, within a defined time period, is known as the Minimum Inhibitory Concentration (MIC).

A volume of bacterial suspension equal to the diluted antibacterial solution volume is added to each antibacterial agent tube or well. The periodic viability counts should be performed on the inoculum suspensions to ensure that the inoculums contain approximately 5x10u CFU/ml (colony forming units per milliliter). This can be performed by removing 10µl from the growth control well of the tube immediately after inoculation and diluting in 10ml of broth. 100µl of this dilution is spread on the agar plate surface, which is then incubated overnight. Fifty colonies are expected from an original inoculum of 5x10u CFU/ml. It is recommended that a purity be performed on the inocula; by placing a sample on a non-selective agar plate and incubating it overnight. The results should be read when the test organism growth is sufficient. The *Minimum Bactericidal Concentration* (MBC) is determined by calculating the relative proportion of live and dead bacteria. The tests were performed in microtiter plates. The bacterial viability determination was performed after exposure to the antibacterial agent (essential oil) for 4 h. The *Minimum Bactericidal Concentrations*(MBCs) calculation was carried out by graphical extrapolation and by the mathematical approximation method (Ericson and Sherris, 1971).

Antioxidant capacity determination

The essential oil antioxidant capacity was established using two different methods: the ferric reducing antioxidant power (FRAP) method as described by (Oyaizu, 1986), and the 2,2-diphenyl-1-picrylhydrazyl DPPH method as reported by Burits and Bucar (2000), with some modifications.

For the FRAP test, six various concentrations of methanolic-aqueous solutions (0.66-16.66 mg/ml) and the essential oils were mixed with 2.5 ml of 0.2M phosphate buffer (pH 6.6) and 2.5ml of potassium ferricyanide solution $K_3Fe(CN)_6$, 1% (w/v). The mixtures were incubated for 20 minutes in a water bath at 50°C. The incubated mixtures were then cooled at room temperature. Once cooled; 2.5 ml of 10 % (w/v) of TCA solution (*Trichloroacetic acid*) was added. The absorbances were measured at 700 nm. The positive control was represented by a standard antioxidant solution; the ascorbic acid was measured under the same conditions as the sample. An increase in the absorbances corresponds to an increase in the essential oils reducing power tested (Oyaizu, 1986).

For the DPPH test; the analysis was realized with 300 µl of six different methanolic aqueous solutions concentrations (0.66-16.66 mg/ml) with the essential oils, then they were mixed with 2.7ml of radical DPPH ($6x10^{É5}$mol/l in methanol). A control solution was also prepared with 300 µl of Milli-Q water and 2.7ml of DPPH solution. The mixture was vigorously vortexed and left to stand for 60 minutes in the dark at room temperature. A colometric evaluation was then performed using a spectrophotometer at 517 nm. The DPPH free radical inhibition by ascorbic acid was also tested for comparison with the same concentration. The reaction kinetics and the parameters determination for the ascorbic acid antioxidant activity and the essential oils inhibition percentage determination (%), and IC_{50} were performed in triplicate (Imelouane *et al.,*2009).

Inhibition percentage determination

According to Sharififar *et al.* (2007), The free radical DPPH reduction as percentage (I %) was calculated as follows: I % =(A control - A sample) / A control) x 100.

A control: the control absorbance, and **A Sample:** the test absorbance. The essential oils and the ascorbic acid kinetics reactions with the DPPH are registered at each studied concentration. The essential oils and ascorbic acid rates, as a function of the inhibition percentage, were noted at the reaction end to obtain the index. The essential oils concentration providing 50% of inhibition was calculated. A lower IC_{50} value signified a higher antioxidant activity (Rather *et al*., 2012).

Statistical analysis

Data were submitted to the correlation analysis; it was realized using the SPSS statistical package program (SPSS 17.0 for windows, SPSS Inc. Chicago, IL., USA).

RESULTS AND DISCUSSION

According to AFNOR (2000), the essential oils (EOs) are generally liquid at room temperature and volatile, which differentiates them from the so-called fixed oils. They are more or less colored; their density is generally lower than that of water (Nostro *et al.,* 2000). The studied essential oils yields were moderate values. The essential oils chemical compositions are presented in Table 1. For the *T. vulgaris* E.O, thirty two different compounds were identified representing 98.26% for the Chlef sample and thirty one components for that of Sidi-Bel-Abbes region, which representing 94.92% with proportion of monoterpene hydrocarbons and oxygenated monoterpenes. The γ-Terpineme was the predominant compound in the Chlef region sample with the percentage of 27.09% followed by Thymol (21.07%), then p-Cymene (21.04%) and Camphor (13.05%).The Sid-Bel-Abbes sample region was also principally composed by γ-Terpineme 24.02%, but with a lower content, than the noted sample from Chlef region. The Thymol presented a high percentage (24.89%) compared to that of Chlef region. In addition, the two samples showed equal percentages of p-Cymene (21.04% and 21.09%). By referring to the literature, a number of important compounds were identified from this essential oils plant; a previous studies confirm the γ-Terpineme predominance (AFNOR, 2000), while others have identified the Thymol as dominant compound (Kulisic *et al.,*2004) . Based on previous works that concentrated on this plant from Algeria, it appeared that there are differences in the composition of the major compounds, which were attributed to a various factors, such as, the climate, the development stage and even the species genetic profiles (Silva *et al.,* 2015).

Antioxidant activity

Generally, the unique method is not suggested for the essential oils antioxidant activity determination due to their complex composition (Grigore *et al.,* 2010). Therefore, two different tests were used for the essential oils antioxidant activity determination (the FRAP and DPPH scavenging activity). These tests have various mechanisms. The DPPH method is based on the antioxidants ability operate as radical scavengers; while the FRAP method measures the antioxidants ability to act as reducing agents (Rustaiyan *et al.,* 2000).

DPPH test

The DPPH test measured the sample ability to provide protons; the DPPH radical scavenging activities tests of both samples on the EOs are reported in Table 2, these results are defined as the substrate concentrations that causes 50% loss of the initially introduced DPPH free radical concentration. In comparison to the reference antioxidants (Ascorbic acid and BHA), the EO samples demonstrated a low DPPH radical scavenging activities. These results suggest that; the *T.vulgaris* EO antioxidant activity from Chlef region are twice time more active than that of the Sidi-Bel-Abbes region, the antioxidant activity was higher with lower DPPH value, which could be explained by the different active chemicals in each case. It can be concluded that the EOs samples possessed the powerful antioxidant substances, which that may be responsible for its anti-inflammatory and chemo-protective mechanism, as well as justify the basis of using this plant as popular medicine (Mansour *et al.,* 2021).

FRAP test

As observed, both essential oils exhibited antioxidant capacity. In comparison with the ascorbic acid (Table 2), the EOs exhibited a moderate antioxidant activity. The highest antioxidant capacity was reported for the *T. vulgaris* from Chlef region, whereas the EO with the lowest activity was assigned to *T. vulgaris* the from Sidi-Bel-Abbes region. A high FRAP value indicates a higher antioxidant capacity. It's considered that, the *T. vulgaris* antioxidant capacity is associated to its Thymol and Camphor content, two phenolic compounds with a recognized antioxidant capacity (Marino *et al.,*1999). In a previous study, FRAP values of the EO obtained from six different *T. vulgaris* were found to be in the range 16.23 and 27.84 mmol/l. The results of the present study are

Table 1: ***Thymus vulgaris*** **essential oils samples chemical composition from Chlef and Sidi-Bel-Abbes region**

Compound	*Thymus vulgaris* (Chlef)		*Thymus vulgaris* (Sidi -Bel -Abbes)	
	IK (m/m)	%	IK (m/m)	%
á-Thujen	923	1.01	925	1.03
á-Pineme	929	0.71	926	0.86
Camphene	942	0.04	944	0.08
Sabineme	967	0.03	963	0.01
â-Pineme	980	0.21	978	0.19
Myrcene	987	0.60	982	0.65
á-Phellandere	999	0.17	992	0.14
ä-Carene	1011	0.08	1009	0.07
P-Cymene	**1022**	**21.04**	**1020**	**21.09**
Limoneme	1021	0.015	1019	0.013
Eucalyptol	1038	0.11	1032	0.19
ã-Terpineme	**1057**	**27.09**	**1059**	**24.02**
Terpinoleme	1089	0.17	1084	0.14
Linalool	1097	1.08	1091	1.01
â-Thujone	1117	0.002	1114	0.001
Allo-ocimene	1113	0.08	1117	0.09
Camphor	**1147**	**13.05**	**1143**	**10.07**
Neo-isopilegel	1167	0.013	1161	0.012
Pinocarvone	1160	0.52	1163	0.40
Isopulegol	1168	0.45	1166	0.51
Borneol	1163	1.22	1169	1.02
Terpinene-4-ol	1171	0.58	1172	0.45
á-Terpineol	1174	0.031	1181	0.015
Naphtalene	1177	0.71	1187	0.80
Thymol methyl-l-Ether	**1234**	**7.84**	**1230**	**6.72**
Nerol	1235	0.31	1233	0.39
Pulegone	1239	0.014	1236	0.011
Carvone	237	0.01	1240	0.02
Linaly Acetate	1247	0.003	1249	0.021
Thymol	**1291**	**21.07**	**1293**	**24.89**
Carvenol	1297	0.005	1297	0.01
Total identified		**98.26**		**94.92**
Essential oil yield		**1.69**		**1.56**

IK: Kovats retention index.

Table 2: ***Thymus vulgaris*****essential oils samples antioxidant activity from Chlef and Sidi-Bel-Abbes regions using the DPPH ,the FRAP methods and** IC_{50}

E.O (Chlef)	IC_{50} (mg/mL)	DPPH%	FRAP(mmol/L)
	10.01±0.7	33	21.21±0.04
E.O (Sidi-Bel-Abbes)	5.3±0.01	67	15.02±0.09
Ascorbic acid	0.080±0.003	83	61.07±0.23
BHA	0.061±0.002	75	46.01±0.63

E.O: Essential Oil BHT: Butyl Hydroxy Anisole.

Table 3: *T. vulgaris*essential oil samples antibacterial activity from Chlef and Sidi-Bel-Abbes regions by the well method

Strains	*T.vulgaris* (Chlef)	*T.vulgaris* (Sidi-Bel-Abbes)
	Inhibition zone diameters (mm)	Inhibition zone diameters (mm)
Enterobacter cloacae	35±0.01	28±0.05
Proteus mirabilis	24±0.001	22±001
Citrobactercoseri	20±0.03	17±0.01
Acinetobacterbaumanii	28±0.07	18±0.05
Seratiaodorifera	23±0.05	21±0.09
Enterobactergergoviae	17±0.02	15±0.06
Staphylococcus .ssp	22±0.08	21±0.02
E.coli	25±0.06	22±0.05
Klebsiellassp	37±0.01	32±0.06
Enterococcus faecium	22±0.05	25±0.09
Pseudomonas.ssp	9±0.002	8.2±0.08
Citrobacterfreundii	30±0.01	27±0.01

Table 4: MIC and MBC for the *T.vulgaris*essential oils samples from Chlef and Sidi-Bel-Abbes regions

Strains	*T.vulgaris* (Chlef)		*T.vulgaris* (Sidi-Bel-Abbes)	
	MICs (mg/mL)	MBCs (mg/mL)	MICs (mg/mL)	MBCs (mg/mL)
Enterobacter cloacae	0.109	1.022	0.111	1.041
Proteus mirabilis	0.101	0.429	0.122	0.508
Citrobacter coseri	0.538	1.057	0.432	1.061
Acinetobacterbaumanii	0.508	1.077	0.490	1.051
Seratiaodorifera	0.112	1.070	0.101	1.063
Enterobactergergoviae	0.125	1.015	0.131	1.032
Staphylococcus.ssp	1.019	1061	1.002	1.021
E.coli	1.001	1.063	0.985	1.052
Klebsiella .ssp	0.099	1.001	0.078	1.095

MICs: Minimum Inhibitory Concentrations; **MBCs**; Minimum Bactericidal Concentrations

similar to those in the above literature with some differences. These differences may be related to the regional conditions, the active substance quantity, the extraction methods and the solvent type (Pina-Vaz *et al.,* 2004).

Antibacterial activity

The antimicrobial activity essential oils results against 12 microorganisms responsible for urinary tract infections are reported in Table 3, both EOs exhibited an inhibitory effect against all the microorganism with an inhibition zone ranging from 13 to 37 mm (Values are the mean of 3 replicates).The EOs exhibited a high activity (when the inhibition zone diameter was ≥ 20mm) and a moderate activity (when the inhibition zone diameter was <12- 20-mm). Among the EOs investigated, the highest antibacterial activity against *Klebsiella* ssp. was established with *T. vulgaris* from the Chlef region with an inhibition zone diameter of 37mm, followed by *Enterobacter cloacae* with an inhibition zone diameter of 35mm with also *T. vulgaris* of Chlef region; whereas the lowest activity was assigned to *Pseudomonas ssp.* with an inhibition zone diameter of 8.2 mm with *T. vulgaris* of Sidi-Bel-Abbes region. This *Pseudomonas ssp* remarkable resistance was reported by Cosentino *et al.* (1999) and by Hussain

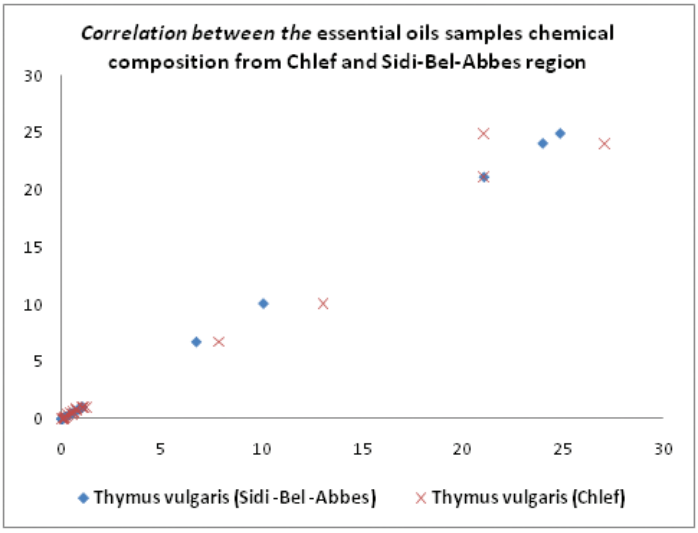

Fig. 1*:* ***Thymus vulgaris*** **essential oils samples chemical composition from Chlef and Sidi-Bel-Abbes region**

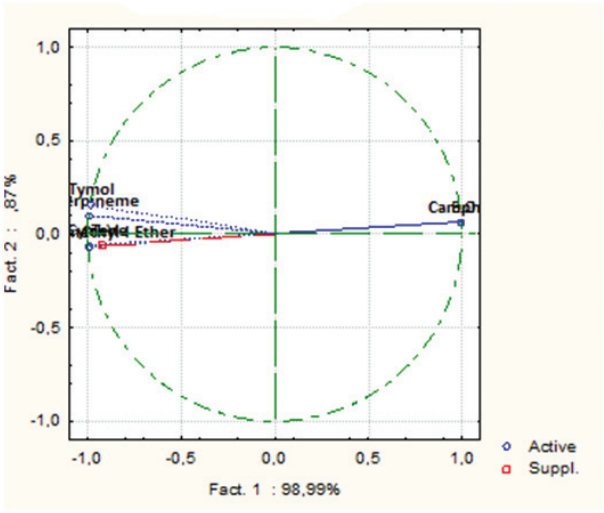

Fig. 2: Two dimensional plot on the F1 and F2 axes of the essential oils samples and their antioxidant activity using principal component analysis.

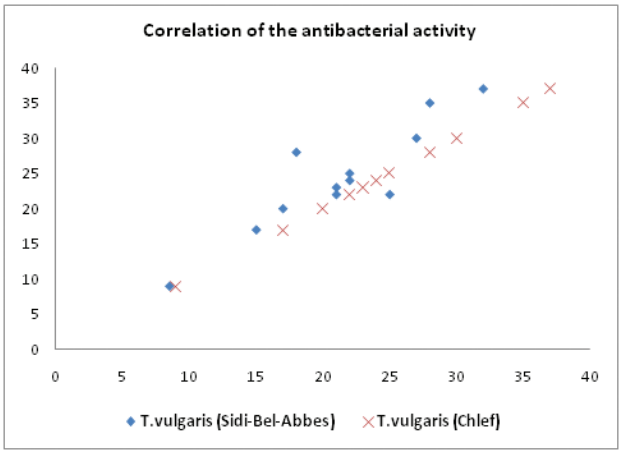

Fig. 3: The correlation between *T.vulgaris* essential oil samples and the antibacterial activity from Chlef and Sidi-Bel- Abbes regions

et al. (2011) for three species of *Thymus* genus, and six plants from the *Lamiaceae* family. These results revealed a significant variation in the antimicrobial properties of EO samples. In general, the EOs contained a phenolic compounds such as Thymol at high level and camphor, which exhibited a strong antimicrobial activity effect against pathogenic microorganisms (Simandi *et al.,*2001).These compounds can inhibit the essential enzymes, interfere with the cell membrane activity or disturb the genetic material's function and perturb the energy production and structural compounds synthesis (Sokoviæ *et al.,*2008).

Minimum inhibitory concentrations (MICs) and Minimum bactericidal concentrations (MBCs); tests determination

The MIC values obtained ranged from 0.099 to more than 2.004 mg/mL for *Pseudomonas ssp*. The same resistance was observed for *Pseudomonas ssp* towards to both EOs samples (Table 4).The Chlef *T. vulgaris* exhibited a MICs values of 0.099 mg/ml for: *Klebsiella* ssp*, Enterococcus faecium, Enterobacter gergoviae, Seratia odorifera, Acinetobacter baumanii, Citrobacter coseri, Proteus mirabilis, Enterobacter cloacae* and *Citrobacter freundii* and 1.019 mg/ml for *Staphylococcus* ssp*,* and *E.coli,* thus samples of Sidi-Bel-Abbes region, were from 0.101 mg/ml for *Seratia odorifera, Enterobacter cloacae, Proteus mirabilis, Citrobacter coseri, Acinetobacter baumanii, Seratia odorifera, Enterobacter gergoviae, Klebsiella* ssp*, Enterococcus faecium, Citrobacter freundii* and *E. coli* and 1.002 mg/ml for *Staphylococcus* ssp. The *Pseudomonas* resistance found at a concentration of 2.004 mg/ml. Imelouane *et al*. (2009) found that for this same species, the EOs MIC had a value equal to 1.33 mg/ml for *E. coli* and *Staphylococcus ssp*, Kaloustian and Hadji-Minaglou, (2012) found that the MIC_S for *E.coli* and *Staphylococcus ssp* are equal to 1 and 2 mg/ml respectively. For the MBCs for *Proteus mirabilis* was the most sensitive with a MBC equal to 0.429 mg/ml for the Chlef region samples, and 0.508 mg/ml for the Sidi-Bel-Abbes region samples. Numerous previous research confirmed the *Thymus* genus EOs antimicrobial proprieties (Sokoviæ *et al.,*2008).The comparison of the *T.vulgaris* essential oils under different geographic conditions revealed that there are some qualitative and quantitative differences between both Algerian localities (Chlef and Sidi-Bel-Abbes), which may have been influenced by genetics differences and different environmental factors. Others results on other *Lamiaceae* plants have indicated that, their chemical composition variation is assigned to the geographical conditions (Baba-Aissa, 2011).

Determination of the *correlation coefficient*

A correlation coefficient is a number between -1 and 1, that tells us the strength and direction of a relationship between variables: $-1 \leq r \leq +1$ .There is a strong positive correlation between the two species in terms of chemical composition of their essential oils, r=0.99 (Fig.1).

In the aim of searching the correlation between the essential oils chemical compositions of the studied plants and their antioxidant capacity, along with the antimicrobial activity, it was performed to use a statistical methods based on the Principal Components Analysis; PCA, which was used graphically. It described the relationship between the different parameters studied. Both axes represent 90.98% of the total variation. The first axis (98.99%) expressed the largest variation percentage (more than 97%) (Fig. 2).The objective of these both figures is to search the correlation between the essential oils chemical composition and their antioxidant properties, the correlation between the *Thymus vulgaris* essential oils chemical composition and the antioxidant activity, as expressed by the IC_{50} and the antibacterial activity with inhibition zones of the different strains.

The Figure 2 presents the factors projection (F1xF2) of the *T.vulgaris* chemical composition data and the antioxidant activity, which is expressed by the IC_{50} values, which are determined as the substrate concentration that produces the loss of 50% of the initially introduced DPPH free radicals. The antioxidant activity results revealed a great variation in the IC_{50} values and it's related to the plant nature studied. Based on PCA, it was revealed a positive correlation between the EOs chemical composition and its pre-identified main compounds: Cymene, γ-Terpineme, Thymol and Thymol methyl-l-Ether with a significant correlation coefficients higher than 97%.The

results also demonstrate a negative correlation between the EOs compounds (P-Cymene, γ-Terpineme, Thymol and Thymol methyl-l-Ether) and the IC_{50}, with a significant correlation coefficients (-0.97 to-0.99). When the IC_{50} is low, the antioxidant activity is higher and *vice versa*, when the IC_{50} is higher, the antioxidant capacity is lower; we observed that there was a negative correlation between the EOs compounds and the IC_{50}. This provided us the possibility to determine: first, a direct relationship between the antioxidant activity and the EOs compounds; second, each of the antioxidant activity and the EOs compounds have an inverse relationship with the IC_{50}. It considered that the *T. vulgaris* EOs antioxidant capacity associated with its thymol and phenolic compounds content with a recognized antioxidant activity (Sokoviæ *et al.,*2008). Many researchers have also reported that EOs extracted from thyme provide an effective antioxidants (Baba-Aissa , 2011). The results of the present study are also similar to those in the many references .

The Figure 3; illustrates the correlation between *T.vulgaris* essential oil samples and the antibacterial activity from Chlef and Sidi-Bel-Abbes regions**.** From this figure, the EOs sample exhibited a significant resistance against the majority of the tested bacteria; this activity is attributed to the camphor presence. There is a positive correlation between this compounds and the antibacterial activity with a correlation coefficient ranging from 0.70 to 0.99.From these previous results, we concluded that this antibacterial activity is principally based on the Camphor strong contribution. It could be deduced that the Eos *T.vulgaris* were partially effective as antioxidant activity, based on the presence of synergistic relationships that occurred between the EOs constituents such as Cymene-Terpineme, Thymol and Thymol methyl-l-Ether, and partially as antibacterial activity as a result of the Camphor presence. A precedent research (Nikoliæ *et al.,*2014) on the EOs antimicrobial activity of some *Thymus* species revealed that, most of the species which possessed a large quantities of terpenoids compounds, indicated the activities against viruses, bacteria and fungi (Adams, 2007). However, no significant investigations on the relationship between the bacterial inhibition and the total Camphor contents.

CONCLUSION

In the present study, we presented the powerful *T.vulgaris* EOs antibacterial activity from the both Algerian regions against twelve pathogenic *microorganisms*, involved in urinary tract infections, which explains the important use of this plant in the traditional medicine. It is appeared that, modern medicine should give more consideration to the synergistic effect of plant secondary metabolites; as they can help to resolve several problems, especially the microbial resistance to synthetic antibiotics. The tested EOs exhibited also an interesting antioxidant capacity, the relationship study between the EOs chemical composition and the antioxidant or the antibacterial activity indicated the presence of different strong correlation with some identified major compounds in each case of study.

Abbreviations: FRAP: Ferric Reducing Antioxidant Power; DPPH: 2,2-diphenyl-1-picrylhydrazyl ; GC/MS: Gas Chromatography coupled with Mass Spectrometry.

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Anti-inflammatory and analgesic properties of *Cannabis sativa* L. leaf ethanol extract in animal model

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ABSTRACT

These studies evaluate the anti-nociceptive and anti-inflammatory properties of Cannabis sativa leaf ethanol extract and to determine its activity at graded doses using animal models. The extract was obtained, rinsed, air dried, macerated and prepared into aqueous extract. Swiss albino mice and Wistar rats were sundered into four groups (n=4). The reference drugs (indomethacin, diphenhydramine, dexamethasone aspirin, and pentazocine), the negative control, and the test groups were administered 15 and 30 mg/kg of the extracts. The results for the analgesic study, elicited a significant decrease in the number of writhes triggered by acetic acid compared with the control ($p< 0.05$). The hot plate-induced test has a significant increase in the percentage inhibition of pain at 30 and 60 minutes when compared with untreated control ($p< 0.05$). Graded doses of the extract (15 and 30 mg/kg) exhibited a significant reduction in formalin-induced pain when compared to the control ($p<0.05$). The anti-dematogenic effects of the extract at dose 15 and 30 mg/kg had a significant reduction in paw-edema carrageenan-induction at 1, 4, 5 hours and dextran-induced inflammation compared with the control. More so, graded doses of the extract inhibited xylene ear edema with a significant reduction compared to the control ($p< 0.05$). This study revealed the effect of Cannabis sativa effective as a potent anti-inflammatory and analgesic agent, hence required further investigation of the possible mechanism and compound implicated for the action.

***Keywords*:** Anti-inflammatory, analgesic, *Cannabis sativa*, wistar rats.

INTRODUCTION

Cannabis sativa L. (*Cannabis*) is also referred to as marijuana and usually grown worldwide. It belongs to family *Cannabaceae*. Over time, several varieties of *Cannabis* have been discovered, from selection and breeding programmes. Although, the *Cannabis* derived from these method opens room for further controversies in botanical classification. The genus was separated into three species namely: *C. sativa* L., *C. indica* Lam. and *C. Ruderalis*. The plant is an annual plant, which has its male as well as its female reproductive system in different plants. From when it was discovered, its use has been hinged on both recreational use and for the relief of pain. Apart from being a stimulant as well as sedative, the plant has active ingredients found in the resin of the plant. Over 538 chemicals of several classes have been reported to come from *Cannabis* (ElSohly and Slade, 2005). Amongst the several classes, the most relevant of them includes non-cannabinoids, cannabinoids, steroids, simple esters, nitrogenous compounds, terpenoids, phenols, glycoproteins, fatty acids, simple acids, simple aldehydes, simple ketones, hydrocarbons, vitamin; usually vitamin K, sugars and related compounds, lactones, amino acids and proteins, simple alcohols, enzymes, certain elements, flavonoids, pigments. The most important medicinal uses of *Cannabis sativa* are for analgesic purposes and also to ease certain nervous disorders. It is also used in the management of gout, insomnia, glaucoma, epilepsy, rheumatism, neuralgia etc. Tetrahydrocannabinol (THC) can be used to treat certain ailments such as; glaucoma, nausea cachexia, pains, glaucoma and for making child birth easier (Cohen and Andrysiak, 1982; Martin *et al*., 1993).

Despite the numerous importance of *C. sativa*, it is however banned and illegalized worldwide except in countries like Uruguay. It remains a controversy as to whether it should be legalized or not. However, most of the unfavourable claims against the use of marijuana were disproved by some research findings (Cherek *et al.*, 1993; Brick, 1990; Carter, 1980; Carter and Doughty, 1976). It is therefore very necessary and important to explore the good aspects and important uses of *C. sativa*. In this regard, the aim of this research was to access its analgesic and anti-inflammatory properties.

MATERIALS AND METHODS

Plant material and extraction

Cannabis sativa fresh leaves were collected from Ovia North East Local Government Area of Benin City, Edo state, Nigeria, in November, 2018. The plant sample was identified and Authenticated by Dr. Timothy Odaro of the Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, Benin City and a Voucher number was issued (UBW-V528). The leaves were dried using a laboratory oven at a temperature of 40^{o}C, for a period of 8 days till the leaves turned crispy. The dried leaves were then grounded to powder form using an electric mill (Tigmax Petrol Gx160-5.5hp). Five hundred (500) grams of the powdered leaves was cold macerated in 2.5 l of ethanol (Owolabi *et al.,* 2008) for 72hours and was shaken regularly. Afterwards, the solution was decanted and the extract filtered (0.45 micron pore size) from the solution and evaporated to dryness at 40^{o}C. The dehydrated extract was then stored in a clean, dried bottle and kept at 4.4^{o}C for further use.

Experimental animals

The study was performed with both sexes of albino mice (20-28 g) and Wistar rats of 16 weeks old (180-210 g). Wistar rats and albino mice were received from the Animal house, Faculty of Pharmacy, University of Benin, Nigeria. They were fed using standard rodent feed procured from Top Feed Palletized Finisher and had clean drinking water *ad libitum*. Before the start of each study, the animals were fasted during the night. The animals were uncovered to a day lighting and handled according to the standard experimental procedures accepted by Institutional Ethical Committee, Faculty of Life Sciences, University of Benin, Edo state, Nigeria with an ethical number of LS20187.

Anti-inflammatory activity

Carrageenan-induced paw edema

For this study, four groups of four Wistar rats of 16 weeks old (180-210 g) each were used. The extract at 15 and 30 mg/kg doses were administered orally to the test groups. Ten (10) mg/kg of indomethacin was orally administered to the positive control while distilled water (3 mL/kg) was administered to the negative control group. After an hour, 0.1 mL, 1 % w/v carrageenan suspension in normal saline injected into the subplantar tissue of the right hind paw of the animal. The thickness of the paw was measured using Vernier caliper at 0 hour and after every one hour interval for 5 hours (Thambi *et al.*, 2006).

Dextran-induced paw edema

Wistar rats weighing between 135-180 g were randomly divided into four groups of four animals each for this study. The extract (15 and 30 mg/kg) were administered to the test group orally, while distilled water (3 ml/kg) was administered orally to the negative control group. The reference group were given 60 mg/kg of diphenhydramine. The animals were treated one hour before they were injected with 1.5 % w/v dextran in normal saline of 0.1 mL into right hind paw subplantar tissue (Glauce *et al.*, 1998). Vernier calipers were used to measure the thickness of the paw at (0, 1 to 5 hours).

Xylene-induced ear edema

For this study four groups consisting of randomly selected four Swiss albino mice were used. The extract (15 and 30mg/kg) were administered orally to the test groups. The method described by Akindele *et al.* (2007) was adopted. Distilled water (3 ml/kg) was given to the untreated control group while the reference group was administered dexamethasone (1 mg/kg). After thirty (30) minutes, ear-edema was induced in the mice by the application of a drop of xylene to the interior surface of the right ear. About 15 minutes after, they were sacrificed in a mild chloroform, by maintaining a good animal ethics for ethical purpose, and the left and right ears were isolated, sized and weight taken and recorded.

Analgesic activity

Mouse writhing assay

Four groups consisting of randomly selected four male Swiss albino mice were used for this study. The test groups were administered the extract (15 and 30 mg/kg) orally, while distilled water (10 ml/kg) was administered to the group induced without treatment (negative control). The reference drug used was aspirin (100 mg/kg).Thirty (30) minutes after administration of reference drug and extract, acetic acid in normal saline (0.6% v/v) was given to the mice intraperitoneally with an injection and writhes were counted for 30 minutes at five minutes interval.

Formalin test

Male Wistar rats were randomly separated into four groups of four animals each for this experiment. The extract (15 and 30 mg/kg) were administered orally to the test group while the reference group was administered aspirin (100 mg/kg, subcutaneously). Distilled water was administered orally to the negative control group. At 30 minutes after they were administered, 1% of 20µl formalin was subcutaneously administered into the right paw of the rats. As a sign of response to pain, the time spent in biting and licking responses of the paw injected was recorded in seconds. The responses were measured for five (5) minutes subsequent to formalin injection (ûrst phase) and 15 to 30 minutes after injected with formalin (second phase) (Shibata *et al*., 1989)

Hot plate test

The method of the hot plate test described by Eddy and Leimback (1953) was used to evaluate the latencies of pain response. Swiss mice were randomly divided into four groups (n=5). The albino mice were independently positioned on a hot plate kept at constant temperature of 55 ^{o}C (Okpo *et al.,* 2001) the interval of time from placement and shaking or licking of the paw or jumping was recorded as an index of response latency. The pre drug latency period for each animal was determined and recorded. The negative control group was treated orally with distilled water at 10 mL/kg. The test groups of mice were administered with 15 and 30 mg/kg of the extract. Pentazocine (15 mg/kg) was given intraperitoneally and used as a standard. The animals were placed on the hot plate at 15, 30, 45, 60 minute, 15 minutes after treatment and the time recorded for either paw shaking or jumping was recorded.

Statistical analysis

Data from this study were analysed as Mean ±SEM. Means of the various groups were compared by ANOVA, using 2009 version of Graph Pad Prism computer software package. P-values <0.05 (95 % confidence interval) were regarded significant.

RESULTS AND DISCUSSION

Table 1 shows the result of carrageenan induced paw edema. The ethanol leaf extract of *Cannabis sativa* at 15 and 30 mg/kg, significantly inhibited paw edema in comparison to the negative control group. The 15 mg/kg extract dose was not significantly different from the indomethacin treated group. The 30 mg/kg extract at the first hour showed higher level of inhibiting paw edema as compared to the indomethacin and the 15 mg/kg extract. However, as compared to both groups, the 30 mg/kg had a non-significant effect in the fourth hour but showed paw edema inhibition at fifth hour.

The study investigated the antinociceptive and anti-inflammatory potential of *Cannabis sativa* leaf ethanol extract to determine its activity at different doses. Our study has shown that ethanol leaf extract of *Cannabis sativa* had anti-inflammatory activities. Inflammation is one of the processes utilized by the body to fix damaged tissue as well as eliminate injurious stimuli. Continuous inflammation would result in gradual tissue injury (Shin *et al*., 2008). Evidence has shown that inflammatory processes results in tissue damage, which eventually leads to several diseases like inflammatory bowel disorder and rheumatoid arthritis (Monaco *et al*., 2004; Akaogi *et al*., 2006). Therefore, research and the development of anti-inflammatory drugs have remained a necessary scientific pursuit (Gautam and Jachack, 2009).

This study employed carrageenan-induced paw edema, an already established protocol for the study of acute inflammation. As an agent for testing anti-inflammatory drugs, carrageenan has been proven to be a non-comparable choice due to its non-antigenic nature and elimination of subsequent systemic effect (Chakraborty *et al.*, 2006). Edema resulting from carrageenan occurs in two phases

Table 1: Effect of *Cannabis sativa* leaf ethanol extract on carrageenan-induced paw edema

Groups	Doses (mg/kg)	Pre-drug	0hr	1hr	2hr	3hr	4hr	5hr
Control (Dw)	0.5 ml/kg	-	0.60±0.03	0.68±0.02	0.70±0.03	0.63±0.01	0.67±0.02	0.65±0.01
Indomethacin	10	0.37±0.03	0.59±0.05	0.55±0.03*	0.72±0.01	0.53±0.03	0.55±0.02*	0.53±0.01**
C. sativa	15	0.33±0.01	0.52±0.05	0.57±0.03*	0.63±0.01	0.60±0.03	0.57±0.03*	0.54±0.03**
C. sativa	30	0.34±0.01	0.57±0.03	0.54±0.03**	0.70±0.04	0.62±0.04	0.59±0.03	0.56±0.02*

Values are means ± SEM; n=4; *= $p<0.05$. Dw – Distilled water

Table 2: Effect of *Cannabis sativa* leaf ethanol extract on xylene-induced ear edema in rats

Groups	Doses (mg/kg)	Left ear (g)	Right ear (g)	Differences in both ear (g)
Control (Dw)	0.5ml/kg	0.010±0.00	0.019±0.00	0.009±0.00
Aspirin	100	0.065±0.01	0.098±0.02	0.033±0.02***
C. sativa	15	0.058±0.00	0.092±0.01	0.034±0.01***
C. sativa	30	0.064±0.01	0.117±0.0	0.058±0.01**

Values are means ± SEM; n=4; **$p<0.05$. Dw — Distilled water.

Table 3: Effect of *Cannabis sativa* leaf ethanol extract on acetic acid induced writhing with time dependent

Groups	Doses (mg/kg)	0-5 mins	5-10 mins	10-15 mins	15-20 mins	20-25 mins	25-30 mins
Control (Dw)	0.5 ml/kg	18.0±6.19	28.7±2.40	25.0±2.58	20.8±3.17	18.0±2.38	15.0±1.29
Aspirin	100	5.25±1.32*	18.0±1.00**	19.5±1.56	18.5±1.26	17.5±2.18	15.3±1.18
C. sativa	15	3.50±1.19*	15.7±0.33**	13.8±0.75**	11.0±1.35*	7.75±1.49*	5.75±1.11***
C. sativa	30	0.0±0.0**	15.3±1.86***	14.0±2.16**	9.50±1.94**	8.50±2.18*	6.50±1.04***

Values are means ± SEM; n=4; $p<0.05$. Dw — Distilled water

Table 4: Effect of *Cannabis sativa* leaf ethanol extract on hot plate-induced pain with time dependent

Groups	Doses (mg/kg)	0 sec	15 mins	30 mins	45 mins	60 mins
Control (Dw)	0.5 ml/kg	3.00±0.33	4.17±0.75	3.00±0.12	3.20±0.32	2.67±0.46
Pentazocine	100	2.35±0.13	7.75±2.00*	8.40±1.82**	10.92±0.89***	9.00±1.68***
C. sativa	15	2.83±0.24*	5.83±2.33	6.68±2.20*	6.83±2.18*	6.94±2.19**
C. sativa	30	2.70±0.43	6.12±2.57	6.70±2.38*	8.55±1.69**	8.64±1.75**

Values are means ± SEM; n=4; $p<0.05$.Dw — Distilled water.

(Vinegar *et al.*, 1987). The first phase takes place during one hour of carrageenan inflammation and it is as a result of cytoplasmic enzymes, serotonin and histamine discharge from the mast cells.

Arachidonic acid metabolites and platelet activating factor also have their distinct roles to play (Boughton-Smith *et al.*, 1993). Second phase of carrageenan-induced edema takes place after an hour and is mediated by release of proteolytic enzymes, prostaglandins, release of oxygen, free radicals arachidonate metabolites, migration of neutrophil and some neutrophil-acquired mediators (Boughton-Smith *et al.*, 1993; Bouriche *et al.*, 2003) and kinins are responsible for the continuity between the two phases (Vinegar *et al.*, 1987). Our findings have demonstrated that *C. sativa* ethanol leaf extract (15 and 30 mg/kg doses) had a significant ($p<0.05$, 0.01) inhibition of paw edema producing an inhibitory effect in the first and second phases of the inflammation (Table 1).

The antihistamine potential of the extract is demonstrated in the first phase which may be as a result of the extracts ability to reduce carrageenan-induced leakage of the microvasculature (Kuriyama *et al.*, 2000). Histamine can induce vascular permeability of endothelial cells thereby resulting

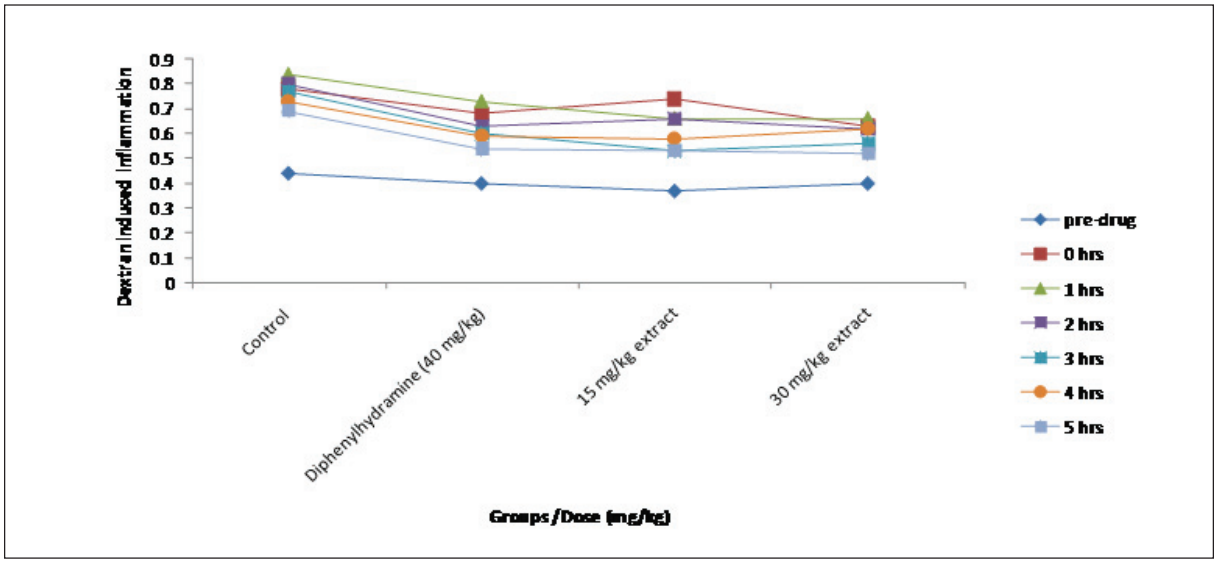

Fig. 1: Effect of *Cannabis sativa* leaf ethanol extract on dextran-induced inflammation in rats paw

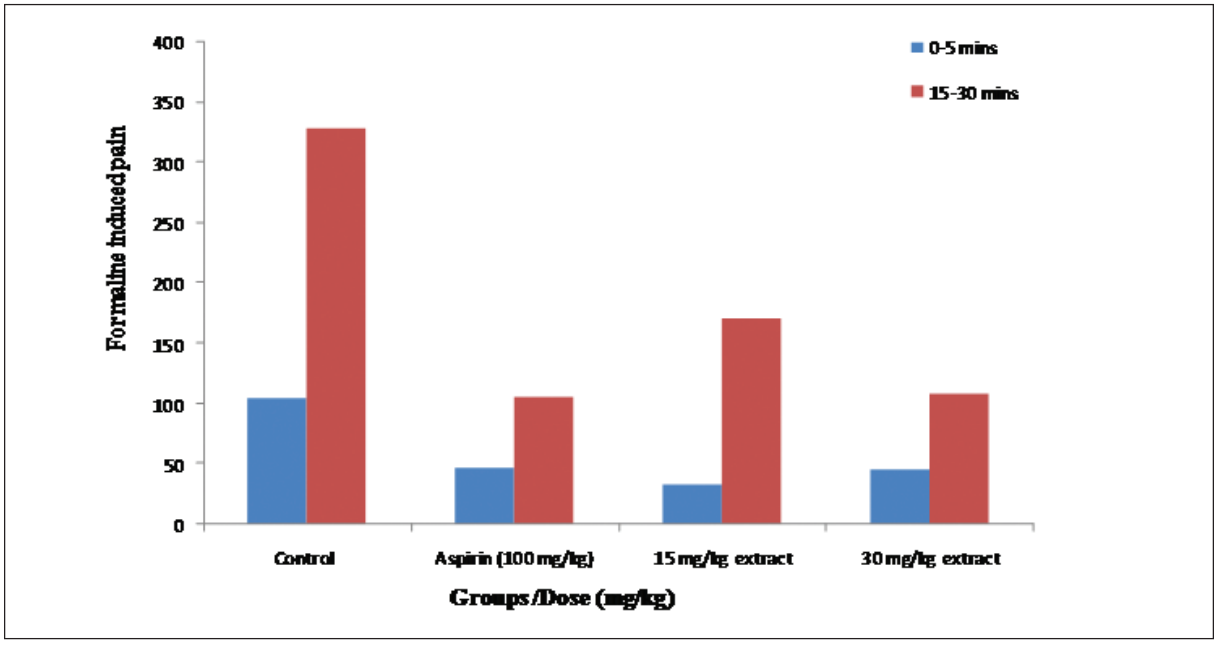

Fig. 2: Effect of Cannabis sativa leaf ethanol extract on formalin induced pain in rats

in outpouring of fluid and cells (Kuriyama *et al.*, 2000). Our study is in agreement with Musa *et al.* (2011). In this study carried out using *Cannabis sativa* seeds in petroleum oil extract in albino rats, the authors reported a decrease in edema. The edema ameliorating potential of *C. sativa* at second phase, suggesting a potential cyclooxygenase synthesis inhibited due to the fact that the carrageenan inflammatory model reveals prostaglandins actions (Di Rosa *et al.*, 1971; Ferreira *et al.*, 1974).

Figure 1 presents the results for dextran induced paw edema. The ethanol leaf extract of *Cannabis sativa* (15 and 30 mg/kg) treated groups hindered paw edema, in comparison with the negative control. *C. sativa* extract (15 mg/kg) had greater

paw edema inhibitory properties when compared to the positive control at the first, third, and fourth hour of the experiment, but had no inhibiting effect at zero hour in comparison to the positive control. At 30 mg/kg, the extract had a greater inhibitory effect of paw edema in comparison to the positive control at zero and one hour. More so, dextran-induced inflammation is mediated via serotonin released from mast cells (Lo *et al*., 1982).

These inflammatory mediators released bring about marked vascular changes such as vasodilatation, higher permeability and slow blood flow, finally leading to paw inflammation. Our study have demonstrated that ethanol leaf extract of *Cannabis sativa* inhibited significantly ($p<0.05$, 0.01) dextran-induced paw edema at both doses (15 and 30 mg/kg) (Figure 1). These observations were in agreement with the study reported by Wade *et al*. (2004) where they showed that the ethanol extract of *Leptadenia arborea* demonstrated an inhibitory effects in the edema size.

The ethanol leaf extract of *Cannabis sativa* (15 and 30 mg/kg) inhibited xylene-induced ear edema in comparison to the negative control (Table 2). At 15 mg/kg dose, *C. sativa* had inhibitory activity comparable to the positive control. Xylene induced ear edema is used to investigate the anti-inflammatory steroids to reduce the sensitivity of non-steroidal anti-inflammatory mediators (Zannir *et al.*, 1992). The indicators on acute inflammation after topical applications of xylene detected were; infiltration of inflammatory cells, severe vasodilation and edematous skin changes. This study showed that the xylene ear edema was inhibited by the extract at both doses significantly ($p<0.01$, 0.001), which suggests that *C. sativa* possess antiphlogistic effects. Our findings corroborates with the study conducted by Okpo *et al.* (2001) where they demonstrated the inhibitory potential of *Crinum glaucum* in xylene induced ear edema.

Acetic acid induced writhing (Table 3) shows that ethanol leaf extract of *Cannabis sativa* (15 and 30mg/kg) inhibited writhing in comparison to the negative control. The extract showed a higher inhibitory effects compared with the positive control drug.

In the formalin induced test (Figure 2), the extract (15 and 30 mg/kg) ameliorated formalin induced pain as compared to negative control group. This observed reduction was seen in both phases. However, at 15 mg/kg dose, a higher inhibitory effect in the first phase was observed when compared to (100 mg/kg) dose of aspirin. The 30 mg/kg dose had effect in both phases similar in comparison to that of aspirin. Furthermore, our study on the analgesic properties of *Cannabis sativa* showed the relationship between *Cannabis* and pain (Figure 2). Some random, controlled clinical studies have reported the effective quality of *Cannabis* as a pharmacotherapy for pain (Hill, 2015).

Several studies have reported the analgesic properties of tetrahydrocannabinol using animal models (Lim *et al*., 2003; Johanek *et al*., 2001), also experimental research carried out on investigating the properties of *Cannabis sativa* on human response to nociceptive has been on clinical pain samples or healthy adults. For instance, Wallace *et al*. (2007) validated the activities of smoked *C. sativa* at different doses (low, medium, or high doses vs. lethargic placebo) on intradermal capsaicin-induced pain reactions by means of a randomized, double-blind, verge trial in fifteen (15) healthy volunteers (mean age of 28.9; 58% male). They observed a significant reduction in pain at medium dose and increase pain at higher dose. The results showed a signiûcant reduction in pain at medium dose of *C. sativa* and a significant increase in pain at high dose.

The study also reported no significant difference was seen with the low dose of *C. sativa*. The conclusion derived was that medium dose of smoked Cannabis could be a good therapy for pain. Another experimental study by the same authors was carried out with 18 healthy female volunteers who tested the effect of orally administered *C. sativa* extract (vs. potent placebo) on sunburn and intradermal capsaicin pain reactions using a double-blind, cross over trial (Wallace *et al*., 2007).

They observed that the extract of *Cannabis* did not result in any analgesic effect. They concluded that the lack of understanding of the dose-dependent therapeutic and psychotropic effects may limit the use of *C. sativa* as an analgesic agent. A review of cannabinoids for medical usage among patients with chronic pain has shown excellent reduction in pain and numerical pain rating (Whiting *et al*., 2015). The authors therefore concluded that

moderate evidences are available to support the analgesic properties of cannabinoids (Whiting *et al*., 2015). Our findings revealed that ethanol leaf extract of *Cannabis sativa* possessed analgesic properties.

One of the widely used methods to evaluate anti-nociceptive activity is the writhing on animal models caused by acetic acid (Gene *et al.*, 1998). This method is highly sensitive even at lower dose when compared to the tail-flick test in detecting the anti-nociceptive potentials of bioactive agents (Collier *et al.*, 1968; Bentley *et al.*, 1981). Our study demonstrated that *C. sativa* ethanol leaf extract caused a significant ($p < 0.005$, 0.01, 0.001) reduction in acetic acid induced-writhes at all doses. *C. sativa* extract generally was more effective than 100mg/kg of aspirin dose, which was the control drug used and it was sustained throughout the 30 min period suggesting peripheral mediation for the extract's analgesic effect.

The extract (15 and 30mg/kg) inhibited hot plate induced pain in mice from the 30th minute all through to the 60th minute in comparison to the negative control (Table 4). Pentazocine (15mg/kg), a known centrally active drug had a better inhibitory effects than the extract treated groups.

The findings from our study showed that *C. sativa* ethanol extract (15 and 30 mg/kg) was able to cause a significant ($p<0.005$, 0.01) inhibition in the two phases of formalin-induced pain. However we observed a significant inhibition in the first phase in the group administered 15 mg/kg in comparison to the group administered aspirin (100 mg/kg), while the 30 mg/kg dose had effects in both phases similar to that of aspirin as shown in Figure 2. In addition, Formalin induced pain in rats is a model that is of great importance in explaining the mode of action of analgesia as well as pain (Tjolsen *et al*., 1992). Centrally mediated bioactive agents like the narcotics are able to inhibit both phases (Santos *et al*., 1994). This suggests that analgesic activity was centrally mediated.

Hot plate tests are usually employed to evaluate nociceptive effects mediated centrally. We observed in our study that *C. sativa* (15 and 30 mg/kg) was able to cause a significant extension of reaction latency to pain in the hot plate as shown in Table 4which suggests that the analgesic activity was centrally mediated. The abnormal calmness some minutes after administration of the extract observed in the animal models suggests psychotropic effects of the extract.

CONCLUSION

The study showed that *Cannabis sativa* had a dose-dependent anti-inflammatory and analgesic activities. Hence, this study elicited a scientific validation the ethno-medicinal uses of the plant.

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Phytochemical screening of *Moringa oleifera* Lam. and histo-protective evaluation of cadmium chloride toxicity in rats.

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ABSTRACT

The aim of this experiment was to determine the phytochemical screening and histopathological property of Moringa oleifera Lam. root ethanol extract in Wistar rats. The qualitative phytochemical screening was experimented using standard protocol. The animals were randomly divided into groups seven (n=5). Cadmium chloride (3.6 mg/kg) was administered across the groups for 7 days to established bio-toxicity. There after graded doses of the treatment groups (100, 200 and 400 mg/kg) and the control groups were treated for another two (2) weeks to remediate the deposition of cadmium toxicity. The animals were sacrificed and the targeted visceral organs (heart, lungs, liver and kidney) were isolated and prepared for histopathological analysis. The phytochemical results showed the presence of alkaloids, tannins, saponnins, flavonoids, phenol, anthraquinone and carbohydrate. The histopathological study elicited from the treatment revealed an ameliorative effect from the damage organs triggered by cadmium chloride, enhance displayed cardio-protective, reno-protective, hepato-protective and brochio-protective effect when compared with untreated control. The study showed that graded doses (100, 200 and 400 mg/kg) of the extract had a significant effect in the targeted organs against cadmium chloride toxicity.

***Keywords*:** Cadmium Chloride, histopathological, *Moringa Oleifera*, phytochemistry, rats.

INTRODUCTION

Moringa oleifera Lam. is a small, fast-growing evergreen or deciduous tree, having soft and white wood with corky and gummy bark and grows up to a height of 9 m (Garima *et al.,* 2011; Khawaja *et al.,* 2010). It is one of the most studied and used species with various use categories stretching from food and medicinal uses to water purification, bio-pesticide and production of biodiesel. It is used and valorized through food fortification. For instance, in Nigeria, it is used to fortify food formulations of corn, soy and peanut and as food fortificant in amala (stiff dough), ogi (maize gruel), bread, biscuits, yoghurt, cheese and soup making (Shiriki *et al.*, 2015; Oyeyinka and Oyeyinka, 2016; Gandji *et al.,* 2018). *M. oleifera* possess antispasmodic, expectorant, diuretic and stimulant activities. Whole plant exhibited cardiac circulatory tonic and antiseptic. Pods possess anthelmintic; diabetes and antipyretic effect. The root juice is utilized as cardiac tonic, antiepileptic, nervous debility, treat hepatic and spleen enlargement, asthma, detox toxins, inflammation and as a good diuretic agent. It is also use as anti-paralytic, anti-cholesterolemic, mosquito larvicidal activity (Garima *et al.,* 2011; Okwari, 2013; Tom and Benny, 2017).

The immune system is a host defense system that comprises of biological structures and processes organisms that protects against diseases (Beck and Habitat, 1996). It protect the immune system by detecting a wide variety of agents, known as pathogens, from viruses to parasitic worms, and distinguish them from the organism's own healthy tissue. In humans, the blood-brain barrier, blood-

cerebrospinal fluid barrier, and similar fluid-brain barriers separate the peripheral immune system from the neuro-immune system which protects the brain (Thomas, 2010; O'Byrne and Dalgleish, 2001). The immune system could be affected by environmental and dietary habits and it is believed a rich diet in antioxidants and micronutrients can boost the immune system (Sudha *et al*., 2010). This study evaluates the phytochemical screening and histopathological properties of *Moringa oleifera* root ethanol extract.

MATERIALS AND METHODS

Collection and identification of plant materials

Fresh samples of *Moringa oleifera* plant was collected in the rain forest at Obe, Sapele road, Benin City, Edo State, Nigeria. The plant was collected in July, and was identified and authenticated with the code (X690) by Dr. H. Akinnibosun, in the Department of Plant Biology and Biotechnology, University of Benin, Benin City, Edo State, Nigeria. A voucher number was issued (UBX-M517).

Plant preparation

The samples of *Moringa oleifera* plant was washed and air-dried at room temperature for 3 weeks and further oven-dried at a temperature of 40^{o}C. It was then pulverized to powder using the British milling machine. 800 g of the plant was macerated using 1400 ml of absolute ethanol solvent (Mukherjee, 2002). The maceration was done for 72 hours with periodic shaking and stirring. The filtrate was then concentrated into paste using water bath to give % yield of 10.6 %. The extract was store for further use.

Phytochemical screening

Phytochemical screening was carried out to check the presence of bioactive agent in the *M. oleifera* root ethanol extract. This was carried out using the standard method described by Trease and Evans (1989). The confirmatory tests were on alkaloid, tannin, saponnin, flavonoid, phenol, anthraquinone, carbohydrate and cardiac glycoside, using the following test protocol; saponins frothing test; saponins fehling's test; test for anthraquinones; test for flavonoids; flavonoids shinoda test; phenolic compounds test for ferric chloride; test for tannins; tannin ferric chloride test; test for alkaloids Dragendorff's; Wagner's; Hager's; Mayer test; test for glycosides; glycosides fehling's solution test; glycosides ferric chloride test; terpenoids salkowski's test.

Experimental animals

Thirty five (35) *Wistar* rats of 12 weeks old, weighing (190-220) were obtained from the Department of Biochemistry, University of Benin and allowed to acclimatize in the Animal House, of the Department of Animal and Environmental Biology, University of Benin, Edo State, Nigeria, for a period of 14 days with 12 hours day and 12 hour night in a well ventilated cages. With constant feed and water *ad libidum*. The Animal House was well ventilated throughout the course of the experiment. The ethical community of Life Sciences, University of Benin certified the use of animals after evaluating the protocol, the ethical number issued was LS20383 (MacDonald *et al*., 2020).

Inducement of cadmium chloride

Cadmium Chloride ($CdCl_2$) (3.6 mg/kg) stock was prepared with the highest weight of the animals, weighed and dissolved in distilled water. The prepared $CdCl_2$ was administered across the groups for 7 days via intra-peritoneal route to induce toxicity prior to the commencement of treatment.

Experimental design

After 7 days of inducing toxicity via cadmium chloride, the treatments were administered to the various groups. Group 1 to 3 were administered with (100, 200 and 400 mg/kg *M. oleifera* extract root ethanol extract). Group 4 received only clean water and Group 5 was administered with 3.6 mg/kg Cadmium Chloride only. The treatment lasted for 14 days afterwards the rats were anesthetized in a mild chloroform to sedate the rats and was dissected to isolate the visceral organs. The kidney, liver, heart, and lung were isolated, rinse in normal saline and fixed in 10% buffered formal-saline in preparation for tissue processing.

Histopathological analysis

The isolated heart, liver, kidney and lungs of *Wistar* rats were fixed in neutral buffered formalin.

Affixed organs were utterly dehydrated with 99.9 % ethanol along with 70 % ethanol, and 96 % ethanol washed using distilled water. 4 µm sections were prepared, and stained in hematoxylin-eosin dye. Stained tissues were optical photomicroscope (Leica MC170 HD, Leica Biosystems, Germany) viewed at x 400 magnification. This involves the preparation of tissues and organs into a slide for easy interpretation.

RESULTS AND DISCUSSION

The results obtained for the phytochemical screening showed the presence of alkaloid, tannin, saponin and carbohydrate as the phytoconstituents found in *M. oleifera* root ethanol extract as shown in Table 1. Phytochemicals are secondary plants metabolites responsible for many bioactivity of plant extracts. They are known to possess antioxidant, anti-inflammatory, antibacterial, immunomodulatory and anti-sickling activities concurred with the work of Egba *et al.* (2012) and Fuglie, (2001). The phytochemicals observed in *M. oleifera* ethanolic and aqueous root extract in the present study undoubtedly, are responsible for the cardiovascular and immunomodulatory effects of the plant extracts. Flavonoids have been shown to have antibacterial, anti-inflammatory, antiallergic antiviral and antineoplastic activity. This agreed with the report of Suaib *et al.* (2012).

The amelioration of the perivascular infiltrates of chronic inflammatory cells (vasculitis) induced in the heart of experimental Wistar rats by cadmium chloride, undoubtedly, had been made possible by the presence of flavonoids present in the extract. Alkaloids in *M. oleifera* leaves have been found to possess analgesic, antimalarial, antibacterial and antihypertensive properties (Dangi *et al*., 2002; Lockett and Calvert, 2000). The present study which shows alkaloids as one of the phytochemical constituents of *M. oleifera* ethanolic root extract as seen in Table 1, and which upholds antihypertension as one of the properties of *M. oleifera* ethanolic root extract corroborates the claim by Dangi *et al.* (2002). Results obtained from an experiment conducted by Matsui *et al.* (2009) indicated tea-leaf saponnins to be the active components in Fr2-3 and that these saponnins exhibited anti-hypercholesterolemic activity by inhibiting cholesterol absorption in the intestines. The fact that saponnins is one of the phytochemicals of the ethanolic root extract of *M. oleifera* is certainly a reason for the hypotensive property of the extract in the present study (Seriki *et al.,* 2015).

The heart, liver, kidney and lungs showed the histoprotective effect of graded doses of the extract as shown in Figure 1-4. Cadmium chloride induced patchy vascular ulceration, congestion and perivascular infiltrates of chronic inflammatory cells (vasculitis) in the heart (Figure 1). This is in line with the study of Bowen (2003). Graded doses of *M. oleifera* extract protected the heart and dilated the blood vessels against toxicity, compared to the control. Cadmium chloride is capable of causing vascular injury toxicity (erosion) in the bronchial vessels of the lungs. However, there was no obvious damage to the alveoli in the control group compared to graded doses of the extract, to activate local immune system of the lungs (bronchiolo-alveolar lymphoid aggregates) to a mild degree as shown in Figure 2. The extract activated the local immune response, producing florid effect.

Cadmium chloride induced severe vascular ulceration and steatosis in the liver. The extract elicited a protective measure of the liver cells as well as dilated its blood vessels also triggered the activation of the local immune response (kupffer cell) in liver cells compared with untreated control (Figure 3) (Owolabi *et al.,* 2012).

Cadmium chloride induced patchy tubular necrosis in the kidney. Treatment administered with graded doses of the methanol extract, showed no defect compared to the control. The heart, lungs and liver revealed absent parenchymal damages (Figure 4). The renal cells triggered no tubular necrosis in the cortex of the treated groups compared with the control as shown by Owolabi and Ogunnaike, (2014) that worked on the histological evaluation of the effects of Moringa leaf extract treatment on vital organs of murine models.

According to Bowen (2003), kupffer cells are a type of fixed macrophage. Molawi and Sieweke (2015) in their findings stated that liver kupffer cells (KCs) are self-maintained tissue-resident macrophages, this is similar with this present study in graded doses. The activation of kupffer cells in the liver and bronchiolo-aveolar lymphoid aggregates in the lungs of the Wistar rats, in this

Table 1: The Phytochemical Screening of *Moringa oleifera* Root Ethanol Extract

Parameters	Degree
Alkaloids	+++
Tannins	+++
Saponins	+++
Flavonoids	+
Phenol	+
Anthraquinone	++
Carbohydrate	+++
Cardiac Glycoside	-

Key: +++ Very highly present; ++ highly present; + fairly Present; - Absent

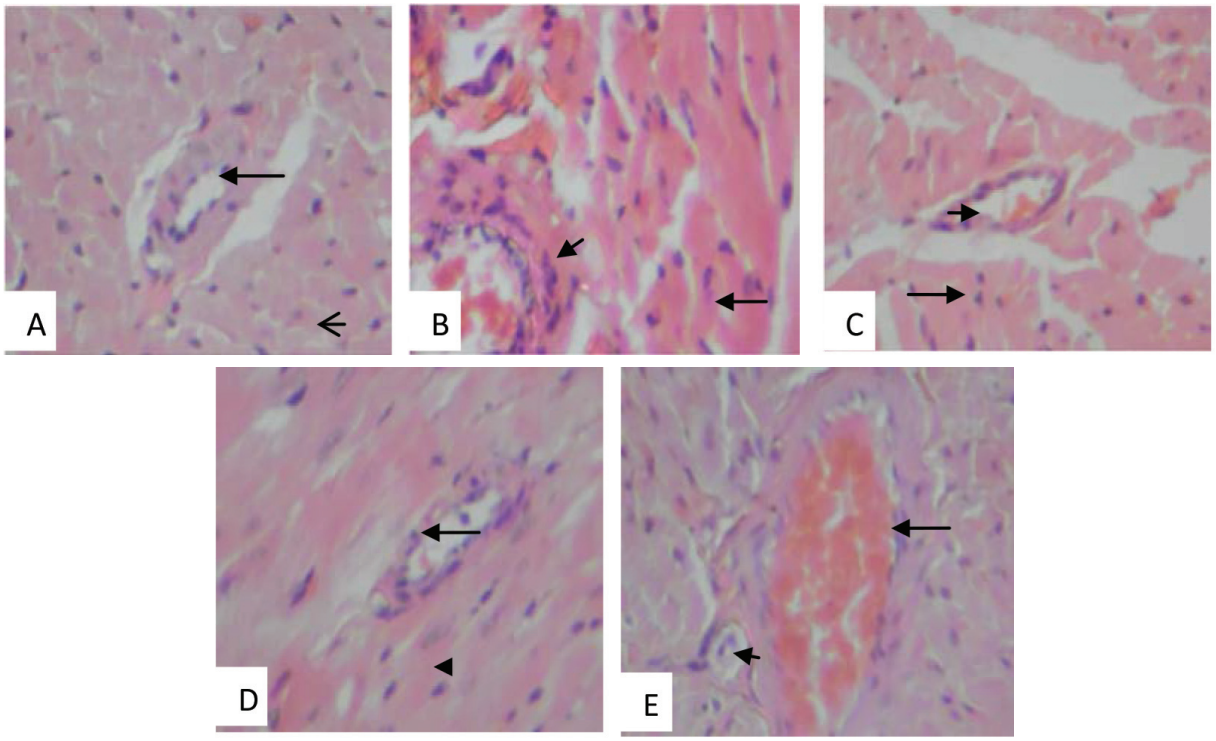

Fig. 1: Effects of *Moringa oleifera* root ethanol extract on cardiac cells

(A). Cadmium chloride 3.6 mg/kg showed long arrow, mild vascular congestion and short arrow, moderate vasodilatation (B). *M. oleifera* root ethanol extract 100 mg/kg: showed long arrow, normal coronary artery and short arrow, normal myocardium. (C). *M. oleifera* root ethanol extract 200 mg/kg: showed long arrow, mild vascular congestion and short arrow, normal myocardium. (D). *M. oleifera* root ethanol extract 400 mg/kg: showed long arrow, mild vascular congestion and short arrow, normal myocardium. (E). Normal control: showed long arrow, normal coronary artery and short arrow, normal myocardium (H&E x 100).

present study, elicited the effect of *M. oleifera* root ethanol extract with the capability to trigger the immune response. A similar study conducted by Edith *et al.* (2016) that showed the protective effect of the root ethanol extract of *M. oleifera* Lam., revealed on cardiac muscles and vessels in Wistar rats.

CONCLUSION

The present study showed the phytochemicals responsible for the histoprotective effect of *M. oleifera* root ethanol extract in dose dependent manner against toxicity in the targeted tissues had a progressive protection at graded doses. Hence

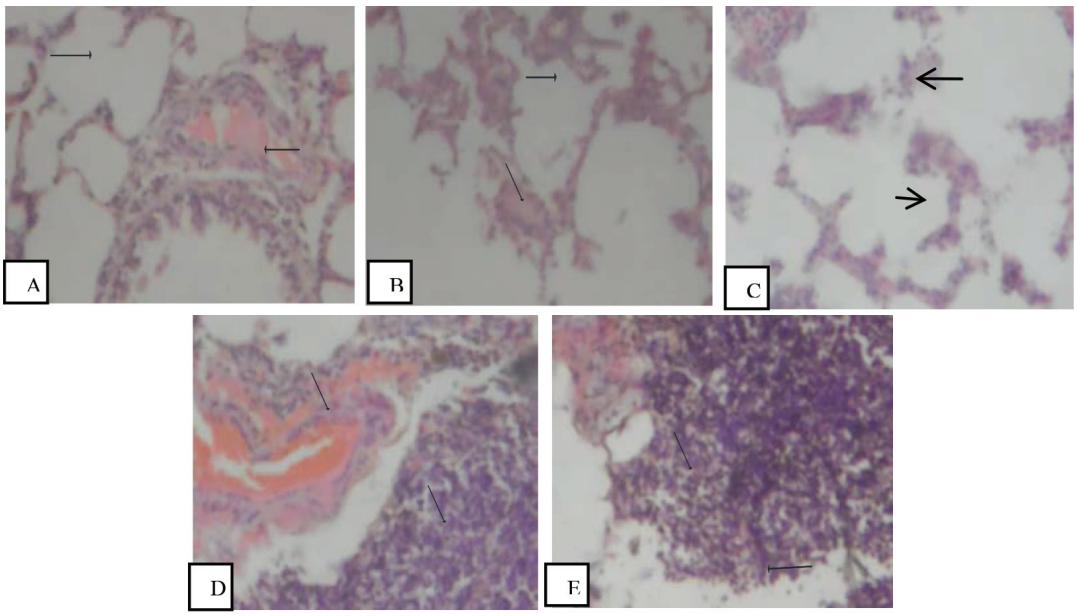

Fig. 2: Effects of *Moringa oleifera* root ethanol extract on the lungs cells
(A). Cadmium chloride 3.6 mg/kg showed: long arrow, normal alveoli and short arrow, patchy vascular intimal erosion. (B). *M. oleifera* root ethanol extract 100 mg/kg: showed long arrow, normal alveoli. (C). *M. oleifera* root ethanol extract 200 mg/kg: showed long arrow, moderate interstitial congestion and short arrow, moderate activation of bronchiolo-alveolar lymphoid aggregates. (D). *M. oleifera* root ethanol extract 400 mg/kg: showed long arrow, mild activation of bronchiolo-alveolar lymphoid aggregates (E). Normal control: showed long arrow, normal alveoli (H&E x 40).

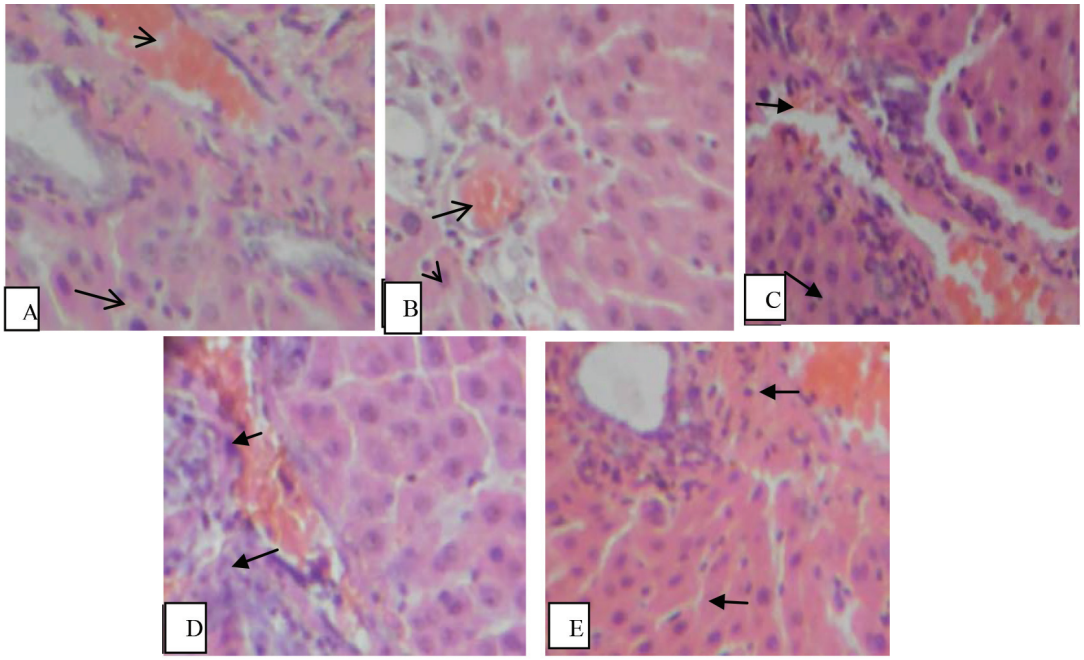

Fig. 3: Effects of *Moringa oleifera* root ethanol extract on the hepatic cells
(A). Cadmium chloride 3.6 mg/kg showed: long arrow, moderate vascular congestion and short arrow, moderate kupffer cell activation. (B). *M. oleifera* root ethanol extract 100 mg/kg: showed long arrow, mild vascular congestion and short arrow, moderate kupffer cell activation. (C). *M. oleifera* root ethanol extract 200 mg/kg: showed long arrow, mild vascular congestion and short arrow, mild kupffer cell activation. (D). *M. oleifera* root ethanol extract 400 mg/kg: showed long arrow, mild vascular congestion and short arrow, moderate kupffer cell activation. (E). Normal control: showed long arrow, mild vascular congestion and short arrow, mild kupffer cell activation (H&E x 100).

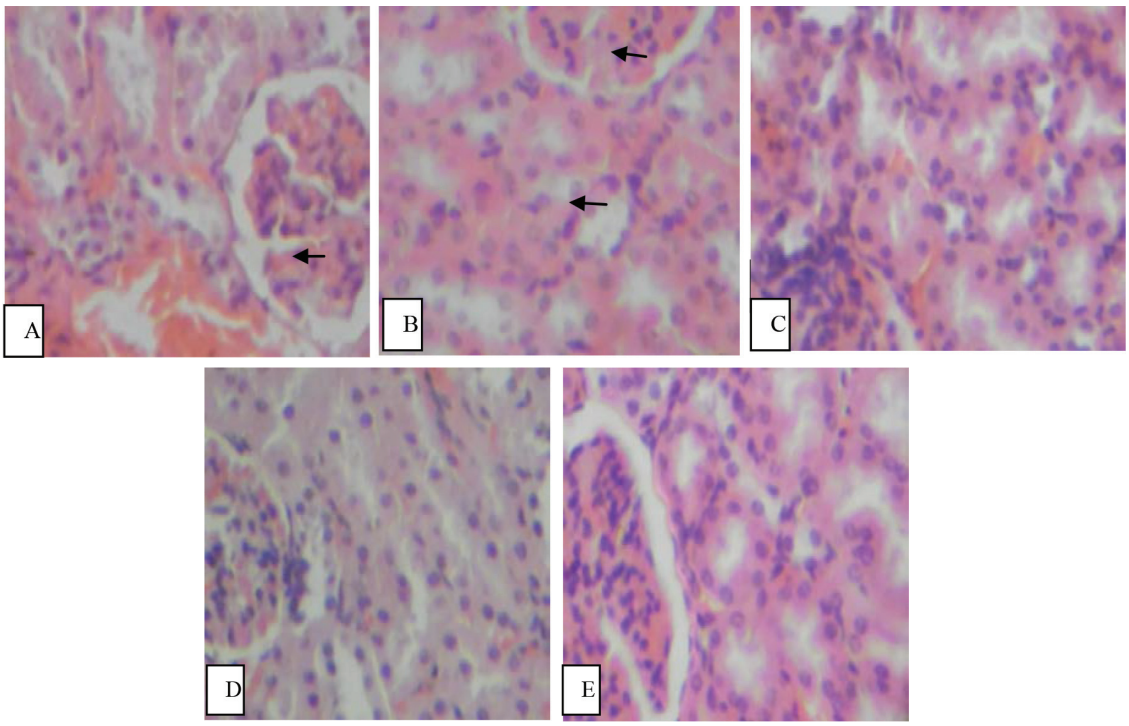

Fig. 4: Effects of *Moringa oleifera* root ethanol extract on the renal cells

(A). Cadmium chloride 3.6 mg/kg showed long arrow, focal tubular necrosis and short arrow, mild congestion. (B). *M. oleifera* root ethanol extract 100 mg/kg: showed long arrow, normal glomerulus and short arrow, tubules. (C). *M. oleifera* root ethanol extract 200 mg/kg: showed long arrow, normal tubules. (D). *M. oleifera* root ethanol extract 400 mg/kg: showed long arrow, normal tubules. (E). Normal control: showed long arrow, normal tubules (H&E x 100).

required further study to validate its ethnomedicinal benefits.

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Valorization of dry coffee grounds (DCG) and their oily extract: *Coffea arabica* and *Coffea canephora* and their mixture

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ABSTRACT

The coffee grounds CG are considered as a solid residue generated by consumers of ground coffee.For this purpose, three samples of dry coffee grounds DCG and its oily extract (Sample 1: Arabica, Sample 2: Robusta, Sample 3: mixture of two varieties) were subjected to a physicochemical characterization, quantification of primary and secondary metabolites and the mineral salts determination.The results show that the DCG of the three samples is a good source of carbohydrates with a max of 65.31±0.67%. In fact, the oil DCG extraction provided a yield with a maximum of 15.01±0.9%. The obtained values for the different physicochemical quality indices (refractive index RI , acidity index AI , ester index EI , saponification index SI and peroxide index PI),showed the conformity of the studied oils to the standards. Laquantification of secondary metabolites showed that the oily extract of mixture of two varieties is the richest in polyphenols with a value of 50.28±13.32 mg GAE/gr, on the other hand, the Robusta variety was found to be the richest in flavonoids (337.12±22.66 mg CE/gr).

***Keywords*:** Dry coffee grounds (DCG), dry coffee grounds oil (DCGO), physicochemical analysis, primary metabolites, quality index. secondary metabolites

INTRODUCTION

In the present day, the coffee became a major economic richness and presents the first agricultural product exchanged in volume,and the second most important commercialized resource in the world after petroleum. Its annual global production is constantly increasing and it exceeds 8 million tons per year (Stanislav *et al.,* 2014). Almost 50% of the coffee produced in the world is destined for the preparation of ground coffee (Ramalakshmi *et al.,* 2009), which generates significant quantities of a solid residue called coffee grounds (CG).The latter is usually evacuated to landfills with the environmental and economic consequences that this involves.

In recent decades, the growing awareness of the necessity to waste reduce, in order to protect the environment, has stimulated the search for valorize coffee grounds methods in direct use , in composting (Liu and price*.,* 2011) , or for energy production in the agropellets form by combustion (Jeguirim *et al.,*2014).Some studies have demonstrated the adsorptive properties of CG towards colorants (Shen and Gondal, 2017), such as methylene blue in an aqueous solution (Franca *et al.,*2009), as well as heavy metals (Kim *et al.,*2014) such as lead ions in potablewater (Tokimoto *et al.,*2005).

Other work confirmed the CG using possibility as a chelating agent to increase the iron availability in the soil for plants (Morikawa and Saigusa, 2008). Furthermore, other studies have shown that it is possible to extract up to 15% of CG oil by using an organic solvent (Kondamudi *et al.,*2008). This oil can be used for many applications; due to its richness in high value added molecules.In fact, the presence of diterpenes (Hugo *et al.,*2013), polysaccharides, galactomannans and arabinogalactans allowed the CG oil use as a source of dietary fiber (Simões *et al.,*2013). The CG is

rich in lipids (Couto *et al.,* 2009) and free fatty acids, which would make it convertible to biodiesel and bioethanol (Rocha *et al.,* 2014). It can be used to extract phenolic compounds which suggests the possibility of its use as a natural source of antioxidants for the cosmetic and pharmaceutical industry (Zuoroo *et al.,*2012).The free radical scavenging activity of CG is probably related to the presence of brown pigments (melanoids and polyphenols) formed during the coffee beans roasting process.

Moreover, it has been shown that coffee grounds also possess anti-allergenic properties and in a lesser extent of the anti-inflammatory properties (Ramalakshmi *et al.,*2009). An attractive approach for a country like Algeria of this valuable food residue, can be developed by creating apractical ideas with an innovative character to valorize the national agricultural patrimony and the development of a market or a durable agriculture as an example.

In the context of these data, our study is aimed to evaluate some physicochemical and biological properties of coffee grounds and its oily extract of the two varieties *Coffea arabica* (Arabica) and *Coffea canephora* (Robusta) as well as their mixture.

MATERIALS AND METHODS

Biological material and preparation

The CG (Arabica variety, Robusta variety, mixture of two varieties) used was collected daily after infusion using a MOKA Italian type coffee machine (coffee marker). In order to prevent the microbial degradation during the storage, the coffee grounds were air dried in the open air for a few days and then steamed at 40°C for 24 hours to obtain a dry coffee grounds (DCG). Once dried, the DCG is transferred to an opaque container and stored in the dark at room temperature, the samples were distributed as follows:

Sample 1 (DACG): dry Arabica coffee grounds

Sample 2 (DRCG): dry Robusta coffee grounds.

Sample 3 (DMCG): dry mixed Arabica and Robusta coffee grounds

Extraction of the DCG oil

This method consists of an extraction of the oil by an organic solvent (Hexane) on a solid matrix (coffee grounds) for 3 hours. The extraction was carried out in a closed chamber according to a semi-continuous process from 10 g of grindings, introduced in the soxhlet cartridge placed inside the apparatus, a flask filled with 200 ml of hexane was heated to 60°C for 3 hours. The hexane containing the dissolved lipids was then evaporated to recover the oil.

Three oil extracts of DCG are obtained: DACGO: dry Arabica coffee grounds oil; DRCGO: dry Robusta coffee grounds oil; DCGOM: dry coffee grounds oil of two varieties mixture.The oil yield is determined after extraction. It expresses the percentage of oil obtained in relation to the quantity of coffee grounds used for extraction.

The yield is calculated according to the following formula:

Yield (%) = (H/A) × 100 (I)

H: oil quantity (g) obtained after extraction; A: test sample (g) of coffee grounds used.

Analyses performed on the DCG

Physicochemical parameters

The various samples of DCG were subjected to several assays, namely moisture content (NFV 03-903), ash content (Horwitz and Latimer, 2005), pH measure (NFV 05-108, 1970), and titratable acidity measure (NFV 05-101, 1974).

Macronutrients

The protein determination was performed according to the method described by Bradford (1976), the protocol according to NF ISO 8262-3, 2006 was adopted to determine the fat content, and the total carbohydrates were calculated according to the equation:

Total carbohydrates (g/100 g) =100- (m fat+ m ash+ m proteins) (II) (Bazile *et al .,* 2016).

Mineral content

The flame spectrometry was used to determine the Na^+ and K^+cations with a CORNING 400 spectrophotometer. It is a method that depends on the fact that atoms excited by a flame can emit radiation of a characteristic wavelength whose intensity can be measured by spectrometry.The cation initial concentration to be determined is deduced from the intensity absolute value of the measured spectral emission.

Analysis performed on DCGO

Physicochemical characterization

The oil obtained from the DCG (DCGO) of the three samples cited previously had been subjected to several physicochemical analyses .

Refraction index IR (ISO 6320, 1983)

RI is determined by direct reading on a conventional Abbe refractometer (Bellingham & Stanley, BEL-44-501) . After the prism surfaces cleaning, and calibration of the instrument, a few oil drops are deposited in the prism middle.The value is indicated by the range reading.

Acid index AI (ISO N 660, 1990)

Oil (2g) is introduced into a 100 ml flask, 5 ml of ethanolic solution of neutralized potash is added, and a few drops of phenolphthalein indicator are added. Mix and titrate the liquid with the 0.1N potassium hydroxide solution contained in the burette, until the color of the solution changes and persists for 30 seconds. AI is calculated by the following formula:

AI = 56.11 x N x V / P (mg KOH / g oil) (III)

Where: P: mass (g) of the test sample; 56.11: molar mass, expressed in g/mol, of potassium hydroxide; V: volume in ml of KOH (0.1 N) required for titration; N: the potash solution normality (0.1 N).

Ester index EI (AFNOR, 2000)

In a flask, we introduce 2g of oil that we added with a burette of 25 ml, a solution of ethyl KOH (0.5 M), and pumice stone fragments.The flask is placed in a flask heater adapted to a refrigerator, with a duration of 1 hour from boiling. This time is sufficient to allow the acids release by the esters hydrolysis. We let it cool down, then we added 20 ml of distilled water with 5 drops of phenolphthalein. Finally, the excess of KOH is titrated with a HCL solution (0.5M) for the blank test, the same procedure is performed, but without the test sample. The EI determination is calculated by the following formula: EI = 28.05 /m (V0-V1) –AV .Where: V0: volume in ml of the HCl solution used to titrate the blank test; V1: volume in ml of the HCl solution used to titrate the excess KOH; m: mass in grams of the test sample; AV: the acid index determined.

Saponification index SI (NF ISO 3657)

The SI is the number in mg of potash (KOH), necessary to saponify the fatty acids contained in one gram of fat.This value is all the higher as the fatty acids are of low molecular weight. We can calculate SI from this formula: SI = EI – AV.

Peroxide Index PI (NF T 60-220)

1g of oil was dissolved in 25ml of solvent mixture, 15ml of acetic acid and 10ml of chloroform, then 1ml of potassium iodide solution (KI), mix and protect from light for 5 min. Add 25ml of distilled water, and titrate the released iodine with 0.01N sodium thiosulfate solution by vigorous mixing in the presence of starch (1g / 100ml) as a color indicator.

PI is determined by the following formula: PI = V-V0/P x10 (milliequivalents /Kg).With : V0: volume (ml) of $Na_2S_2O_3$ (0.01 N) required to titrate the blank; V: volume (ml) of $Na_2S_2O_3$ (0.01 N) required to titrate the sample; P: sample intake (g) of test.

Evaluation of secondary metabolites of phenolic extract

Preparation of phenolic extracts

For the phenolic compounds extraction, we adopted the protocol of Pirisi *et al*. (2000), 2g of oil are introduced in a tube, supplemented with 1ml of n-hexane and 2ml of 60% methanol. After agitation for 2 min, the mixture was centrifuged at 3000 rpm/5 min.The supernatant was collected, this procedure is repeated 2 to 3 times to exhaust the oil, then are combined and concentrated in dry conditions under vacuum at 40°C, then collected in 1ml of methanol 50%.

Polyphenol content

Total phenolic compounds were determined according to Vazquez Roncero *et al.*(1973). The prepared extract (100 ul) was mixed with 2ml of 2% Na_2C0_3 and then incubated for 2 minutes at room temperature. After incubation, 100µl of Folin-Ciocalteau reagent was added. The reaction mixture was left to stand for 30 minutes at room temperature in the dark.

The absorbance of all sample solutions was measured at 725 nm.The results are expressed as mg gallic acid equivalent per gr of dry plant material with reference to the gallic acid calibration curve.

Flavonoid content

Each extract (0.1ml)is added to 0.4ml of distilled water and 0.03ml of sodium nitrite (5%), all mixed with 0.02ml of aluminum chloride (10%). After incubation for 5min at room temperature, 0.2ml of sodium hydroxide (1M), and 0.25ml of distilled water were added. The absorbance was measured at 510 nm, the flavonoid content is expressed as mg catechin equivalent per g plant dry matter (Kim, 2003).

Statistical analysis

The results are expressed as means and their standard error (X±ES). The data were statistically analyzed using Microsoft Excel version 7.0 software. In all cases, a value of $p<0.05$ was considered significant.

RESULTS AND DISCUSSION

Physicochemical analysis of DCG

The different physicochemical parameters of the three DCGsamples are shown in (Figure 1). The moisture contents of DACG, DRCG and DMCG were in the range of (7.83±1.25% vs 7 ±1.5% vs 8.5±1.5%) respectively. According to Ballesteros *et al.*(2014), the moisture content of DCG of Arabica variety is 7.43% which is close to our study, while, the work of Panusa *et al.*(2013) remain higher (9.50%) than our study. On the other hand, according to Ravindranath *et al.*(1972), the moisture content of DRCG varied from 7.7 to 9.9%, while Martinez-Saez *et al.*(2017) reported a low rate of 3.6%. On the other hand, the moisture contents of the DMCG varied from 7.12% to 7.84% (Panusa *et al.,*2013), which remains lower than our results.

Several researchers have evaluated the ash content of DCG , Ravindranath *et al.,*1972 (1.8 to 2.4%), Martinez-Saez *et al.* (2017) (0.5%), Bastelleros *et al.*(2014) (1.71%), (Pujol *et al.,* 2013; Caetano *et al.,* 2012; Campos-Vega *et al.,*2015) (0.19 to 1.6%). These works synchronized with our results.

According to our results the pH of three samples is situated in the range of 5.5 to 5.85 which is in agreement with the work of several researchers who have found the values in a range of 4.2 to 5.7 (Go *et al.,*2016; Todaka *et al.,* 2013; Somnuk *et al.,* 2017).

Primary metabolites

The content of DCG in carbohydrates, lipids and proteins is represented in (Figure 2). Our results confirm that DRCG is the richest in protein 14.69±1.13% compared to DACG13.68 ±0.21% and the mixture of two varieties 13.91±0.61%.These results synchronize with those of: Ravindranath *et al.* (1972) with a rate of 14.8% for DRCG; (Ballesteros *et al.,*2014 and Mussatto *et al,.*2011b), reported a rate in the order of 17.44% and 14% protein in DMCG respectively. While the works of Lago *et al.*(2001) and Martinez-Saez *et al.*(2017) are not synchronized with our results, with values of 6.7 to 9.9% and 11.20% respectively. According to Arya and Rao (2007), the roasted coffee contains an average of 3.1% (w/w) of protein. The protein content in CG is higher than in coffee beans due to the concentration of the non-extracted components during the preparation of instant coffee. However, these estimates may be over-estimated due to the presence of other nitrogen-containing compounds such as caffeine as well as several amino acids (Delgado *et al.,* 2008). In fact, 17 amino acids are present in CG, including the nine essential amino acids. These represent almost 50% of the amino acids present (Ballesteros *et al.,*2014), mainly leucine contributing 13 or 21% of the total content. This suggests that the protein content may be over-estimated, depending on the coffee variety.

Regarding fat content, our results varied between 11.2% - 15.01%. It has been reported that CG contained 10-15% (Jenkins *et al.,* 2014), (9.3-16.2%) (Cruz *et al.,* 2012), 20%) (Lago *et al.,* 2001; Martinez-Saez *et al.,* 2017), 15.6% (Haile, 2014), 14.70% (Jorge *et al.,* 2017) of fats. The variations in content compared to our work are probably related to extraction methods and coffee varieties. In addition, since fats are not efficiently extracted in an aqueous medium, it is normal to find most of them in the CG. Also, the CG fats are composed of 84.4% of triglycerides. These are represented by linoleic acid (C18:2), palmitic acid (C18:2), oleic acid (C18:1) and stearic acid (De Melo *et al.,* 2014).The CG fats also contain sterols, including sitosterol (10-14% of coffee grounds), stigmasterol (4-5%) and campesterol (3-4%) (Campos-Vega *et al.,* 2015).

Table 1: Organoleptic characteristics and different CG oils yields

Characteristics	DACGO	DRCGO	DCGOM
Appearance and color	Yellowish brown	Chocolate brown	Dark color (brown)
Smell	Strong and agreeable coffee flavor		
Touch	Viscous, unctuous		
Taste	Lightly bitter and pungent but very sweet and persistent, as a good black coffee		
Yield (%)	**15.01±0.9**	**11.2±0.26**	**13, 06 ± 0.32**

Each value was expressed as means ± Standard deviations for triplicate experiments ,DACGO: dry Arabica coffee grounds oil; DRCGO: dry Robusta coffee grounds oil; DCGOM : dry coffee grounds oil of two varieties mixture

Table 2: Physicochemical indexes

Indexes	DACGO	DRCGO	DCGOM
Refractive Index	1.4667 ±0.004	1.4694±0.002	1.4720±0.0005
Peroxide Index meq d'O_2/Kg	7±0.25	12±0.33	8±0.15
Acid Index mg KOH/g	11.22±0.1	7.85±0.17	8.97±0.05
Saponification Index	180±0,08	190±0,07	174.6±0,31
Ester Index mg KOH/g	168.8±2,24	182.15±1.74	165.63±0,35

Each value was expressed as means ± Standard deviations for triplicate experiments ,DACGO: dry Arabica coffee grounds oil; DRCGO: dry Robusta coffee grounds oil; DCGOM : dry coffee grounds oil of two varieties mixture

Table 3: Secondary metabolite levels of different CG oils

	TPC (mg GAE/gr DM)	*TFC (mg CE/gr DM)*
DACGO	10.4 ± 3.13	110,9 ± 14,73
DRCGO	38.63 ± 1.88	337,12 ± 22,66
DCGOM	50.28 ± 13.32	169,28 ± 12 ,27

Each value was expressed as means ± Standard deviations for triplicate experiments. CE: Catechin equivalent; DM: Dry Matter; GAE: Gallic acid equivalent; TFC: Total flavonoid content; TPC: Total phenolic content; DACGO: dry Arabica coffee grounds oil; DRCGO: dry Robusta coffee grounds oil; DCGOM : dry coffee grounds oil of two varieties mixture

The carbohydrates are the most abundant elements in the CG. Indeed, it is rich in sugars of polymerized cellulose and hemicellulose structures. Specifically, the CG contains 46.8% of mannose, 30.4% of galactose, 19% of glucose and 3.8% of arabinose. This data varied from study to study, which may be due to the coffee beans variety used. In CG expresso, galactomannan is the most abundant and represents 50% of carbohydrates (Ballesteros *et al.,*2014). From our results, we can distinguish that the carbohydrate content DRCG (65.31±0.67%) is slightly higher than DACG and DMCG, which have a similar content (63.17±0.36% ,63.67±0.54%). This difference is not significant.

Mineral salts

According to our results, the Na^+ content varied between 2.22 and 3.14 mg/l for the 3 samples (Fig.3), these results are lower than the study carried out by Ballesteros *et al.*(2014) with a content of 33.70 (mg/Kg dry material). On the other hand, the different samples of CG are rich in K^+ with contents that varied from 8.85 to 9.58 mg/l (Fig .3). These results are not synchronized with those obtained by Ballesteros *et al.*(2014) and Cruz *et al.*(2012) who found K+ contents in the range of 11.7 mg/kg and 3.12 to 21.88 mg/kg respectively.

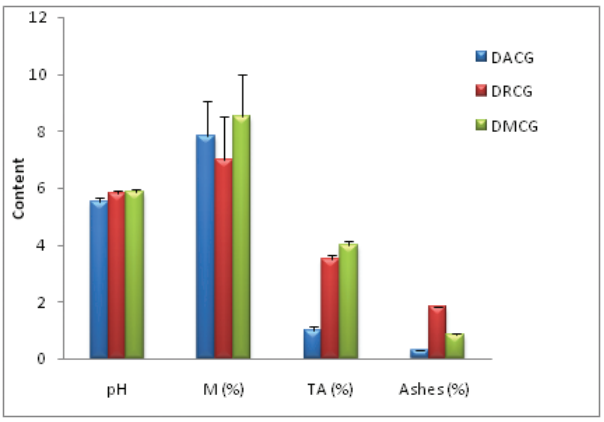

Fig. 1: Physicochemical parameters of the different DCG

Each value was expressed as means ± Standard deviations for triplicate experiments , DACG : dry Arabica coffee grounds , DRCG : dry Robusta coffee grounds , DMCG: dry mixed Arabica and Robusta coffee grounds, TA : Titratable Acidity ; M : Moisture

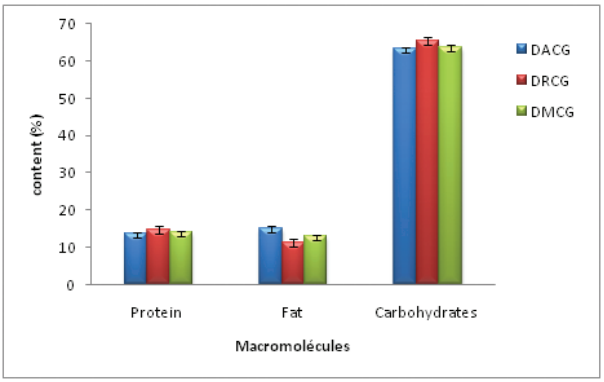

Fig. 2: Macronutrients contents

Each value was expressed as means ± Standard deviations for triplicate experiments , DACG : dry Arabica coffee grounds , DRCG : dry Robusta coffee grounds , DMCG: dry mixed Arabica and Robusta coffee grounds

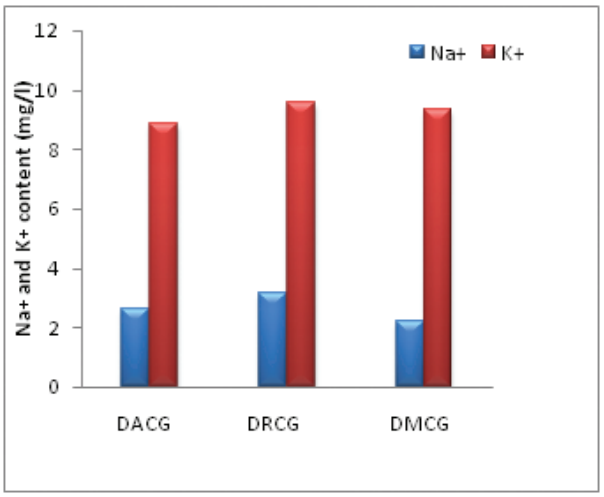

Fig. 3: Na^+ and K^+ content of the different DCG

DACG: dry Arabica coffee grounds, DRCG: dry Robusta coffee grounds, DMCG: dry mix coffee grounds of Arabica and Robusta .

Evaluation of different characteristics of DCGO

Organoleptic characterization

The organoleptic characteristics of different oils are quite similar, with a maximum yield of around (15.01±0.9%) in the Arabica variety. DACGO is in agreement with that of Haile.(2014) with a value of 15.6% and Jorge *et al.*(2017) with a value of 14.70%.While the yield of DRCGO is not in agreement with the study of Ravindranath *et al.*(1972) with a value of 7.9%. The result of DCGOM does not synchronize with the results achieved by Ballesteros *et al.*(2014) with 2.29 ±0.30% and Haddoudi *et al.*(2014) with 11% (Table.1).

Physicochemical characterization

The different physicochemical indexes of the three oil samples are shown in Table 2. The Refractive Index (RI) is a good indicator of the fat conservation state. It measures the total hydro-peroxides, which are the first oxidation products (Kiritsakis, 1998). It varied with the wavelength of the incident light and with temperature.It is proportional to the fatty acids molecular weight and their unsaturation degree, which provides a good appreciation on the oxidation possibility. The results showed that RI of the different varieties studied is comparable to that reported by Ravindranath *et al.*(1972) with a value of 1.460, and that of the codex alimentarius (1.4720-1.4750).

The results reported a high Peroxide Index (PI) for DACGO (12 meqO_2/Kg oil) compared to that of DRCGO**,** which is in the order (7 meq O_2/Kg oil) and DCGOM, which is (8.97 meq O_2/Kg). The low peroxide index of the Arabica variety may indicate that its rancidity is low and that it can be stored for a long time.

The Acid Index (AI) indicated the degree of ester alteration (mainly triglycerides) present in the fat. According to our results we noticed that the acid index is 11.22 mg KOH/g; 7.85 mg KOH/g and 8.97 mg KOH /g for DACGO, DRCGO and DCGOM respectively, our results are comparable to those reported by Ravindranath *et al.*(1972) with values in the range of (7.3-10.1 mg KOH/g).

For the Saponification Index (SI) of different varieties of DCGO ranged between174.6 to 190 (mg KOH/g), according to Ravindrarth *et al*.(1972), the SI of DRCGO varied from 180-200 mg KOH/ g. Similarly, a study performed by (Haile ,2014) revealed a value of 167.28 mg KOH/g for DACGO.

According to our results, the Ester Index (EI 0 values of three samples varied from 165.63 - 182.15 mg KOH/g, these results remained similar to the scientific literature with values of : 172.2-189.9mg KOH/g (Ravindrarth *et al.,*1972); 157.43 mg KOH/ g (Haile, 2014)

Phytonutrients

According to our results, the oil extract is relatively rich in polyphenols with a maximum of 50.28 ± 13.32 mg GAE/gr DM, this value remain higher than other works namely those of (Haddoudi *et al*., 2014) with a value of about 28 mg GAE /g; (Choi and Koh, 2017) with a level of 25.5 mg GAE/ g and (Panusa *et al*., 2013) with 12.58 -17.45 mg GAE/g using ethanol at (60%).

Similarly, the content of flavonoids of the oil extract of the three samples varies between 11.09 ± 1.47 to 33.71 ± 2.26 mg EC/gr DM, these values remain comparable with other studies, namely those Samar *et al*.(2018) with a value of 34.32 mg EC/g.

In general, several factors can influence the rate of secondary metabolites namely: the method of extraction, temperature, time of extraction (Samar *et al*., 2018), climatic conditions and area of harvest (Elkadi *et al.*, 2021).

CONCLUSION

Our results confirm the richness of DCG in macromolecules, namely carbohydrates, and the oil extracted from two varieties *viz*., Arabica and Robusta, as well as their mixture showed a conformity of the different quality indexes to the standards, with a richness in phytonutrients. On the other hand, we detected a quantitative differences in yield and in some physicochemical and biological parameters between the two varieties Arabica and Robusta and their mixture. According to our results, the rate of some primary and secondary metabolites is influenced by the origin and the variety of the coffee beans.

This study promotes the use of CG oil as an economic and ecological alternative to the use of many common cosmetic oils such as coconut or jojoba oil often imported from tropical countries.

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Assessment of grapevine genetic resources based on physiological data

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ABSTRACT

One of the most important breeding priorities is the assessment and improvement of the resistance of cultivated fruit crop genotypes to environmental stressors. Abiotic factors often do not allow realizing the productivity potential of fruit plants, which entails a decrease in the efficiency of their cultivation and financial losses for producers. In this regard, varieties with increased resistance to extreme environmental conditions are of great importance. Therefore, it is necessary to develop and use practical approaches to study and isolate genetic sources of high stress resistance for use in plant breeding and introduction.

The aims of the studies were identifying the potential for resistance of grapevine genetic resources to abiotic stressors (through physiological diagnostics) and assessing new highly adaptive genotypes. We studied varieties and wild forms of grapevine differing in ecological and geographical origin to determine the main photosynthetic indicators (the total amount of chlorophyll, chlorophyll "a" / "b") in connection with their drought resistance. As a result of the study, grapevine varieties - (Ag Shany, Ag kishmishi, Tozlaiyjy, Girmizi kishmishi) and wild grapevine samples No.71, 17 No. 78, 25 No. 43, No. 74, 3 No.34 were identified as the most drought resistant. As a result of the study, relatively drought-resistant samples were identified, which are recommended for use in breeding work. Determination of the degree of plant resistance to extreme environmental factors will make it possible to assess the prospects of varieties of various fruit crops, including grapevine, for different soil and climatic zones of Azerbaijan.

***Keywords*:** Abiotic stress, chlorophyll, drought, environmental factors, grapevine, resistance.

INTRODUCTION

In Azerbaijan Republic the wild grape samples spread widely in large areas and on the banks and shores of rivers, lakes and sea and mountain slopes of Absheron, Nakhchivan AR, Ganja-Gazakh, Garabagh, Mil-Mughan, Shirvan, Talysh and etc. A number of researches were implemented in Khachmaz, Guba, Khudat, Nabran, Gusar, Shamakhi, Ismayilli, Aghsu, Oghuz, Gabala, Shaky, Zagatala, Lankaran, Fuzuli, etc. regions for studying the genetic resources of grape. At the same time as it may be noted that wild grape spread on the whole territory of Azerbaijan is very ancient formation. Wild grape - *V. vinifera* L. subsp. *sylvestris* (C. C. Gmel.) Hegi. of Azerbaijan is distinguished with specific characters. It is spread on the territory of Azerbaijan from 12 m below sea level (Kyur riverside, Salyan region) to 2000 m above sea level (Gusar region). There are two kinds of wild grapes in Azerbaijan: *typica* Negr. (with hairs) and *aberrans* Negr. (hairless). Hundreds (according to some information about more than 600) of landraces of grapevine are grown in the Republic (Musayev and Huseynova, 2016). White, red, black, pink colored table, technical and seedless grapevine varieties are cultivated here (Musayev *et al*., 2013; Maghradze *et al*., 2012; Musayev *et al*., 2019)

One of the most important breeding priorities is the assessment and improvement of the resistance of cultivated fruit crop genotypes to environmental stressors. The maximum productivity of cultivated crops is possible with an increase in their resistance to climatic stress. Abiotic stressors are the main limiting factor in agricultural productivity. There is a wide variety of fruit plants in Azerbaijan, including grapevines, which have great value as donors of high quality fruits and productivity and resistance to adverse external conditions. The usefulness and value of these crops lie in the fact that many of these plant species are adapted to growing in such difficult agricultural conditions as soil salinity, climate aridity and degraded or hilly field relief.

The meteorological conditions of the territory of Azerbaijan are often characterized by an insufficient amount of precipitation, saline soils, and a lack of moisture in the air and in the soil leading to a decrease in the activity of enzyme systems, disruption of water exchange, negatively affects photosynthesis, the assimilation of mineral nutrients by the plant organism. The violation of the basic physiological and biochemical processes led to the reduction of the productivity of plants. Abiotic factors more and more often do not allow realizing the productivity potential of fruit plants, which entails a decrease in the efficiency of their cultivation and financial losses for producers. In this regard, varieties with increased resistance to extreme environmental conditions are of great importance (Musayev *et al*., 2017).Therefore, it is necessary to develop and use effective approaches in order to study and isolate genetic sources of high stress resistance for use in plant breeding and introduction.

The main role in identifying the traits that determine its resistance to unfavorable environmental factors belongs to the physiological characteristics of plants. Determination of the degree of resistance to drought will make it possible to assess their prospects for different soil and climatic zones and to identify genetic sources with high stress resistance for their use in breeding and crop production. The use of physiological methods in combination with agro biological observations in fruit plantations makes it possible to reliably assess the degree of drought and salt tolerance of fruit trees, and to select stable and productive samples. The problem of adaptation of such valuable plants as sea buckthorn, grapevines, and other fruit crops deserves attention. The aim of our work was to study the content and ratio of one of the photosynthetic pigments - chlorophyll in the leaves of grapevine plants in connection with their drought resistance.

MATERIALS AND METHODS

The experiments were carried out in the department of fruit crops and physiology of the Genetic Resources Institute of the National Academy of Sciences of Azerbaijan. The objects of research were the leaves of the grapevine varieties and forms: Ag kishmishi, Gyrmyzy kishmishi, Tabrizi, Nakhichevan sary kishmishi, Hafizeli, Bayanshire, Shamakhy merendisi, Sary gile, Gush ureyi, Gizil uzum, Ag shany, etc., as well as wild grapevine samples.

The studies were carried out according to the methodological guidelines (Udovenko, 1988). One of the diagnostic methods of plant resistance to drought stress is to study changes for chlorophyll *(a + b)* in plant leaves under the influence of stress and to determine the degree of stress-depression of the pigment complex. Determination of the peculiarities of the pigment-protein complex of chloroplasts in plants under the influence of drought formed the basis of the method for diagnosing drought resistance.

The determination of plant resistance to drought was carried out according to some physiological indicators: the content and stress depression of the photosynthetic pigment complex (the content of the total amount of chlorophyll, chlorophyll *a, b*) in a sucrose solution simulating a lack of moisture. The assessment of plant resistance to drought by the magnitude of the decrease in the concentration of pigments was carried out using leaf cuttings placed in test tubes with sucrose solution (experiment) and water (control), after which, for the extraction of pigments, the material was placed in test tubes with 10 ml of 96% ethanol. Using a modern spectrophotometer (UV-3100PC, Japan), the optical density (D) of chlorophyll a and b in the total mixture of pigments was determined at two wavelengths (D665, 649), corresponding to the absorption maxima of pigments in this solution. Based on the data obtained, the ratio (in percent) of the concentration of pigments in leaf cuttings in an osmatic solution (experiment) to their concentration in water (control) was calculated. This ratio is a measure for determining the relative drought resistance of the compared objects - it is the higher, the greater the drought resistance of plants. Cluster analysis was determined by the coefficient of genetic proximity among the samples using the PAST computer program (Hammer *et al*., 2001).

RESULTS AND DISCUSSION

One of the indicators of the physiological state of plants associated with their productivity is the determination of changes in the pigment complex

Table 1: Physiological assessment of drought tolerance of grape samples

No.	The name of the variety and forms	The amount of chl. per unit leaf area, in mcg			
		Chlorophyll a+b		Change in the amount of chl-la under the influence of drought, in percent (%)	Degree of depression, in percent (%)
		Control	Sucrose		
1.	Ag kishmishi	1.38	1.67	121.0	0
2.	Tozlaiyjy	2.52	3.22	127.7	0
3.	Gyrmyzy kishmishi	2.62	3.13	119.5	0
4.	Tabrizi	1.76	1.88	106.8	0
5.	Misgaly	1.66	1.76	106.0	0
6.	Nakhichevan sary kishmishi	1.95	1.91	98.0	2.0
7.	Hafizeli	1.74	1.77	101.7	0
8.	Bayanshire	1.41	1.43	101.4	0
9.	Shamakhy merendisi	1.92	1.82	94.8	5.2
10.	Sary gile	2.07	1.92	91.8	8.2
11	Gush ureyi	2.03	2.16	106.4	0
12	Gizil uzum	2.75	2.40	87.3	12.7
13	Ag shany	1.19	1.79	150.4	0
14.	Madrasa	2.78	2.14	77.0	23.0
15.	Gara shany	1.80	1.31	72.8	22.2
	Wild grape samples				
1.	No 71	1.93	2.00	103.6	0
2.	17 No 78	2.08	2.32	111.5	0
3	No 74	2.77	2.97	107.2	0
4.	3 No 34	1.81	1.88	103.9	0
5.	18 No 13	2.92	2.98	102.0	0
6.	25 No 43	2.68	2.94	110.0	0
7.	10 No 79	2.84	2.81	99.0	1
8.	29 No 10	3.04	3.45	113.5	0
9.	27 No 80	3.16	2.81	89.0	11
10	1 No 4	2.57	2.13	83.0	17
11.	26 No 9	3.25	3.08	94.8	5.2
12.	No 72	2.25	2.28	101.3	0
13.	12 No 90	2.46	2.46	100.0	0
14.	5 No 32	1.30	1.24	95.4	4.6
15.	16 No 17	2.56	2.73	106.6	0
16.	31 No 13	2.94	2.99	101.7	0
17.	No 87	2.17	1.86	85.7	14.3

of the photosynthetic apparatus of leaves and the assessment of plant resistance to unfavorable factors, in particular to drought and salinity. The use of physiological methods in combination with agro biological observations in fruit plantations makes it possible to reliably assess the degree of drought resistance of fruit trees, and to select stable and productive samples.

In the adaptation and resistance of plants to unfavorable factors belongs to the structural elements of the plastid apparatus, as a material basis, providing its functional activity in changing

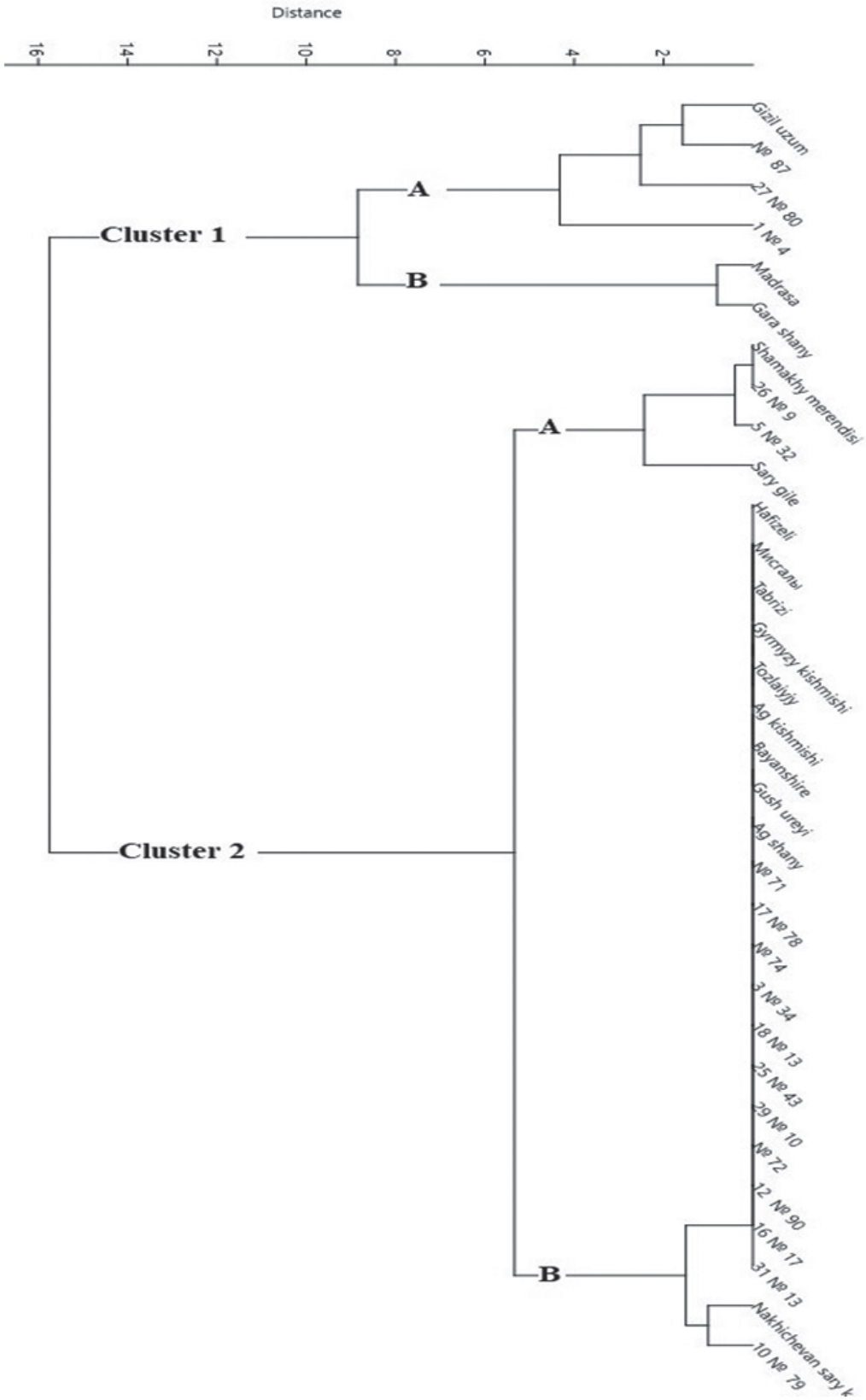

Fig. : Grouping of grapevine samples by grade change depression.

environmental conditions. The pigment system of plants is the basis for the photosynthetic conversion of solar energy into the energy of chemical bonds. The process of adaptation of plants to environmental factors of the environment is carried out at the level of cell organelles, in particular, chloroplasts, and the mechanisms of adaptation to these unfavorable factors in plants are different. The main photosynthetic pigments are chlorophylls (Chl), and carotenoids (Car) transfer additional energy to chlorophylls, performing a light-collecting function, performing a light-shielding function. The efficiency of the pigment system depends on the compliance of its structure and function with environmental conditions. Adaptation to abiotic stress factors can affect both the content of photosynthetic pigments and the ratio of their forms. At the same time, there are few data on changes in the pigment complex of plants under extreme abiotic conditions (Musayev and Huseynova, 2017; Ivanov *et al*., 2013). The inherent resistance of plants to regularly manifested unfavorable environmental factors is a mandatory feature and is carried out at the level of cell organelles, in particular, chloroplasts, and the mechanisms of adaptation to these unfavorable factors in plants are different. The pigment complex of the photosynthetic apparatus of plants is highly sensitive to changing environmental conditions. The efficiency of the pigment system depends on the compliance of its structure and function with environmental conditions (Musayev *et al*., 2017).

The research was aimed at identifying the influence of environmental extreme factors (drought) on the state of the main photosynthetic pigments, chlorophyll of grapevine plants in varieties that differ in ecological and geographical origin, as well as wild forms. The work evaluates the response to stressful actions of some varieties and wild forms of grapevines in order to study and isolate genetic sources with high stress resistance in terms of physiological parameters. The study of correlations between the amount of chlorophyll sum (a + b) and in the ratio (a / b) and changes in climatic factors in grapevine plants revealed changes in the content of the studied samples under the influence of drought stress. The following results were obtained (Table 1): 4 grapevine varieties - (Ag shany, Ag kishmishi, Tozlaiyjy, Girmizi kishmishi) were identified as highly drought-resistant. The degree of chlorophyll depression in the sucrose solution is completely absent in these varieties. The resistance of the studied samples to drought is confirmed by indicators that fluctuate within the range of 119.5% - 150.4%. The varieties of Tabrizi, Hafizeli, Misgaly, Bayanshire, Shamakhy merendisi, Nakhchivan sary kishmishi proved to be drought-resistant. Wild grapevine specimens, collection numbers No.71, 17 No. 78, 25 No. 43, No. 74, 3 No. 34 have been identified as highly drought tolerant.

It is known that the ratio of chlorophyll forms (*"a" / "b*") is an indicator of resistance to adverse environmental factors. Abiotic stress factors significantly affect the change in the pigment complex and the dynamics of the accumulation of the amount of chlorophyll. Changes in the pigment complex are explained, first of all, by a decrease in the labile form of chlorophyll, i.e. the amount of chlorophyll "*a*", while the amount of chlorophyll *"b*" is stable under these conditions. The change in the chlorophyll ratio ("*a*" / "*b*") observed in the research work can be considered an adaptive response (nonspecific) of the assimilation apparatus of plants to stress effects and, as a result, a decrease in the amount of the main photosynthetic pigment - chlorophyll "*a*" and an increase in the amount of the auxiliary form of chlorophyll "*b*" (Maghradze *et al.,* 2012).

The work also carried out a cluster analysis reflecting the physiological and genetic relationships between the samples studied by us (Fig.). As is known, cluster analysis is used to determine physiological and genetic similarities in terms of stress resistance and grouping of samples, i.e. creation of a database of the grapevine genetic resources studied by us. The results of the grouping of the samples obtained by us made it possible to distinguish 2 main clusters and to reveal the nature of the distribution of genotypes in the varieties and wild forms of grapevines studied by us. In each cluster, genotypes from different regions of Azerbaijan were grouped. The first cluster is Sub divided into 2 subgroups. Each subgroup consists of samples - variety Gizil uzum, form ^{1}87, 27^{1}80, 1 ^{1}. 4, varieties Madrasa and Gara shany, which were identified as moderately resistant: the degree of chlorophyll depression during drought is 12.7 – 23.0%. The second cluster turned out to be numerous, in which most of the samples studied by us are localized. This cluster contains 22 samples with high stress resistance, *i.e.* these samples were

assessed as highly resistant to drought stress (depression stress is 0%). Most of these samples, which are presented in the 2nd cluster, were taken from Nakhichevan. This cluster houses the grapevine form - 5 No. 32, Sary gile variety, form No. 87, varieties-Hafizeli, Misgaly, Tabrizi, Girmizi kishmishi, Ag kishmishi, Bayanshire, Ag shany and others. The second subgroup b included - forms 16 No.17 and 31 No. 13, variety Nakhichevan sary kishmishi and form 10 No. 79, that is, the first subgroup follows from the second, the second from the third. These samples are highlighted as resistant. Thus, from the constructed scheme, on the basis of the obtained results of cluster analysis, it can be concluded that 60% of the studied samples were resistant, 40% resistant and moderately resistant to stress. The presence of stress-sensitive samples was not recorded in our experiments.

The revealed results of the analysis indicate the presence of a number of general patterns in the deviation of the structural and functional parameters of the photosynthetic apparatus of plants under the influence of such unfavorable environmental factors as drought. Qualitatively the same type, common for all plants, the nature of the negative response of their photosynthetic apparatus to stress is expressed in a decrease in the content of pigments and in a general drop in the photochemical activity of chloroplasts. The magnitude of these deviations directly correlates with the level of tension of the stress factor and depends on the resistance of plants.

If we take into account the opinion that the response of the photosynthetic apparatus to an unfavorable external influence is nonspecific, common for plant organisms, character (Musayev *et al.,* 2017; Shishkanu and Titova, 1985), this suggests that the differences between genotypes that we have established are the levels of the response of the photosynthetic apparatus to the action of external factors, being reflection of unequal rates of internal structural and functional adjustment of plants to an unfavorable situation and characterize their adaptive ability and resistance to changing environmental conditions.

CONCLUSION

As a result of studying the genetic resources of wild and cultivated forms of the grapevine, the most drought-resistant samples were identified. These samples will later be used as donors in the breeding of new drought-resistant grape cultivars with high productivity and quality of berries.

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Short communication

Development of Eco-friendly Smart Bio-food Wrapper using Undersized *Heliconia bihai* leaves

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ABSTRACT

Two consecutive experiments were conducted with the objectives of developing eco-friendly food wrappers with Heliconia leaves. First experiment was conducted to attach narrow leaves of Heliconia bihai together along with the outer packing paper introduce as a user friendly smart bio food wrapper since those are narrow and not in adequate size for wrapping food. Sago solution, gelatin, wheat flour and rice starch solutions were tested as sticking agents to attach leaves. There were twenty replicates in each treatment and performances of the product was evaluated using a taste panel comprised of twenty un trained numbers. "Kruskal Wallis H Test" was used to analyze the data as non-parametric test ($p>0.05$). There were significant differences between the treatments on smell, adherence and overall acceptability up to ten days after the production. Texture and colour showed significant changes after four and six days respectively. It was observed that up to four days of time all the sticking agents gave good results in all the tested parameters. Sago and gelatin solution showed good in all qualities up to six days. Based on the results of first experiment, second experiment was conducted to determine the quality of food wrapped inside the wrappers. Food wrappers prepared with sago and gelatin were compared with Banana leaves as a food wrapper. There were no significant differences ($p<0.05$) between the tested food wrappers on taste, smell, appearance and overall acceptability. User friendly food wrappers can be produced as two in one (wrapping and packing) wrapper by sticking narrow Heliconia bihai leaves in fairly good size.

***Keywords*:** Bio food wrapper, friendly user, *Heliconia bihai*, sticking agent.

INTRODUCTION

Plastics are extensively favored and used due to their resistance to corrosion, light in weight, moisture proof, and adaptability. Use of plastic food packing is hard to environment and damage the human and animal health. Disposable plastic packages take several decades to degrade and they remain in the environment and leading to environmental pollution (Verma *et al.,* 2016). These are preferred only due to their low cost and short term convenience. At present, many industries are moving towards developing biodegradable packaging materials, leading to zero waste in the environment. Biodegradable means the materials can be easily decomposed within a shorter duration under the action of microorganisms (Babu *et al.,* 2013). It is considered that biodegradable packaging materials can substitute for synthetics at a low cost, thereby making positive effect on both environment and ecosystems.

Several plant-based packaging materials can be used as an alternative to plastics and safe for environment (Khazir and Shetty, 2014). As reported by Guillard *et al.* (2018) 50% packaging waste production can be reduced by 2050, if one in two food packs are made. As a trend in creating sustainable and eco-friendly packaging materials, attention is turned towards the plant products such as leaves and agro-based waste materials. Several materials are joined to produce food packaging; this method generally uses each of the materials' functional or aesthetic characteristics (Marsh and Bugusu, 2007). These two characteristics are linked, helps to determine things like shelf life, product protection, and the packages insulation properties. Finding the ideal material or combination of materials aids to retain product quality and originality during storage, supply, and consumption (Fellows and Axtell, 2002).

The home gardens in Sri Lanka are abundant in different species of medicinal plants. Since almost every component of the plant has therapeutic potential, it is employed in conventional Ayurvedic procedures. In contrast to other plant organs, however, leaves, roots, flowers, bark, fruits, and rhizomes have greater therapeutic potential (Mirihagalla and Fernando, 2021). Banana leaves are the commonly and popularly using leaf material for wrapping foods. Weerasinghe *et al*. (2007) has introduced banana leaves as food wrappers in cured form, which last long under refrigerated conditions without affecting the quality. However, there are certain limitations eg. especially under dry zone conditions, most of the banana leaves are subjected for tearing due to blowing winds that limits the availability of pieces of leaves in desired size. Therefore, the identification of an alternative suitable leaves regardless of the size is of prime importance. *Heliconia*, ornamental herbaceous shrubs, has a high potentiality of utilizing *its* leaves for alternative purposes in various household applications due to their close relationship to bananas, cannas and ginger. Among Heliconia species, *Heliconia bihai*, well known as Parrot's beak has drawn a considerable attention for its medicinal value. Leaf extracts of *H. bihaii* is used as a diuretic and as a astringent while the root extract is used to ease the expulsion of the fetus at the birth (Awodele *et al*., 2015). Moreover, their large paddle-like leaves, shows similarity to banana leaves and hence shows potential to be used as food wrappers. This has scientifically been proved by Weerasinghe and Madhushani in 2019 by producing cured food wrappers with *heliconia bihai* leaves in adequate size for wrapping food. Heliconia is grown in wet zone in Sri Lanka and they produce large leaves under shade conditions. Under dry zone condition they grow well but produce somewhat narrower leaves compared to the wet zone. Hence, the present study was conducted to develop food wrappers with small Heliconia leaves but in adequate size by attaching narrow leaves together along with the outer packing paper as a user-friendly smart bio food wrapper.

Two experiments were conducted at the Institute for Agro-Technology and Rural Sciences, Weligatta New Town, Hambantota, Sri Lanka where the climatic condition was warm and belongs to the Agro Ecological Zone of DL5. The suckers of *Heliconia bihai* collected from the wet zone, Gannoruwa, were planted in a well-prepared land in the experimental location with 30cm x 30cm spacing. Plants were maintained under well managed conditions. Harvesting of leaves was done four months after establishment in the field, just before the flowering. Leaves with well grown, fresh, fully expanded, and good look were cut and separated using a sharp knife. Just after harvesting of leaves, they were washed, cleaned and ashes adhered to then lower surface of the lea were removed carefully using a piece of cotton cloth. They were dipped in boiling water for few seconds. Thereafter, they were air dried under room temperature for 4 – 5 hours for curing. Midrib was removed using a sharp blade. Heliconia leaves were joined together by adhering them with a natural sticking agent to achieve the adequate size. They were placed on pieces of demy papers in desired size for wrapping a pack of food.

The different types of sticking agents were used as the treatments. There were 5 treatments and 20 replicates in each. The treatments were;

T1 - Sago solution (50 g of sago granules added with 100ml of boiling water)

T2 – Gelatin solution (50 g of gelatin powder added with 100ml of boiling water)

T3 – Wheat flour solution (50 g of wheat flour added with 100ml of boiling water)

T4 – Rice starch solution (50 g of rice added with 100ml of boiling water)

T5 -No sticking agent used (Control)

Prepared food wrappers were kept inside the refrigerator and taken out at two days interval for the sensory evaluation. The observations were made using a panel comprised of twenty members For Colour, Texture, Odor, Adherence and Overall acceptability of the food wrapper. A Likert scale ranging from 0-5 (Very bad to very good) was used for the sensory analysis.

Based on the observations from 1st experiment, experiment 2 was conducted with following treatments;

T1 – Food wrapper with Banana leaf

T2 – Smart food wrapper prepared with sago solution as sticking agent

T3 – Smart food wrapper prepared with gelatin solution as sticking agent

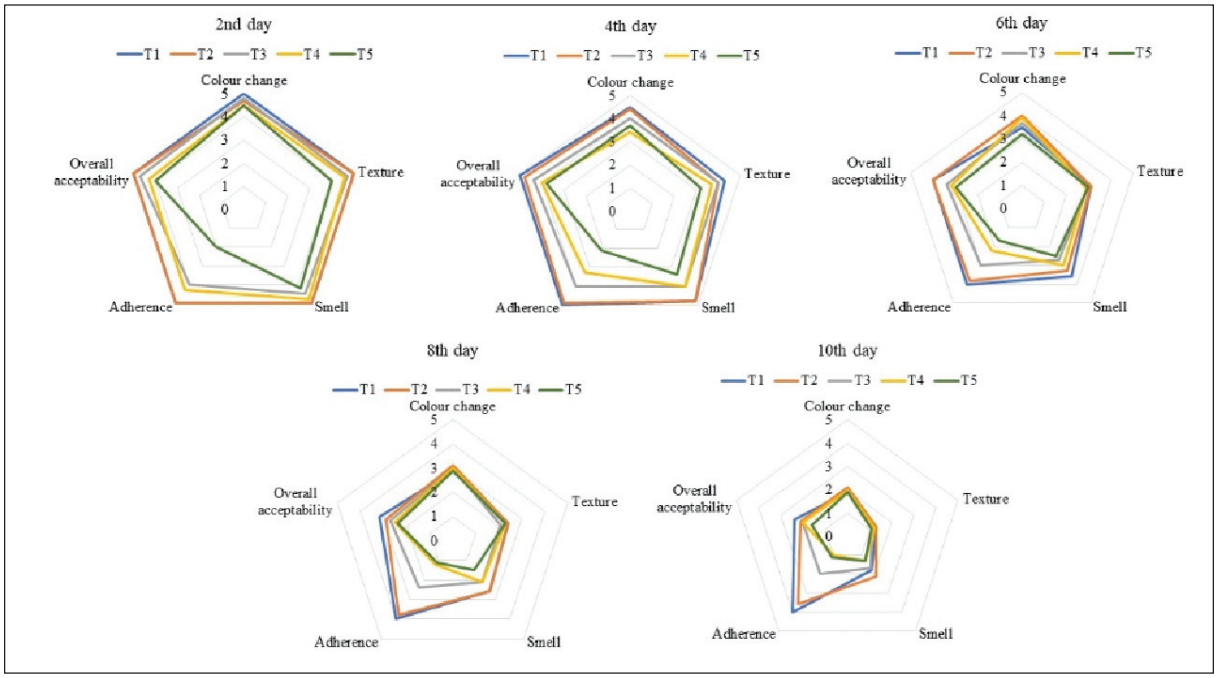

Fig. 1: Performances of food wrappers up to 10 days of production

Fig. 2: Prepared food wrappers

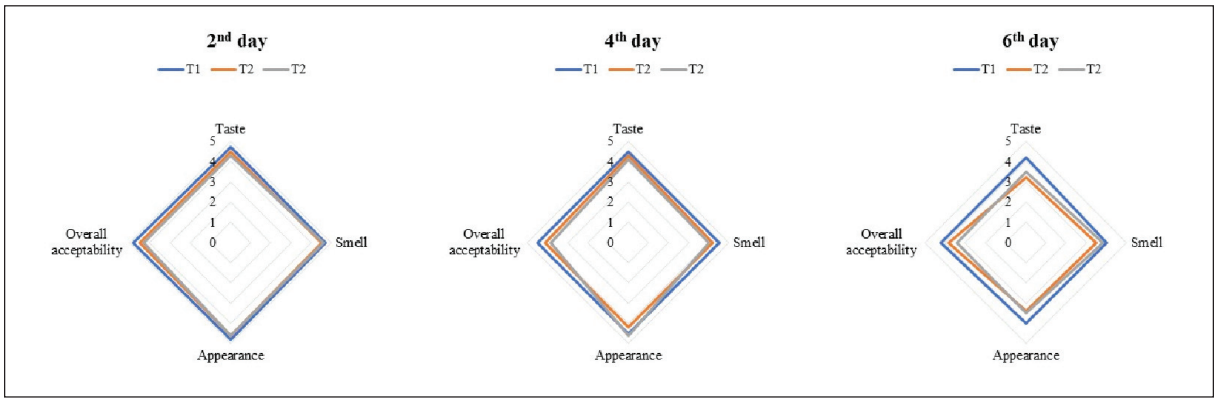

Fig. 3: Performances of food wrapped up to 6 days of production

One serve of food was packed inside the prepared food wrapper and taste, smell, appearance and overall acceptability were evaluated up to 6th day at 2 days interval after preparation of food wrapper using a panel comprised of twenty untrained members. A Likert scale ranging from 0-5 (Very bad to very good) was used for the sensory analysis. Collected data were analyzed using Minitab statistical software. Each response were given numerical value as a rating system and "Kruskal Wallis H Test" used to test the significance as non-parametric test ($P>0.05$).

Food wrappers were stored under refrigerated conditions to observe the maximum period that can be stored without affecting the quality. The results at different time periods were shown in figure 1. There were statistically significant ($p<0.05$) differences among treatments in odor, adherence, and overall acceptability up to 10 days. Further, Colour showed significant ($p<0.05$) change up to 6 days, as texture showed significant change up to 4 days. The food wrappers produced in all techniques can be stored up to four days without affecting the quality. There were significantly low performances in all parameters of wrappers developed using wheat flour starch and rice broth than that of in wrappers produced with sago solution and Gelatin solutions. Therefore, this revealed that there is no use of applying wheat flour starch and rice broth if we expect the shelf life more than four days.

Results confirm that only sago and gelatin can maintain the quality of wrapper up to six days. However, the wrappers prepared with sago and gelatin solution reduced in all qualities except adherence. Hence, it showed better adherence up to 10 days. Adherence is an important factor while considering the production of smart food wrapper. Mohamed *et al.* (2008) mentioned that Starch in the Sago is generally used as an efficient component such as thickener, stabilizer, and gelling agent in the food industry. Gelatin is used in many foods production processes as a binding agent and as a source for texture (Abdelfadeel, 2012). As revealed by Nishimoto *et al.* (2005); Karim and Bhat (2009) the distinct hydrocolloidal feature of gelatin has facilitated it to find various applications in the food industry including providing chewiness, texture, water binding, mouthfeel, etc. Food wrappers were stored under refrigerated conditions up to 6 days, wrapped with the food and results taken at 2 days interval were shown in figure 3.

It was found that, there were no statistically significant ($p>0.05$) differences among treatments in all the tested parameters taste, smell, appearance and overall acceptability up to 6 days. Quality of food packed in wrappers having the shelf life up to 6 days were evaluated with a sensory panel revealed the quality of packed foods were in good quality and good in appearance, taste and flavor. Newly developed food wrappers with Heliconia leaves showed not significant differences with the generally used Banana food wrappers. As indicated by Ng (2015) one of the better visual differences between the flora of the tropics and the flora of other climates is the huge difference in the size of leaves. In the tropics, the leaves of bananas, heliconias, gingers, palms, bamboos, macarangas, cordylines, water lotus, dipterocarpus and many others are much bigger and many are put to outstanding use wherever sheets of waterproofed materials are required.

It was concluded that *Heliconia* leaves in fairly good size can be produced by sticking under sized leaves together. For that Sago and Gelatin solutions could be considered as appropriate sticking agents. Hence, using *Heliconia bihai* leaves it is easy to produce bio food wrappers coupled with outer packing papers in more user friendly manner for busy consumers as smart food wrappers to replace synthetic wrapping materials.

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