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Research Article

EXHAUSTIVE EXTRACTION AND SCREENING THE BIOLOGICAL ACTIVITIES OF *HELIOTROPIUM HIRSUTISSIMUM* (HAIRY HELIOTROPE): A MEMBER OF PALESTINIAN FLORA

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ABSTRACT

Objective: Heliotropium hirsutissimum Grauer. is one of the important folk plants that grow wildly in the mountains of Palestine and widely used for several purposes by tribal peoples and traditional practitioners for treatment of various skin inflammations as a paste, for that our study aimed to evaluate antibacterial and antifungal activities for this plant.

Methods: In our experiments, the entire plant exhaustively extracted by n-hexane and ethanol mixed with an equal volume of triple distilled water to yield 2.36 g crude aqueous extract and 0.42 g crude organic extract from 25 g of the plant and then screened for antifungal and antibacterial activities.

Results: The plant aqueous extract showed antibacterial activities against all Gram-positive bacteria with the greatest activity against Staphylococcus epidermidis, its inhibition zone diameter (DIZ) was 16 mm equivalent to 50% of the DIZ of imipenem a broad spectrum antibiotics. Furthermore, the plant aqueous extract activity against Bacillus subtilis and Staphylococcus aureus were 21.7% of imipenem activity (10 mm) and 26% imipenem activity (12 mm), respectively. The minimum inhibition concentration (MIC) of the aqueous extract was 30 mg/ml or less against all bacterial strains as well as against the fungi Candida albican. More specifically, the MIC ranged between 2.7 mg/ml and 30 mg/ml with the lowest MIC value was 2.7 mg/ml against S. epidermidis, while Pseudomonas aeruginosa, Escherichia coli, and C. albican was all inhibited at the highest concentration of 30 mg/ml. There is no bacteriocidal or fungicidal effect of its extract at 30 mg/ml the highest concentration tested in our experiments. The organic extract showed identical antibacterial activity of aqueous extract except in that it had no activity against B. subtilis, as well as it had antibacterial activity against E. coli with DIZ of 50% of DIZ of imipenem (18 mm) and antifungal activity against C. albican with DIZ 80% of DIZ of nystatin broad spectrum antifungal compound (16 mm).

Conclusion: The plant aqueous and organic extracts have antibacterial, antifungal active compounds.

Keywords: Heliotropium hirsutissimum, Antibacterial, Antifungal, Hairy heliotrope.

INTRODUCTION

Most areas of the hills and mountains of Palestine and the Golan heights are covered with more than 2600 plant species of which more than 700 are noted for their uses as medicinal herbs or as botanical pesticides [1]. Medicinal natural products, especially medicinal plants contribute significantly to primary health care in many countries and utilized as the starting materials for all semisynthetic medications. A lot of plants and natural components have demonstrated antibacterial and wound healing properties as well as anticancer activity, which illustrates the potential for novel agents to be identified from uncharacterized natural plant resources [2-6]. In general and on a global scale, they are still the most popular source of natural products of therapeutic importance to three-quarters of the world's population. All the countries with highly developed pharmaceutical industries are mainly interested in plants as a source of biologically active and medicinal important compounds which might lead to the discovery of new and better drugs with pharmacological potency [7,8].

Heliotropium hirsutissimum Grauer. is an annual herbaceous plant, hermaphrodite, belonged to boraginaceae family with erect or spread out, generally branched stems, whitish or yellowish, covered in long spread out hairs. The plant leaves alternate, simple, elliptic to oval, hairy, dull green, petiolate, from 20 to 50 mm long and from 15 to 25 mm across. Its petiole is shorter than the half of the blade. The fowlers are radically symmetrical, white with yellow center, odorous, from 5 to 8 mm in diameter, with scales in the throat, joined together by their short peduncle in long scorpioid unilateral cymes. Corolla with 5 spreads out, fused petals, forming a tube at the base. Calyx consists of 5 erect linear (oblong) and very hairy sepals. 5 stamens fused with the corolla. Stigma very hairy, pointed at the top. The plants ovaries are superior, and their fruits have 4 achenes and in sometimes have only 2 achenes.

The plant H. hirsutissimum Grauer. flowering time is from April to November [9]. Globally this plant distributed in Libya, Egypt, East Mediterranean region and Greece. Locally it is growing and spreading in all regions in Waste ground, cultivated, and fallow fields in all the Palestinian territories. Middle East countries have a long tradition in utilizing plants containing biological active compounds that can produce therapeutic effects [10]. The development of microbial resistance to different antibiotics used today has caused a trend globally to search for drugs of natural origin to solve this problem.

Scientifically the H. hirsutissimum Grauer. plant is classified [11] as in (Table 1).

There are no studies before about this plant species (hirsutissimum) except the plant aerial parts was shown to contain toxic pyrrolizidine-N-oxide alkaloid [12,13]. The specific name of these pyrrolizidine alkaloids (PAs) is subulacine-N-oxide, as in (Fig. 1) [14]. PAs are naturally occurring heterocyclic compounds, most of them derived from esters of basic alcohols, known as the nicene bases. PAs are produced by plants as a defense mechanism against insect herbivores [15]. More than 660 PAs and PA N-oxides have been identified in over 6000 plants, and about half of them exhibit hepatotoxicity, nevertheless, some of them show promising pharmaceutical properties as potential antitumor [16,17].

Table 1: The scientific classification of hairy heliotrope

Kingdom	Plantae - Plants
Subkingdom	Tracheobionta - Vascular plants
Super division	Spermatophyta - Seed plants
Division	Magnoliophyta - Flowering plants
Class	Magnoliopsida - Di cotyledons
Subclass	Asteridae
Order	Lamiales
Family	Boraginaceae - Borage family
Genus	Heliotropium Grauer. - heliotrope
Species	Heliotropium hirsutissimum Grauer

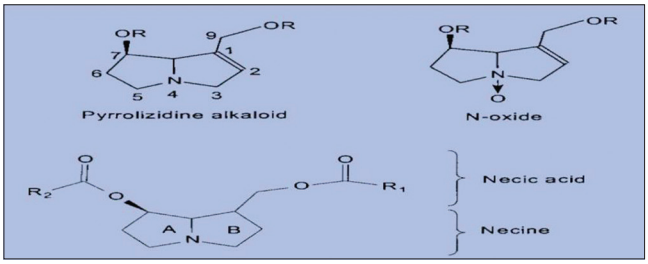

Fig. 1: Pyrrolizidine alkaloids subulacine-N-oxide. Adapted from Coulombe (2003) [18]

H. hirsutissimum Grauer. was used in the Palestinian folk medicine for the treatment of various skin inflammations [19]. Our study is the first to evaluate the biological activity of the aqueous and organic extracts of this plant against Gram-positive and Gram-negative bacteria and the fungi represented in Candida albican.

METHODS

Plant material

Authenticated hairy heliotrope plant was collected by Prof. M. Abu-hadid from Jerusalem area in March, April and October 2012. The taxonomic identity of the plants is confirmed by comparing our plant sample to pictures available from the internet and by group of experts lead by Prof. M. Abu-Hadid.

Plant extraction

The plant extraction was carried out as follows. First, the entire plant was washed well, then dried in the shade at room temperature for 2 weeks, and then powdered in a mechanical grinder moulinex model, France. Second, 25 g of the powder of hairy heliotrope was weighed on the balance (Adventure OHALIS balance, China) and exhaustively extracted by adding 50 ml of n-hexane and 125 ml of 50% ethanol in triple distilled water. The mixture was placed in a shaking incubator (Daihan Labtech. Co. Ltd India) for 72 hrs at 25°C with continuous shaking at (200 rpm). Then filtered by Whitman's No. 1 filter paper using suction flask and Buchner funnel. The resulting liquid filtrate was separated by separatory funnel into 2 phases the lower phase which is the aqueous phase representing the first aqueous extract and the upper phase which is the organic phase representing the organic extract. The remaining solid filtrate was extracted again by adding 125 ml of 50% ethanol in triple distilled water and was placed in the shaking incubator for 72 hrs at 25°C with continuous shaking at (200 rpm) as before. After that, it was filtered with Whitman's No. 1 filter paper to obtain the second aqueous extract. Both first and second aqueous extracts were pooled together and placed in the rotary evaporator (Heidolph OB 2000 ml, Heidolph VV 2000 ml, Schott Duran 1000 ml Germany) for 1 hr at 40°C to evaporate any leftover organic solvents. Then they were dried completely in freeze dryer (Millrock technology, model BT85B, Danfoss, America) for 24 hrs and they were dissolved with 30% ethanol in phosphate buffered saline (PBS) at 100 mg/ml concentration and stored in brown bottle in the refrigerator at 4°C. Third, the organic phase was evaporated completely in the fume hood at room temperature for 5 days followed by dissolving the solid organic extract in pure dimethyl sulfoxide (DMSO) at 100 mg/ml and was kept in a brown bottle in the refrigerator at 4°C.

Antimicrobial assay

In vitro, the antimicrobial evaluation of H. hirsutissimum extracts were carried out against five bacterial strains, three Gram-positive Bacillus subtilis (ATCC-6633), Staphylococcus aureus (ATCC-6538P), and Staphylococcus epidermidis (ATCC-12228) and two Gram-negative.

Escherichia coli (ATCC-8739) and Pseudomonas aeruginosa (ATCC-9027) and against one fungi (yeast) C. albicans (ATCC-10231).

Screening of antibacterial and anticandidal activity of the *H. hirsutissimum* extracts

Hairy heliotrope extracts were screened for antimicrobial activity using the well diffusion method which was carried out according to National Committee for Clinical Laboratory Standards Guidelines (NCCLS, 1993) [19].

Well diffusion method

Bacterial and candidal suspension prepared by picking a single colony of overnight agar culture of the test organism, and adding it to a bottle containing nutrient broth, then incubated overnight at 37°C. The growth turbidity in nutrient broth was compared with that of a McFarland nephelometer tube No. 0.5 ($1.5*10^8$ cfu/ml). A sterile wire loop was dipped into the bacteria or C. albican suspension, and then it was streaked in a zigzag pattern on Mueller-Hinton agar (MHA) plate in all directions and around the agar margin to ensure even distribution of the inoculum. After that, the plate was left to dry in a laminar flow bench for 4-5 minutes. Then holes (wells) were made using a sterile straw (diameter 6 mm) on the seeded MHA plate and 100 µL of hairy heliotrope aqueous or organic extracts 100 mg/ml stock solutions 100 µL of 30% ethanol in PBS as a negative for aqueous extract and 100 µL of pure DMSO as a negative control for the organic extract. This was followed by incubating the seeded MHA plates for 24 hr for the bacteria and 72 hr for C. albican at 37°C. The positive controls were the antibiotic Imipenem 10 µg/disc and the anti-candidal nystatin 10 mg/disc were placed onto the seeded MHA plate and incubated for 24 hr at 37°C for comparison with hairy heliotrope extracts. After 24 hrs, the inhibition zone around each hole or antimicrobial disc was measured using transparent ruler.

Determination of minimum inhibitory concentration (MIC)

The MIC is the lowest concentration of an antimicrobial agent that inhibits the growth of a microorganism after 24 hr incubation at 37°C. The extracts that showed antimicrobial activity were subjected to the serial broth dilution technique to determine their MIC. The MIC was carried out according to the NCCLS, 2000 [20] as the following. First five tubes contain 700 µL of nutrient broth was set up. Then, 300 µL of the hairy heliotrope aqueous extracts (stock concentration 100 mg/ml) transferred by a micropipette into to the first tube and mixed well. Followed by transferring, 300 µL of the prepared solution in the first tube to the second tube and mixed well, then 300 µL from the second tube was transferred into the third tube and so on till we reached, the last tube then 300 µL of solution was discarded from it. All the tubes were inoculated with 50 µL of overnight bacterial/Candida suspension. A positive control tube containing 50 µL inoculums and 700 µL nutrient broth, and negative control tube containing 300 µL plant extract and 450 µL nutrient broth without inoculums were prepared. The tubes were incubated at 37°C for 24 hrs. After 24 hrs, the tubes that appear clear (not turbid) means that no detectable growth of the bacteria or C. albican were considered as MIC tubes.

Determine of minimum bactericidal-fungicidal concentration (MBC-MFC)

An MBC-MFC is the lowest concentration of an antimicrobial agent required to kill the microorganism. The MBC-MFC was determined by

sub-culturing 10 µL of the test dilutions from MIC tubes on to fresh MHA plates. Plates were incubated at 37°C for 24 hrs. The highest dilution that yielded no single bacterial colony on the plates was recorded as MBC.

RESULTS

Extracts yield

The total yield of H. hirsutissimum 2.78 g out of 25 g (11.12% w/w) that we started with. The yield of the first aqueous extract was 1.74 g (73.7% of all aqueous extracts w/w), whereas the second aqueous extract was 0.62 g (26% of all aqueous extracts w/w). The yield of organic extract was 0.42 g (15% of all extracts w/w) (Table 2).

Antimicrobial activity

Both the aqueous and organic extracts of hairy heliotrope showed varying degree of antimicrobial activity against the tested organisms in the well diffusion method using 100 µL/well (stock concentration was 100 mg/ml) from both stocks of extracts (Table 2). The organic extract DIZ ranged from (0 to 18 mm) was found to be stronger than the aqueous extract DIZ that ranged from (0 to 16 mm) against all the test organisms. The aqueous extract showed antibacterial activity against three Gram-positive bacteria (S. aureus, S. epidermidis, and B. subtilis), but no effect on Gram-negative bacteria or on C. albican a representative fungus used in the experiments, while the organic extract showed antibacterial activity against two Gram-positive bacteria (S. aureus, and S. epidermidis), one Gram-negative, E. coli, and anti-fungal activity against C. albican.

For the aqueous extract S. epidermidis, was more susceptible than any other tested organism with zone of inhibition of 16 mm, about 50% of the diameter of inhibition zone (DIZ) of the imipenem, while the zone of inhibition of S. aureus was 12 mm 26% of Imipenem DIZ and B. subtilis showed the least susceptibility with zone of inhibition of 10 mm 21.7% of Imipenem DIZ. In the organic extract, E. coli was highly susceptible to the extract 18 mm, 50% of Imipenem DIZ followed by S. epidermidis 16 mm, 50% of imipenem DIZ and C. albican 16 mm, 80% of nystatin DIZ and S. aureus showed the least susceptibility 12 mm, 26% of imipenem DIZ.

The aqueous and organic extracts of hairy heliotrope showed antimicrobial activity less than the antimicrobial activity of the standard antibiotic (imipenem) and anti-candida (nystatin), as presented in Table 3.

MIC and MBC-MFC evaluations

The MIC values were evaluated for hairy heliotrope aqueous extract at concentration 30 mg/ml (stock concentration was 100 mg/ml) using serial dilution method (Table 4). The MIC values at concentration 30 mg/ml ranged from 2.7 to 30 mg/ml, this concentration of aqueous extract inhibited the bacterial strains and C. albican at different values. The lowest value of MIC was 2.7 mg/ml against S. epidermidis, followed by 9 mg/ml against S. aureus, and B. subtilis, while the MIC of P. aeruginosa, E. coli and C. albican occur at the highest concentration tube 30 mg/ml. There is no MBC-MFC of the hairy heliotrope extracts at a concentration 30 mg/ml.

DISCUSSION

Herbal products considered important source of potentially useful compounds for the development of new phytotherapeutic agents. The first step toward this goal is the in vitro antibacterial activity assay [21]. Hairy heliotrope as a member of wild Palestinian flora and grow in the Upper Jordan valley, Northern valleys, Samarian mountains, Samarian desert, Judean mountains, Judean desert, and Dead Sea valley considered one of the main subjects of screening its activities [22]. The plant aerial parts contain the alkaloid pyrrolizidine group founded in all other plants aerial parts, most of these compounds are hepatotoxic, but some of them show promising therapeutic properties as potential antitumor agents [17].

Table 2: Yield of hairy heliotrope extracts

H. hirsutissimum. extracts	Weight (g)	Percentage of each extract
Total extract	2.78	11.12%
First aqueous extract	1.74	73.7% of all aqueous extracts
Second aqueous extract	0.62	26% of all aqueous extracts
Organic extract	0.42	15% of all extracts

H. hirsutissimum: Heliotropium hirsutissimum

Table 3: Antimicrobial activity of aqueous and organic extracts of hairy heliotrope (100 µL/well) and the standard antibiotic and anticandidal

Plants extracts, negative control, and antibiotics	Diameter zone of inhibition (mm)					
	B.S	S.A	S.E	E.C	P.A	C.A
Aqueous extract	10	12	16	n.i	n.i	n.i
Negative control (30% ethanol)	n.i	n.i	n.i	n.i	n.i	n.i
Organic extract	n.i	12	16	18	n.i	16
Negative control (DEMSO)	n.i	n.i	n.i	n.i	n.i	n.i
Imipenim	46	46	32	36	26	-
Nystatin	-	-	-	-	-	20

DEMSO: Dimethyl sulfoxide, B.S: Bacillus subtilis, S.A: Staphylococcus aureus, S.E: Staphylococcus epidermidis, E.C: Escherichia coli, P.A: Pseudomonas aeruginosa, C.A: Candida albican. Concentration of control drugs: Imipenim 10 µg/disc, Nystatin 10 mg/disc. Diameter of the well/disc, 6 mm, included. n.i: No inhibition

Table 4: MIC values of hairy heliotrope aqueous extract at 30 mg/ml concentration

Microorganisms	MIC (mg/ml)
S. aureus	9
Bacillus subtilis	9
S. epidermidis	2.7
E. coli	30
P. aeregenosa	30

MIC: Minimum inhibition concentration, S. aureus: Staphylococcus aureus, B. subtilis: Bacillus subtilis, S. epidermidis: Staphylococcus epidermidis, E. coli: Escherichia coli, P. aeregenosa: Pseudomonas aeregenosa

The plant aqueous and organic extracts differ significantly in their activity against tested microorganisms. In spite of the crude aqueous extract antibacterial activity against Gram-positive bacteria with maximum diameter zone of inhibition reached 16 mm (against S. epidermidis) which is less than the diameter zone of inhibition of imipenem (32 mm). It did not show any antimicrobial activity against Gram-negative bacteria or C. albican. These differences may be attributed in one hand to the fact that the cell wall in Gram-positive bacteria consists of a single layer whereas the Gram-negative cell wall is a multi-layered structure and quite complex [23,24]. While, on the other hand, the difference in activity against C. albican can be explained by the fact that this organism is a eukaryotic cell.

Furthermore, the organic extract has a broad spectrum of antimicrobial activity as compared to the aqueous extract of the plant. Hence, it effect Gram-positive, Gram-negative bacteria and C. albican, with maximum diameter zone of inhibition reached 18 mm (against E. coli a Gram-negative prokaryote) followed by 16 mm (against S. epidermidis Gram-positive prokaryote and C. albican an eukaryotic organism). The organic extract may have different active compound to inhibit or kill the Gram-negative bacteria rather than acting on the bacterial cell wall, it may inhibit a bacterium ability to turn glucose into energy, inhibit protein, nucleic acid, or cell membrane synthesis [25].

The aqueous and organic extracts from the same plant showed different activities, the organic extracts showed the same or greater activity than

the aqueous extracts, these results suggest that the interesting active compounds in this plant have a limited solubility in water and are expected to be non-polar, hydrophobic organic compounds.

The DIZ of aqueous and organic extracts is less than the DIZ of the imipenem or nystatin. This may be due mainly to the extract of H. hirsutissimum is a crude extract and the active compounds are not purified, when the active compounds are purified and identified in the future from H. hirsutissimum extracts in an ongoing research in our laboratory, their activity may be the same or more than the activity of antibiotics.

In the literature, it has been suggested that the antibacterial activity is due to different chemical agents in the extract, including essential oils (especially thymol), flavonoids (antioxidants), and triterpenoids and other compounds of phenolic nature or free hydroxyl group, which are classified as active anti-microbial compounds [26-28].

CONCLUSIONS

Wild plants have provided and will continue to provide unique compounds of molecular diversity and biological functionality in medication research and medication development. Based on the obtained results and within the limitations of our study, we suggest that H. hirsutissimum is a phytotherapeutic product that may contribute to tissue healing as this plant has been used before in the folk medicine.

In this research, we can conclude that we confirmed the classification of H. hirsutissimum and established a new exhaustive extraction method for H. hirsutissimum that preserve the biological activity at least against bacteria and fungi. Furthermore, we found that the plant extract have a broad spectrum of activity against Gram-positive, Gram-negative, and C. albican. Finally, this study can be used as a basis for utilizing this plant species for further investigation in drug discovery for potential new natural bioactive compounds.

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