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SCREENING ANTIBACTERIAL AND ANTIFUNGAL ACTIVITIES AND EVALUATION OF THE EXHAUSTIVE EXTRACTIONS YIELDS FOR *VERBASCUM SINUATUM* L.

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ABSTRACT

This study aimed to evaluate antibacterial and antifungal activities and exhaustive extraction yields of the aqueous and organic extracts of *Verbascum sinuatum* L., against possible human pathogens, which are the fungus *Candida albicans*, gram positive bacteria *Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis* and the gram negative bacteria *Eschrichia coli* and *Pseudomonas aeruginosa*. Well diffusion method was used in screening of antimicrobial activity for the plant extracts, in which the diameters of inhibition zones were measured and compared to a positive control. Serial dilution method was used for measuring the minimum inhibition concentrations for each microorganism. In well diffusion method, the plant's aqueous extract has antimicrobial activity to all the tested organisms except *Pseudomonas aeruginosa* and *Candida albicans,* with variable diameters of inhibition zone. The percent inhibition compared to the positive control imipenem was 39.13 % for *Staphylococcus aureus*, 37.5 % for *Staphylococcus epidermidis,* 30.55 % for *Eschrichia coli* and the least 30.43 % for *Bacillus subtilis*. The organic extract exhibited inhibition activity 26.08 % against *Bacillus subtilis* and 50 % against *Eschrichia coli*. In the serial dilution method, the aqueous extract exhibited inhibition for all the test microorganisms. At initial concentration of 20 mg/ml, the lowest MIC value was for *Staphylococcus aureus* 1.28 µg/ml, and highest for *Staphylococcus epidermidis* 4000 µg/ml. The MIC values for *Pseudomonas aeruginosa* 160 µg/ml, 800 µg/ml for *Bacillus subtilis,* 800 µg/ml for *Eschrichia coli* and 32 µg/ml for *Candida albicans* respectively. This study showed that *V. sinuatum* extract has a broad spectrum activity against gram positive and gram negative bacteria, as well as anti candidal activity.

Keywords: *Verbascum sinuatum* L., Exhaustive extraction, *Candida albicans*, *Bacillus subtilis, Staphylococcus aureus*, *Staphylococcus epidermidis, Eschrichia coli, Pseudomonas aeruginosa*.

INTRODUCTION

Natural plant products have been used throughout the human history for different purposes. Having co-evolved with animal life, many of the plants from which these natural products medications were derived are billions of years old. Tens of thousands of these products are produced as secondary metabolites by higher plants as a natural defense mechanism against infections and diseases by animals. Many of these natural products have pharmacological or biological activities that can be exploited in the pharmaceutical drug discovery and drug design. Medicines derived from plants have played a pivotal role in the health care of many cultures, both ancient and modern[1-5]. Medicinal plants were used in the folklore medicine in treating a wide spectrum of ailments and diseases. Folk remedies for treatment of various diseases were prepared as infusions, powders, poultices, ointments, baths, creams, decoctions and teas. The interest in studying the biological effects of traditional medicinal plants or isolating their active components for treatment of illness has been increasing all over the world and comprehensive screening programs have been established[5,6]. The increased emergences of illness caused by pathogens have lead to the curiosity of many researchers and microbiological scientists in the investigation of antibacterial activities of medicinal plants which considered the main source of medicines to many African and Asian cultures since time immemorial. Recently nearly 30 % or more of the modern pharmacological drugs are derived directly or indirectly from plants and their extracts. So they dominate in homeopathic or alternative medicines[7-10]. *Verbascum sinuatum* L. is an annual, biennial or perennial herbaceous or sub-shrubby plant, growing to 0.5–3 m height. The herb first forms a dense rosette of leaves at low level, subsequently sending up a tall flowering stem. Biennial plants form the rosette the first year and the stem the following season. The leaves are spirally arranged and mainly densely hairy with a wavy margin (Figure 1). The flowers have five symmetrical petals; petal colors in different species include yellow (mostly common), orange, reddish brown, purple, white, or blue. The fruit is a capsule containing numerous small seeds[11].

Figure 1: The leaf of *Verbascum sinuatum* L. (Prof. M. M. Abu-hadid)

Figure 2: The flowers of *Verbascum sinuatum* L.

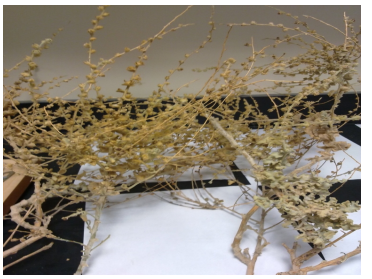

Figure 3: The dried sample of *Verbascum sinuatum* L. including the leaves, roots, stems and flowers. (Prof. M. M. Abu-hadid)

Verbascum sinuatum L. is a form of barbascum, from the Latin barba (a beard), that indicate to the shaggy leaves; the ancient Latin name for this plant. Sinuatum, sinuate, with a wavy margin; wavy edged. Its common names are (العمية in Arabic), Wavy Leaf Mullein and Scallop-Leaved Mullein. It is a species of Verbascum also known as Mullein and velvet plants which are a genus of about 250 species of flowering plants belonging to the Scrophulariaceae family[12]. The plant family and its species are native of Europe and Asia, with the highest species diversity in the Mediterranean regions with numerous small rich yellow flowers (Figure 2). The flowering period from May to October[13,14].

Verbascum species contain biologically active compounds, such as flavonoids, phenylethanoid, neolignan, saponins, iridoids and monoterpene glycosides, in addition to mucilage[15]. Infusions from the leaves and flowers of different *Verbascum* species are still used for their expectorant and demulcent properties to treat respiratory problems such as irritating coughs with bronchial congestion[16]. Because of the presence of mucilaginous constituents and saponins, aerial parts of *Verbascum* sp. have soothing action on mucous membranes and expectorant action[17]. The plant is reported to be mildly diuretic and to have an anti-inflammatory effect on the urinary tract[18]. The leaves, roots and flowers are also used as antiseptic, antispasmodic, emollient, analgesic, antihistaminic, anticancer and antioxidant[19], as well as to the treatment of psoriasis[20]. In Palestine, it's used as anti-inflammatory, soothing inflamed eyes, emollient and for the treatment of bronchitis[21].

MATERIAL AND METHODS

Collection and identification of *Verbascum sinuatum* L.

The plant was collected by Prof. M. M. Abu-hadid from Jerusalem area, between March and April 2012 and October 2012; this specimen was for the plant in its mature phase with its long stems and the flowers. Another sample was collected from Nablus area in January 2013, which was for the dense leaves at the ground level. So both samples with all the plants parts (stems, leaves flowers and roots) (Figure 3) were included in the study. The collected plants were then identified and authenticated by comparing them with pictures available from the internet and by group of experts lead by Prof. M. Abu-hadid (voucher specimen number is: Pharm-PCT-2604)[22].

The first extraction

All parts of the plant samples were dried in the shade for about 2 weeks, at room temperature, until they became completely dry. Then 25 gram including all the parts of the plant were obtained and cut into small pieces, then powdered in a mechanical grinder. The 25 gram of the powdered plant, were suspended in 50 ml n-hexane which is cheap, relatively safe, largely unreactive and easily evaporated non-polar (hydrophobic) solvent and 250 ml of 50 % ethanol in triple distilled water (to ensure sterility) in a bottle, with continuous shaking (200 round per minute) at 25°C for 72 hours in the Shaking Incubator. After that, the mixture was filtered by Whatman's No.1 filter paper using the Buchner funnel. The plant materials that had been accumulated on the filter paper were re-extracted again (2nd extraction). The liquid filtrate was separated by separatory funnel into 2 phases: lower phase which has higher density (aqueous phase) and upper phase which has lower density (organic phase). The aqueous phase was collected first and kept in a volumetric flask at room temperature till the next step (obtaining the powder of aqueous extract). The organic phase was collected second and placed in a pre-weighed glass beaker, which was placed in the hood at room temperature in order to evaporate the solvent (n-hexane), and to obtain the organic extract. The beaker with the organic extract was weighed again after evaporation; the weight of the organic extract was determined by calculating the difference of the weights. Then it was dissolved in dimethyl sulfoxide (DMSO), which is one of the most powerful organic solvents as therapeutic and toxic agents that are not soluble in water are often soluble in DMSO[23], the extract was dissolved at 100 mg/ml concentration and was kept in a sterile brown bottle at 4°C in the refrigerator till further use.

The second extraction

This extraction was only for the aqueous extract, the plant materials that accumulated on the filter paper after the first filtration were re-extracted again, by adding 250 ml of 50 % ethanol in triple distilled water, with continuous shaking for 72 hours in the shaking incubator at 25°C as before. A second filtration for the mixture was done by

using Whatman's No.1 filter paper on the Buchner funnel. The second aqueous phase was collected after filtration and kept in a volumetric flask at room temperature.

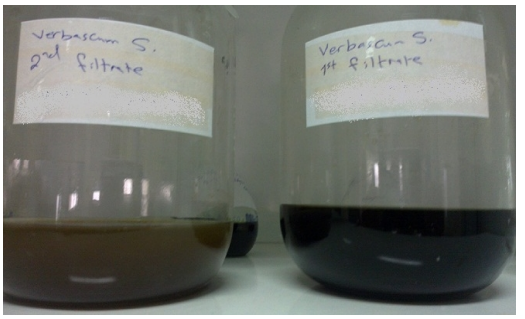

Figure 4: The 1st and 2nd aqueous extracts before ethanol evaporation and water freezing

The rotary evaporator was used for 1 hour at 40°C to evaporate any leftover organic solvents from both aqueous phases obtained from the first and second extraction (Figure 4). Then both aqueous extracts were put separately in pre weighed freeze dryer bottles and placed on the freeze dryer for 24 hours till they dried completely. Then the freeze dryer bottles were reweighed again and the dry weight of both extracts was calculated. The dry aqueous extracts were dissolved (a concentration of 100 mg/ml) in 30 % ethanol in phosphate buffered saline (PBS) which is a buffer solution that maintains constant neutral pH[24]. Then the prepared solutions of the aqueous extracts were placed in amber bottles in refrigerator at 4^0C till we used them for the biological testing.

Antimicrobial assay
Test microorganisms and control

In vitro antimicrobial activities of the aqueous and organic extracts of *Verbascum sinuatum* L. were tested against five potentially human pathogenic bacterial strains, and against one fungus (yeast) (Table 1).

Imipenem 10 µg/ml, a broad spectrum antibacterial antibiotic and nystatin antifungal drug were used as a positive control and the solvents (30 % ethanol in PBS for the aqueous extracts and dimethyl sulfoxide for the organic extract) were used as a negative control.

Preparation of the bacterial and candidal suspensions

The bacterial and the *Candidal inocula* obtained from the ATCC were sub cultured into prepared nutrients broth and incubated at 37°C for 24 h and standardized to 0.5 Mc-Farland Scale (10^8 cfu/mL)[25].

Screening for antibacterial and anti candidal activity of the plant extract

Well diffusion method was used for screening, by determining the zone of inhibition[26]. The prepared cell suspensions were seeded into prepared plates of Muller-Hinton agar. For each strain, 20 µl of the suspension was added on the surface of the plate and then was spread by special spreading tool in all directions and around the agar margins to ensure even distribution. Wells were then bored into the plates of the seeded organism using sterile straw of 6 mm diameter. Wells were filled completely with the plant extracts (the 1st and 2nd aqueous and the organic) with 100 µl in each well. Then the plates were incubated at 37°C for 24 h for the bacteria cultures, and 48 h for the Candida cultures in an incubator. Controls were also set up in parallel, using the solvents as negative control and disks of broad spectrum antibiotic (Imipenem) as positive control. After the incubation, the plates were observed for inhibition zones, which were measured in millimeters. This procedure was carried out three times for confirmation except for the organic extract due to its low volume. All steps were performed in a sterile condition.

Measuring the minimum inhibitory concentration (MIC)

The MIC is the lowest concentration of an antimicrobial that inhibits the growth of a microorganism after 18-24 h[27]. Serial broth dilution technique was used to determine the MIC for all the test microorganisms[28], even those with negative results (exhibit no inhibition) in the well diffusion method. A set of 7 tubes were prepared for each microorganism, 750 µl of nutrient broth was added in all the tubes, then 200 µl of the aqueous extract of *Verbascum sinuatum* L. (its concentration 100 mg / ml) was added in the first tube by the micropipette and was mixed well. Then, from the solution of the first tube, 200 µl were transferred to the second tube, mixed well. Then, 200 µl of the solution in the second tube were transferred to the third, then from the third to the fourth and so on, until the last tube, the 200 µl were discarded. Finally 50 µl of bacterial/candidal suspension standardized to 0.5 Mc-Farland Scale (10^8 cfu/mL) was added to all the tubes after the dilution was done. The extract's concentration in the first tube was 20 mg/ml and five times dilution was carried out. Negative control tubes were prepared, by using 30 % ethanol in PBS instead of the plant extract as a negative control, and tubes containing broth and suspensions as positive control. The tubes were incubated at 37°C for 24 h for the bacteria, and 48 h for the candida. After the incubation, the clear tubes (exhibit inhibitory action) were observed for each microorganism and the least clear tube from each set was considered as the MIC. This test was repeated two times for confirmation and all the steps were carried out under sterile conditions, by working near Bunsen flame, and sterilizing instruments in the autoclave.

Minimum bactericidal/ Fungicidal concentration (MBC/MFC)

The MBC/MFC of the plant extracts, which is the minimum concentration that is required to kills the bacteria/fungi[27], were tested after the results of the MIC. The tubes of the MIC that showed no growth (no turbidity) of the microbes were sub-cultured into nutrient agar plates and incubated at 37°C for 24 hours for the

bacteria, and 48 hours for the candida. The concentration of the extract that did not show any colony growth was labeled as the MBC/MFC.

RESULTS

Twenty five grams of the *Verbascum sinuatum* L. plant powder were subjected to exhaustive ethanolic extraction. The weights of the dried aqueous and organic extracts that were produced from the first and the second extractions are shown in Table 2. The total aqueous extract was 4710 mg (18.84 % of the total starting powder weight); the first extract was 12.88 % and the second extract was 5.96 % of the total starting powder weight. All extracts were dissolved to 100 mg/ml concentration. The aqueous extracts were dissolved in 30 % ethanol in PBS, the first in 32.2 ml and the second in 14.9 ml respectively. The organic extract one was dissolved at 100 mg / ml in DMSO (0.61 ml).

The antimicrobial activities of *Verbascum sinuatum* L. extracts against the selected pathogens examined in this study were qualitatively assessed by the well diffusion method, quantitatively by MIC and MBC, and were compared with the activity of a broad spectrum antibiotic, the imipenem 10 µg/ml. Using the well diffusion method and measuring the inhibition zone including the diameter of the well\disk (6 mm), the aqueous extracts of *Verbascum sinuatum* L. showed an antibacterial activity against all tested gram positive bacteria: *Staphylococcus aureus, Staphylococcus epidermidis* and *Bacillus subtilis* with inhibition zone diameter of 18 mm, 12 mm and 14 mm respectively and against one gram negative bacteria: *Escherichia coli,* with 11 mm diameter of the inhibition zone. The extracts did not show any inhibition zone for *Pseudomonas aeruginosa* and *Candida albican*. Both, the first and second extractions showed the same effects. The negative control did not show any inhibitory zone as shown in (Table 3). The organic extract of *Verbascum sinuatum* L. showed antibacterial activity against *Escherichia coli* (gram negative), 18 mm diameter inhibition zone, and against *Bacillus subtilis* (gram positive), with 12 mm inhibition zone. Imipenem 10 µg/ml is a broad spectrum antibiotic was used as positive control for the antibacterial activity, and nystatin antifungal drug for the anti candidal activity. The results are listed in Table 3 and compared with those of the plant extracts activities.

The minimum inhibitory concentration (MIC)

Quantitative assessment of the antimicrobial activity for the aqueous plant extracts had been carried out by serial broth dilution. Positive results appeared for all the microorganisms, even for *Pseudomonas aeruginosa* and *Candida albican* which were negative in screening by the well diffusion method. The MIC was tested at initial concentration 20 mg/ml with five fold dilution, the lowest value was for *Staphylococcus aureus*: $128*10^{-5}$ mg/ml (1.28 µg /ml), then for *Bacillus subtilis:* 0.8 mg/ml (800 µg/ml) and highest for *Staphylococcus epidermidis*: 4 mg/ml (4000 µg/ml). The MIC for *Pseudomonas aeruginosa,* was 0.16 mg/ml (160 µg/ml) and 0.8 mg/ml (800 µg/ml) for *Escherichia coli* and finally the MIC for *Candida albican,* 0.032 mg/ml (32 µg/ml) as shown in (Table 4).

Table 1: The tested microorganisms and their sources. *ATCC American Type Culture Collection

The microorganism	Category	ATCC* reference no.
Staphylococcus aureus	Gram positive bacteria	6538P
Staphylococcus epidermidis	Gram positive bacteria	12228
Bacillus subtilis	Gram positive bacteria	6633
Escherichia coli	Gram negative bacteria	8739
Pseudomonas aeruginosa	Gram negative bacteria	9027
Candida albican	Yeast	10231

Table 2: The weights of the resulted extracts

The extract	The weight in mg (g)
The organic extract	61 (0.061 g) 0.244 %
The first aqueous	3220 (3.22 g) 12.88 %
The second aqueous	1490 (1.49 g) 5.96 %

Table 3: The diameters of the zones of inhibition in mm, (the diameter of the well/disk is included which is 6 mm)

Microorganism	*Aqueous extract	**Organic extract	Imipenem	Extract/imipenem %	
				*	**
Staphylococcus aureus (G+)	18	0	46	39.13 %	0
Staphylococcus epidermidis (G+)	12	0	32	37.5 %	0
Bacillus subtilis (G+)	14	12	46	30.43 %	26.08 %
Escherichia coli (G-)	11	18	36	30.55 %	50 %
Pseudomonas aeruginosa (G-)	0	0	26	0	0
Candida albican (yeast)	0	0	Nystatin 20	0	0

Table 4: The MIC values of the test microorganisms: Concentration

Microorganism	MIC at Initial Conc. 20 mg/ml
Staphylococcus aureus (G+)	$128*10^{-5}$ mg/ml (1.28 µg/ml)
Staphylococcus epidermidis (G+)	4 mg/ml (4000 µg/ml)
Bacillus subtilis (G+)	0.8 mg/ml(800 µg/ml)
Escherichia coli (G-)	0.8 mg/ml (800 µg/ml)
Pseudomonas aeruginosa (G-)	0.16 mg/ml (160 µg/ml)
Candida albican (yeast)	0.032 mg/ml (32 µg/ml)

Minimum bactericidal/ Fungicidal concentration (MBC/ MFC)

Verbascum sinuatum L. extract did not show any bactericidal or fungicidal activity, as growth occurred in all the sub-cultured pure tubes. So it has inhibitory effect (bacterio-static/ fungi static) activity rather than bactericidal/fungicidal activity at the concentrations used in the experiment.

DISCUSSION

In this study, extraction was performed twice for the powder of the plant and it was found that 12.88 % of the aqueous extract was produced in the first time and 5.96 % in the second time, which is a significant amount, so to obtain most of the plant extract, multiple extractions are required. The aqueous and organic extracts of *Verbascum sinuatum* L. were tested against gram positive and gram negative bacterial strains, in addition to *Candida albicans*. We used well diffusion method on MHA plates as screening for the antimicrobial activity, which was positive for all the test organisms except for *Pseudomonas aeruginosa* and *Candida albicans*. It appeared that, diffusion method could not always be a reliable method for screening the antimicrobial activity of plant extract, and the absence of inhibition zone did not necessarily mean that the extract was ineffective, particularly for the less polar compounds which diffuse more slowly into the medium[27]. In addition to that, in the well diffusion method, the added amount (as a result the concentration) of the plant extract is limited, and that may be less than the required amount to exhibit the antimicrobial activity. Of the positive results obtained in this screening method, the zones of inhibition for the gram positive bacteria (*Staphylococcus aureus, Staphylococcus epidermidis* and *Bacillus subtilis* 18 mm, 12 mm and 14 mm respectively) were higher than that for the gram negative *Escherichia coli,* with 11 mm. This difference may be explained by the differences in the structure of the cell wall in gram positive bacteria which consists of a single layer and the gram negative bacteria which is a multi-layered structure and quite complex[12,29]. By comparing the inhibitory zone of the plant extract with those of the used broad spectrum antibiotic (imipenem) as listed in Table 3, it's obvious that the antibiotic has higher inhibition zone, but the used extract is crude, and the active compound and its percentage are not known, while the antibiotic is purified, so identification and purification of the active antimicrobial agent may produce the same antibiotic activity or even more. The plant extract exhibited a broad spectrum antibacterial activity, as inhibition included both gram positive and gram negative bacteria. In addition to the antibacterial activity, anti candidal effects were also present. It is expected that the substances that produced those activities are different, due to the differences in the structure between prokaryotic bacterial and eukaryotic candidal cells, as the fungal membranes contain sterols, whereas bacterial membranes do not. The antifungal agents act by binding to membrane sterols, make a pore in the fungal membrane and the contents of the fungus leak out. Prokaryotic cells neither bind to polyenes nor are inhibited by polyenes[30,31].

CONCLUSION

From this study, it was concluded that *Verbascum sinuatum* L. has a broad spectrum activity against both gram positive and gram negative bacteria; this may help in discovering new antibiotic, which will be an important addition to the world of antibacterial agents, which is particularly important in facing up the growing resistance human pathogens. This is in addition to its anticandidal activity.

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