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EXTRACTION, FORMULATION AND EVALUATION OF YEMENI *ARGEMONE MEXICANA LINN* AS ANTIMICROBIAL AND WOUNDS HEALING AGENT

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ABSTRACT
Yemen has a rich tradition of plant-based knowledge on healthcare. Traditional medicine in Yemen comprises medical beliefs and practices which are determined by a variety of epidemiology, culture, historical, and economic factor. The Yemeni *Argemone mexicana species* has different traditional uses as treatment of wounds, stop bleeding and enhance wound healing but it is not studied before. As result of this the aim of this search was to evaluate the antimicrobial and the healing activity of Yemeni *Argemone mexicana Linn***.** The stems and leaves were collected, dried and extracted by different solvents differ in polarity from non-polar to higher one starting with petroleum ether, dichloromethane and methanol. The antimicrobial activity was evaluated by well diffusion against two Gram positive bacteria *Micrococcus* luteus and *Bacillus subtilis* and three Gram negative *E. coli*, *P. aeruginosa*, and *S.typhi* bacteria and one candida. Furthermore, the Healing activity of the methanol extracts of the *Argemone mexicana Linn*. by measuring their effects on the rabbit of wound healing were assessed by the rabbit of wound closure, period of epithelialization, wound breaking strength, weights of the granulation tissue. Fusidic acid was used as reference standard for the activity comparison. The methanol stem extract of *A. mexicana* was showed the highest antibacterial activity against *Salmonella typhi* with MIC (20mg/ml) and also it showed activity against (*Micrococcus luteus, Bacillus subtilis* and *Pseudomonas aeruginosa*) with MIC (10mg/ml). All the tried topical preparations were physically stable at room temperature except emulsifying ointment base. Significant differences in the percentage of wound healing from treated animal groups (rabbit) with ointment and untreated groups with p-value 0.03 were achieved. Non-significant differences in the percentage of wound healing from treated animal groups (rabbits) with ointment and treated groups with fusidic acid in either group normal or infected sample with p-value 0.97, 0.825 respectively. Complete healing was done for normal sample as control and 93.57% for infected sample treated with extract as compare with positive control with 97.14%. The wound healing effects and the anti-bacterial of the methanol extract may be attributed to the presence of phyto constituents like alkaloids, triterpenoids, which confirmed by photochemical screening of the extracts.

KEYWORDS: *Argemone Mexicana* luteus *aeruginosa*, and *S.typhi* phyto extracts.

INTRODUCTION
The worldwide use of natural products including medicinal plants has become more and more important in primary health care especially in developing countries.[1] *Argemone mexicana Linn* (Family: Papaveraceae) is a prickly, glabrous, branching annual herb with yellow juice and showy yellow flowers, naturalized throughout up to an altitude of 1500 m[2-5] *Argemone* is a genus of about 30 species of plants in this family. One species of some notoriety is *A. mexicana*, the Mexican poppy. The seeds of this plant used as purgative and sedative (Ayurveda)[5], in skin diseases and leukoderma (Yemeni)[4] and are considered antidote to snake venome and in Homeopathy, and yield non edible toxic oil and causes lethal dropsy when used with mustard oil for cooking and show lots of toxic effect .The leaves are useful in cough, wounds, ulcers and in skin diseases. Also uses as antimalarial activity[6], anti-helmintic, anti-inflammatory, wound healing, anti-bacterial and antifungal activities.[7,8] The tincture of the entire plant is reported to be used orally for bronchitis and whooping cough.[9,10] The fresh juice of the leaves and the latex, both are reported to be used externally as a disinfectant for open wounds and cuts[11] In Yemen, *Argemone*

mexicana Linn called: Baroud , Snafah, Senif. its flower is yellow, annual herb growth on agricultural land and fallow land and valley bottoms such as Taiz, Ibb, Dhamar, Dhale, Maweah, Muwzea, Damt, and Socotra.[12] Traditionally used the liquid of stem in the treatment of wounds to stop bleeding and wound healing. Basing on the tradition uses and importance to find new antibiotics from plants and has healing power as well. This search was evaluated the antimicrobial and healing activity of Yemeni Argemone Mexicana Linn.

METHODOLOGY

Material and equipment

Chemicals

Petroleum ether (Sigma Aldrich, Germany), methanol 99.9% (Sigma Aldrich, Germany), dichloromethane 99.9% (Scharlau, Spain), dimethyl sulfoxide (Scharlau, Spain), DDPH, ferric chloride (Sigma Aldrich, Germany), sulfuric acid (Sigma Aldrich, Germany), ferric chloride 1% and 5% (BDH, Germany), ammonia (Sigma Aldrich, Germany), chloroform (Sigma Aldrich, Germany), sodium bicarbonate (HI media, India), ferrous sulfate (BDH, Germany), white wax, white petrolatum, wool fat, hard paraffin, cetostearyl alcohol, white soft paraffin, cholesterol, stearyl alcohol, sodium lauryl sulfate, propylene glycol, methyl paraben, propyl paraben, liquid paraffin. Mueller Hinton broth, and saboraud dextrose agar (HI media, India) Mueller Hinton agar (Scharlau, Spain).

Reference drugs: Ampicillin (10µg disk), gentamicin (10µg disk) working standers, fusidic acid, gentamicin (ampule).

Test microorganism: The microorganism used included two gram positive bacteria, two gram negative bacteria and one fungus strain, which are common cutaneous pathogens. They either were clinical isolates or of American type culture collection (ATCC), *Escherichia coli* (ATCC 10536) *Pseudomonas aeruginosa* (ATCC 25619) *Micrococcus luteus* (ATCC 9341) *Bacillus stabilis* (ATCC6633) *Salmonella typhi* (locally isolated) *Candida albicans* (locally isolated), the species were collected from Sana'a University, Faculty of Sciences.

Animals: Stander animal (rabbits): their age between (5-7 months) and weight (1.5-2kg).

METHODOLOGY

Plant preparation and extraction

The aerial parts of *Argemone mexicana Linn* plant selected were collected from Damt city, Yemen. The plants were identified by Dr. Abdul Wale Al Khulaidi.[13] The different parts of the *Argemone mexicana* were separated and air dried at room temperature 25C˚. The extraction was carried by using a Sox let extractor using solvents with different polarity. Firstly the extraction was done with the less polar solvent which is petroleum ether. The extracted were filtered, and the filtrate from extraction were combined. The residue was dried over the night and re-extraction with dichloromethane. The residue of the dichloromethane extract was re-extracted with methanol 96% and finally with water. The petroleum ether, dichloromethane, and methanol extracts were dried under vacuum by using rotary evaporator until dryness.

Antimicrobial assays

The antimicrobial activity of these extracts evaluated by well diffusion method on the suitable media for both bacteria and fungi, Muller Hinton agar (MHA) and Sabraud dextrose agar (SDA), respectively. Colonies from overnight growth on appropriate agar plate were suspended in suitable media to a turbidity that matches a 0.5 Mcfarland standard (10^8 CFU/ml for bacteria and 10^6 CFU/ml for the fungus. For agar well diffusion method[14, 15] 25ml of Mueller-Hinton agar medium were added to each of the petri-dishes.

The isolated microorganisms were inoculated on the agar surface. In cylinder plate method, the test organisms were spread on agar plate and a well was prepared in the plates with the help of a cork-borer (6mm). Into the well, 100µl of the test compound was introduced with a concentration of (0.1mg/µl) in dimethyl sulfoxide (DMSO) as solvent.[16] DMSO was used as negative control and ampicillin (10µg/disc) and gentamicin (10µg/disc) is used as positive control for bacteria and ketoconazole (10µg/disc) for fungi. The plates were incubated at suitable conditions for bacteria and fungi. The antibacterial activity determined by measuring the diameter of inhibition zone then MIC and MBC. Mean of the inhibition zone was recorded from triplicate tests. The antibacterial activity was defined as strong when the diameter of inhibition zone was ≥15mm, moderate if a diameter 10 - 15 mm and weak if a diameters of <10mm.[17]

Formulation of the extracts

Topical formulation of the defatted extract of *A. mexicana* were prepared as 4% w/w and 5% v/w, using bases with different polarities. Five ointment bases were prepared as shown in table (1).

Table (1): showed the excipients was added to preparing the ointment formulas.

Ingredient	F1	F2	F3	F4	F5
White wax	1g	-	1.5g	-	-
White petrolatum	19g	-	-	-	-
Wool fat	-	1g	-	-	-
Hard paraffin	-	1g	-	-	-
Cetostearyl alcohol	-	1g	-	-	-
White soft paraffin	-	17g	-	-	
Cholesterol	-	-	0.6g	-	-
Stearyl alcohol	-	-	0.6g	-	-
White petrolatum	-	-	17.3g	5g	-
Sodium lauryl sulfate	-	-	-	0.2g	-

Propylene glycol	-	-	-	2.4g	-
Stearyl alcohol				5g	-
Methyl parapen	-	-	-	0.005g	-
Propyl parapen				0.003g	-
Purified water	-	-	-	7.4g	-
Emulsifying wax					6g
White soft paraffin					10g
Liquid paraffin	-	-	-	-	4g

i. Preparation of topical ointment bases

1) White ointment (hydrocarbon base) (F1)

Preparation

The white wax melted on a water on a water bath, the white petrolatum was added and warmed until liquefied, then heating was discontinued and the mixture was stirred until it began to congeal.

2) Simple ointment (F2)

Preparation:

The ingredient were mixed and heated gently with stirring until homogeneous, then heating was discontinued and the mixture was stirred until cold

3) Hydrophilic petrolatum (absorption base) (F3):

Preparation

The stearyl Alcohol and white wax were melted, and then cholesterol was added and stirred until completely dissolved, white petrolatum was added and warmed until liquefied, then heating was discontinued and the mixture was stirred until cold.

4) Hydrophilic ointment {94} (absorption base) (F4):

Preparation

Stearyl alcohol and white petrolatum were warmed on a water bath to 75 C˚. Other ingredients, previously dissolved in water and warmed to 75 C˚, were added. Then the mixture was stirred until cold.

5) Emulsifying ointment (F5)

Preparation

Emulsifying wax was prepared as follows: cetostearyl alcohol was melted, heated to about 95 C˚ and then sodium lauryl sulphate was added and mixed. Purified water was added, heated to 115 C˚ and this temperature was maintained, with vigorous stirring, until frothing was ceased and product was translucent then the product was cooled quickly.

❖ **Incorporation of *A. mexicana* extract into the prepared base**

Extract of *A. mexicana* were separately incorporated into the already prepared bases at the room temperature by levigation method. The control formulations did not contain the extract. Strength of the final product of the methanol extract was prepared to be 4% w/w (equivalent to 0.424% total alkaloid) by incorporating 0.6g of the viscous paste of *A. mexicana* in 14.4g of the respective bases.

❖ **Preparation of ointment formulations**

Selected bases containing some additives and different enhancers such as DMSO and PEG 400 in concentrations of 2.5%, 5% and 10% w/w of base were prepared. Then extract of *A. mexicana* were separately incorporated into the prepared bases in concentration of 4% w/w and 5% v, respectively by levigation method at room temperature. The selected bases and the respective enhancers with their concentration are listed below in table (2).

Table 2: ***illustrates some enhancers were added to the selected bases containing A. mexicana defatted extract.***

No	Type of base	Type of plant material	Enhancer
1.	White ointment	EO	DMSO/PEG400
2.	Macrogol ointment	EO/ MeOH Ext	DMSO
3.	Macrogol-PG ointment	EO/ MeOH Ext	DMSO
4.	Cetomacrogol emulsifying ointment	EO/ MeOH Ext	DMSO/PEG400
5.	Emulsifying ointment	MeOH Ext	DMSO/PEG400

- **Evaluation of *Argemone mexicana* prepared formulas**

i. Physical examination (visualization of physical changing)

The prepared formulations were inspected visually for their color, homogeneity and phase separation.

ii. pH measurements:

One gram of each formulation was weighted and diluted with 10 ml distilled water then the pH of the sample was measure with pH meter at room temperature.[18, 19]

Study the effect of selected topical ointment preparation on healing of rabbits wounds

Fifteen rabbits were weighed. They had free access to feed and clean drinking water for about 30 days (the experimental period). The animals were divided into 3 groups; the different groups were listed in table (3).

Table 3: shows the different types of rabbit groups used in wound healing activity study.

Group number	Type of treatment
1.	Without treatment (3)
2.	Normal control (3)

3.	Infected control (3)
4.	Normal sample (3)
5.	Infected sample (3)

Wound healing activity the selected extracts of *A. mexicana* were separately evaluated for their wound healing activity in rabbit using the effects of test samples on the rabbit of wound healing were assessed by the rabbit of wound closure, period of epithelialization, wound breaking strength, weights of the granulation tissue, determination of hydroxyproline, super oxide dismutase (SOD), catalase and histopathology of the granulation tissue. fusidic acid was used as reference standard for the activity comparison. The test extracts were mixed with simple ointment (I.P.) (10% w/w) and used in the excision and models. For the dead space wound model, the methanol extract was suspended in water and used.

Excision wound model (Normal wounds)

Animals were anesthetized prior to and during creation of the wounds, with chloroform .The rabbits were inflicted with excision wounds as described by (*Morton and Malone*)[20] and suggested by.[21] An impression was made on the dorsal thoracic region 2 cm away from vertebral column and 10 cm away from ear on the anaesthetized rabbit. The dorsal fur of the animals was shaved with an electric clipper and the anticipated area of the wound to be created was outlined on the back of the animals with methylene blue using a circular stainless steel stencil. A full thickness of the excision wound of circular area of (279 - 300 mm^2) and 2 mm depth was created along the markings using toothed forceps, scalpel and pointed scissors. Hemostasis was achieved by blotting the wound with cotton swab soaked in normal saline. The entire wound was left open.

The control group animals (Group I) without treatment with the positive control (Group II) was applied with fusidic acid in Simple ointment. Other groups of animals were (Group III) were treated of following methanol extract of *A. mexicana* at a concentration of 10% w/w in Simple ointment (I.P.) in a similar manner.

The wound closure rate was assessed by tracing the wound on days 1, 3, 5, 8, 11, 15 and 17 post wounding days using transparent paper and a permanent marker. The wound areas recorded were measured using graph paper. Changes in wound area were calculated, giving an indication of the rabbit of wound contraction.

Statistical analysis

Data were presented as means ± standard deviation (SD). A computer program (excel 2010) was used for statistical analysis. The one-way ANOVA and post hoc multiple comparison tests (Scheffe) were performed to examine the differences among the groups. A P value of <0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

i. Yield of extraction

The results of yield of extraction showed in table (4).

Table 4: illustrates the results of yield of extraction.

Sample	Weight (gm)	Percentage
Petroleum ether leaves extract	1.29	3.68 %
Petroleum ether stem extract	0.68	1.94 %
Dichloromethane leaves extract	0.11	0.31 %
Dichloromethane stem extract	0.91	2.6 %
Methanol leaves extract	2.54	7.25 %
Methanol stem extract	4.19	11.97 %

ii. Antimicrobial activity of *A. mexicana*.

Table (5) showed in vitro antibacterial activity and antifungal activity of Argemone mexicana Linn:

Sample tested	*M. luteus*	*P. aeruginosa*	*B. subtilis*	*S. typhi*	*E. coli*	*C.albicans*
Methanol stem	20 mm	20 mm	24 mm	18 mm	13 mm	-
Dichloromethane stem	15 mm	16 mm	15 mm	16.6 mm	13 mm	-
Methanol Leave	12.3 mm	15 mm	13.3 mm	-	-	-
Petroleum ether stem	-	-	-	-	-	-
Petroleum ether leaves	-	-	-	-	-	-
Dichloromethane leaves	-	-	-	-	-	-

The methanol and dichloromethane stem's extracts showed strong inhibition against the gram positive (*Micrococcus luteus and Bacillus subtilis*) and gram negative (*Pseudomonas aeruginosa and Salmonella typhi*) while it was showed moderate activity against *Escherichia coli*. in compare with methanol leave's extract which showed moderate activity against (*Micrococcus luteus, Pseudomonas aeruginosa and*

Bacillus subtilis), but it has no activity against gram negative (*Escherichia coli, salmonella typhi*) this may be due to presence of saponins that has wide activity as antibacterial[9], but the dichloromethane of leave showed no activity, The *A.mexicana* showed no activity against *Candida*.

iii. Assessment of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Table (6) illustrates the concentration of (MIC and MBC) methanol and dichloromethane extracts of A.mexicana.

Bactria	*Methanolic extract A.mexicana*		*Dichloromethane extract A.mexicana*	
	Conc of MIC	**Conc of MBC**	**Conc of MIC**	**Conc of MBC**
Micrococcus luteus	10	20	10	40
Bacillus subtilis	10	20	10	40
Salmonella typhi	20	0	10	0
Pseudomonas aeruginosa	10	10	20	0

The methanol extract showed high activity against *S.typhi* with MIC (20) and also it showed activity against (*Micrococcus luteus, Bacillus subtilis* and *Pseudomonas aeruginosa*) with MIC (10,10,10) respectively, and MBC for (*Micrococcus luteus, Bacillus subtilis* and *Pseudomonas aeruginosa*) (20,20,10) respectively. While the methanol extract only showed bacteriostatic activity against *Salmonella* because it has no bactericidal activity. The dichloromethane extracts showed high activity against *Pseudomonas aeruginosa* with MIC (20) and also it showed activity against (*Micrococcus luteus, Bacillus subtilis* and *Salmonella*) with MIC (10,10,10) respectively, and MBC for (*Micrococcus luteus, Bacillus subtilis*) (40,40) respectively, while the dichloromethane extracts showed bacteriostatic activity against *(Salmonella typhi* and *Pseudomonas aeruginosa)* and no bactericidal activity

iv. Ointment base stability testes

Table 7: shows ointment bases stability tests.

	Before tests			*At (4C^o and 37C^o) for (48hr cycle no 1)*			*At (4 C^o and 37 C^o) for (48hr cycle no 2)*			*At (4 C^o and 37 C^o) for (48hr cycle No3)*			*At (4C^o and 37C^o) for (48hr cycle No4)*		
Sample	**Color**	**Smoothness**	**Phase Separation**	**Color**	**Smoothness**	**Phase Separation**	**Color**	**Smoothness**	**Phase Separation**	**Colour**	**Smoothness**	**Phase Separation**	**Colour**	**Smoothness**	**Phase Separation**
White Ointment	White	Smooth	**No**	White	Smooth	**No**	White	Smooth	**No**	White	Smooth	**No**	White	Smooth	**No**
Simple Ointment	Pale Yellow	Smooth	**No**	Pale Yellow	Smooth	**No**	Pale Yellow	Smooth	**No**	Pale Yellow	Smooth	**No**	Pale Yellow	Smooth	**No**
Hydrophilic Ointment	White	Presence Granulation	**No**	White	Presence Granulation	**No**	White	Presence Granulation	**No**	White	Presence Granulation	**No**	White	Presence Granulation	**No**
Emulsifying Ointment	White	Smooth	**No**	White	Smooth	**No**	White	Smooth	**No**	White	Presence Granulation	**Yes**	White	Presence Granulation	**Yes**

Hydrophilic Petrolatum Ointment	White	Smooth	**No**	White	Smooth	**No**	White	Smooth	**No**	White	Smooth	**No**	White	Smooth	**No**

\All the bases were stable for the tests of stability in the cycle number 1 and 2 then compare with stability before tests. But showed the emulsifying is change (separation and granulation). In the cycle number 3.

v. Effect of methanol stem extract of *A. mexicana* on percentage (%) wound closure (Excision Wound Model)

Table (8): shows the percentage (%) of wound closure.

Groups	Day0	Day 3	Day 5	Day 8	Day 11	Day 15	Day 17
Group I Without Treatment	0	7.47	11.03	13.88	17.08	28.83	32.74
Group II Normal Control (Fusidic Acid)	0	22.22	28.32	41.22	68.10	91.04	100.00
GroupIII Infected Control (Fusidic Acid)	0	6.81	13.57	33.93	64.29	85.00	97.14
Group IV Normal Sample	0	18.33	28.33	39.67	63.67	89.67	100.00
Group V Infected Sample	0	3.21	10.71	31.43	55.00	74.64	93.57

In the 5th day, the healing activity of *A.mexicana* against normal and infected were closed to positive control fusidic acid with % of healing (28.33 , 10.71) for *A.mexicana* and (28.32 , 13.57) for fusidic acid.

The 17th day, complete healing was done for normal sample as control and 93.57% for infected sample treated with extract as compare with control with 97.14%. Presence of alkaloids, triterpenoids, tannins and flavonoids in the extracts of methanol may be responsible for, and the anti-microbial activity (3).

CONCLUSION

The methanolic stem extract of *A. mexicana* was showed the highest antibacterial activity against *Salmonella typhi* with MIC (20mg/ml) and also it showed activity against (*Micrococcus luteus*, *Bacillus subtilis* and *Pseudomonas aeruginosa*) with MIC (10mg/ml). All the tried topical preparations were physically stable at room temperature except emulsifying ointment base. Significant differences in the percentage of wound healing from treated animal groups (rabbit) with ointment and untreated groups with p-value 0.03 were achieved. Non-significant differences in the percentage of wound healing from treated animal groups (rabbits) with ointment and treated groups with fusidic acid in either group normal or infected sample with p-value 0.97, 0.825 respectively. Complete healing was done for normal sample as control and 93.57% for infected sample treated with extract as compare with positive control with 97.14%.

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