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EXTRACTION, FORMULATION AND EVALUATION OF *ARGEMONE MEXICANA* LINN LEAVES AS ANTIMICROBIAL CREAM AND OINTMENT

ABDULKARIM KASSEM ALZOMOR¹, AHMED SAIF MOHAREM², NAHLAH MANSOUR
NOMAN³, JAMEEL ALAFIFI³, HYTHAM MOKRED³, NASEEM ALAREGI³

1. Department of Pharmacy, Faculty of Medicine and health sciences, Tamar University, Republic of Yemen.
2. Department of Microbiology, Faculty of Medicine and health sciences, Tamar University, Republic of Yemen.
3. Department of Pharmacy, Faculty of Medicine and health sciences, Tamar University, Republic of Yemen.

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Abstract: The aim of the present study was to evaluate the antibacterial and antifungal activities of methanolic and ethanolic leaf extracts of *Argemone mexicana* then formulate the best extract as topical semisolid formulations (cream and ointment). The extracts were subjected to qualitative tests, by screening of phytoconstituents that present in the extracts, and to quantitative tests by evaluation of antimicrobial activity using well agar diffusion method. The evaluation of antibacterial as well as antifungal activity were done against standard bacterial strains (*Staphylococcus aureus* and *Pseudomonas aeruginosa*) and standard fungal strains (*Aspergillus flavus* and *Candida albicans*) respectively. Ciprofloxacin used as antibacterial positive control and fluconazole as antifungal positive control. Phytochemical screening showed the presence of alkaloids, flavonoids, tannins, phenolic, terpenoids, reducing sugars and steroids. The present study suggests the ethanolic leaf extract exhibit more potent antibacterial activity than methanolic leaf extract, and the methanolic leaf extract exhibit more potent antifungal activity than ethanolic leaf extract. Also the best formulas of cream and ointment were selected which confirmed to all pharmacopeial requirements in quality control tests (pH, viscosity, color, odor, texture, spreadability, homogeneity and assay).

Keywords: *Argemone mexicana*, Extraction, Antibacterial, Antifungal, Formulation.



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Corresponding Author: MR. ABDULKARIM KASSEM ALZOMOR

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INTRODUCTION

Argemone mexicana Linn is an exotic weed has wide spread distribution in many tropical and sub-tropical countries ^[1]. *Argemone mexicana* Linn belongs to the family of *Papaveraceae*. Different types and effective compounds were quantitatively conformed. These compounds were alkaloids, flavonoids, glycosides, saponins, tannins, phenol, lignin, etc., which show their high efficacy by which they belong to medicinal plant category.

The *Argemone mexicana* Linn extract exerts a number of pharmacological activities like antioxidant activity comparable to free radical scavenging activity of ascorbic acid ^[2], antimalarial activity ^[3], anti-helminthic, anti-inflammatory, wound healing, antibacterial, antifungal activities ^[4,5]. In vivo experiments hepato-protective activity ^[6]. Das et al., 2009 showed promising anti-hepatotoxic activity of aqueous extract of *A. mexicana* stem in carbon tetrachloride- induced hepatotoxic male Albino Wister rats; anti-diabetic activity^[7]. Wound healing activity ^[8]. It has been used in treatment of skin diseases ^[9] in the treatment of dropsy and jaundice diseases ^[6]. The leaves are useful in cough, wounds, ulcers and skin diseases. Juice is used to cure ophthalmic and opacity of cornea. Seeds are purgative and sedative, also used in vitiated conditions of cough, asthma, pertussis, skin diseases, leprosy, dental caries, rheumatism, antidote to snake poisoning, colic and flatulence. The latex is useful in dropsy, jaundice, skin diseases, leprosy, blisters, conjunctivitis, inflammation, burning sensation and malarial fever. The oil is useful in indolent ulcers, wounds, leprosy and skin diseases, constipation, flatulence, colic and rheumatism ^[8]. The fresh yellow, milky, acrid sap contains protein-dissolving substances and has been used in the treatment of warts, cold sores, cutaneous affections, skin diseases, itches etc.. it also useful as anticancer drug ^[10], anti-HIV drug ^[11].

Plant crude extracts were proved to be higher in antimicrobial activity as a synergistic effect than purified individual constituents. Plants screened for antimicrobial activities have provided modern medicine with abundance of drugs and treatments against various ailments ^[12].

Infectious diseases still represents an important cause of morbidity and mortality among human, especially in developing countries. There are three reasons to interest in topics of antimicrobial plant extracts. First, it is very likely that these phytochemicals will find their way into the arsenal antimicrobial drugs prescribed by physicians; several are already being in humans. It was reported that, on average, two or three derived from microorganisms are launched each year. The scientists realize that the effective life span of any antibiotic is limited ^[13]. Second, the public is becoming increasingly aware of problems with the over prescription and misuse of traditional antibiotics. Third, for the renewed interest in plant antimicrobial has been the rapid rate of (plant) species extinction ^[14]. New sources, especially plant sources, are

being investigated. The use of herbal medicine at the primary health care level is widely spread in Yemen. However, only a few species from Yemeni flora have been scientifically investigated for their biological activity [15,16].

Therefore, in 2005 twenty-five selected plants belong to 19 families were be collected from different localities of the island Socotra, dried and extracted with the solvents chloroform, methanol and hot water to yield 80 extracts. The extracts were tested for their antimicrobial activity against several Gram-positive and Gram-negative bacteria and against one yeast species using agar diffusion method. Antibacterial activity was demonstrated especially against Gram-positive bacteria including multiresistant *Staphylococcus* strains. The greatest activity was exhibited by the methanolic extracts of *Boswellia longata*, *Boswellia ameero*, and *Bucus* displayed significant antifungal activity [17].

MATERIALS AND METHODS

Chemicals: *Argemone mexicana* leaves from Tamar city, Yemen; methanol, DMSO, glycerin, tween80, and ethanol from Scharalab, Spain; emulsifying wax from Global Co., Yemen; Sabouraud agar, Muller Hinton agar, paraffin oil, and sodium hydroxide from Himedia, India; white soft paraffin from Jalap-Bros Co., Yemen.

Instruments: Rotavapor evaporator from BUCHI, Germany; Freeze dryer system Lyph. Lock U.S.; LABCON Co, USA; Autoclave from Kern and Sohn GmbH, Germany; Incubator from LTE. Scientific Ltd., Britain; Blender from Panasonic, Jaban; Mixer from Siemems - Gmbh, Germany; pH meter from SFENGLI, China.

Microorganisms: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Asperigellus flavus*, *Candida albicans* from Central Laboratories, Sana'a, Yemen.

Methodology

Collection and extraction of the *Argemone mexicana* leaves

Argemone mexicana Linn leaves was collected from Tamar city, Yemen. *Argemone mexicana* Linn leaves were thoroughly washed with running tap water 2-3 times and finally washed with distilled water followed by shade-drying for fourteen days [18].

250 g of *Argemone mexicana* Linn leaves was taken into 750 ml conical flask and it was soaked with the solvent (one time using methanol and the other using ethanol) by maceration extraction method [19]. The Top of the conical flask was covered with aluminum foil paper for further prevention of evaporation of solvent and volatile constituents from the mixture. It was kept for seven days with shaking occasionally and stirred with a clean glass rod to extract

maximum amounts of phytoconstituents from the leave's powder. Then the extracts with different solvents were evaporated using rotary evaporator and change to semisolid, finally freeze drier was used to complete drying and the semisolid extract to solid.

Evaluation of Mexican leaves

Characteristics and morphology of leaves where tested visually [20].

Phytochemical analysis of the extracts (qualitative test)

The methanolic and ethanolic extracts of *Argemone mexicana* were subjected to qualitative phytochemical test for the identification of various phytochemical constituents such as alkaloids, tannins, flavonoids, sterols, and reduced sugar following standard procedures [21,22,23].

Evaluation the antimicrobial activity of the extracts (quantitative test)

Quantitative assay was done by well agar diffusion method to determine MIC of the extracts. Muller Hinton agar was prepared for bacteria and sabouroud agar was prepared for fungi according to manufacturer directions. Different concentrations of *A.mexicana* extracts (ethanolic and methanolic) from (0.4 – 50 mg/ml), dispersed in DMSO, and wells were made by cork-borer, 4-5 wells in each plate; 6mm diameter holes were cut in the agar, 20 mm between one and another on agar. Each well was incorporated with 40µl of serially Mexicana extract concentrations. Then these plates were incubated in the incubator for 24 hours at 37°C for bacteria and for 48hours at 28°C for fungi and the result was recorded [24]. Ciprofloxacin was used here as antibacterial positive control, the fluconazole was used as antifungal positive control, and DMSO as negative control [18]. 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 fungi) [25].

Argemone mexicana extract formulations

Different excipients were used in different quantities to prepare cream and ointment bases then the best formula of each cream and ointment was selected to perform the stability study as shown in table (3).

Preparation of Argemone mexicana leaves extract in cream formula

Oil in water (o/w) cream base was prepared by using emulsification technique [26,27]. Firstly, all excipients and the extract were weighed accurately by calibrated analytical balance as shown in table (1). The oil phase was prepared where 9gm of emulsifying wax was heated until molten in water bath then 15gm of white soft paraffin was added to it and heated until molten after that 6gm of levigating agent (paraffin oil was added to them with stirring, all 30gm of this phase was

taken in flask and heated to (60-70°C). the aqueous phase was prepared by add 10ml of glycerin then 0.5ml of tween80 and (0.62gm) of the A.P.I. (*Argemone mexicana* extract) in 60ml D.W. were added. After that, the both phases (oily phase and aqueous phase) were heated to the same temperature (60-70 °C), the aqueous phase was added gradually to the oily phase with continuous stirring until congeal.

Preparation of *Argemone mexicana* leaves extract in ointment

Oleaginous base was prepared by using fusion technique. First, all the excipients and A.P.I. (*Argemone mexicana* extract) were weighed accurately by calibrated analytical balance as shown in table (1). Simple ointment was prepared where 2.5gm of emulsifying wax was heated until molten in water bath then 47.5gm of white soft paraffin was added to it and heated until molten. The 0.62gm of *Argemone mexicana* leaves extract dissolved in 15.5ml of liquid paraffin add to simple ointment gradually with continuous stirring until congeal ^[26].

Accelerated stability study for Argemone mexicana extract cream and ointment

Stability studies were carried out for different formulas (cream and ointment) according to International Conference on Harmonization (ICH) guidelines. The cream formula (F1) and ointment formula (F2), that showed good physical properties, each formula was divided into two samples separately and those samples were kept at different storage conditions i.e. at 25°C and at 40°C with 75%RH (Relative Humidity) with intensive light in stability chambers, and observed for a period of one month at a definite time intervals. A sufficient quantity of each formula in suitable containers was stored in oven under 40±2°C and 75%±5%RH for one month and all physical and chemical tests were done for the samples weekly through one month ^[28].

Quality control of finished product

Physical evaluation

All formulas of *Argemone mexicana* extract were evaluated for physical properties including occlusiveness and washability by usual methods, but grittiness test was done by taking small quantity of product was pressed between the thumb and the index finger and by physical observation. The consistency and the texture of cream and ointment were noticed. A small quantity of the sample was rubbed on the skin of the back of the hand to determine the homogeneity and spreadability ^[29,30].

Measurement of viscosity: The resistance of a substance to flow is called viscosity. It was measured in Pascal seconds or poises. The rheological studies were determined using LV-2 spindle in a Brooke field Viscometer. All measurements were performed in triplicate then shown in tables (6),(7).

Measurement of pH of the formulas

Digital pH meter was used to determine the pH of the formulas (cream and ointment) by immersing the electrode of pH meter in the samples and the results were recorded [31,32].

Antimicrobial assay of finished product

Muller Hinton agar was prepared by dispersing 19gm in 500ml of D.W. for bacterial test and sabouraud agar was prepared by dispersing 65gm in 500ml of D.W. for fungi then boiled and sterilized by autoclave. Small amount of each *Argemone mexicana* extract 's cream and ointment (ethanolic and methanolic), were used at MIC 0.62%, 4 wells in each plate; 6mm diameter holes were cut in the agar, 25 mm between one well and another on agar. Each well was incorporated with 40µl of *Argemone mexicana* extract as cream and ointment. These plates were incubated for 24 hours at 37°C for bacteria and for 48hours at 28°C for fungi after that the results were recorded [24]. Ciprofloxacin was used here as antibacterial positive control and the fluconazole as antifungal positive control.

Microbial test

The formulated cream and ointment were inoculated on the plates of Muller Hinton agar media by streak plate method and a control was prepared by omitting the pharmaceutical sample (cream , ointment). The plates were incubated at 37°C for 24hr for bacteria and at 28°C for 48hr for fungi. After the incubation period, plates were taken out and check the microbial growth by comparing it with the control [33].

Packaging

Cream and ointment were packed in plastic and glass jars because the drug during accelerated stability study remain has good stability.

Results and discussion

Results of evaluation and identification of *Argemone mexicana* leaves

Results of the physical test

Argemone mexicana is an annual herb, growing up to 150cm with a slightly branched tap root. Its stem is branched and usually extremely prickly. It exudes a yellow juice when cut. It has showy yellow flowers. Leaves are thistle-like and alternate, without leaf stalks (petioles), toothed and the margins are spiny. The grey-white veins stand out against the bluish-green upper leaf surface. The stem is oblong in cross-section. Flowers are at the tips of the branches (are terminals) and solitary, yellow. Fruit is prickly oblong or ovoid capsule. Seeds are very

numerous, nearly spherical, covered in a fine network of veins, brownish black and about 1mm in diameter [20].

Results of the chemical tests

The phytochemical analysis was done for both ethanolic and methanolic extracts showed presence of many phytoconstituents alkaloids, tannins, carbohydrates, saponins, phenolics, terpenoids, and steroids as shown in table (1).

Table (1) shows the phytochemical screening of *Argemone mexicana* leaf extracts:

Active constituent	Test	Ethanolic	Methanolic
Alkaloids	Wigner test	+	+
Tannins	Gelatin test	+	+
Reducing sugar	Fehling test	+	+
Saponin	Foam test	+	+
Flavonoids	NaOH test	+	+
Phenolics	FeCl ₃ test	+	+
Triterpenoids	Salkowski test	+	+
Steroids	Liebermann- Burchard test	+	+

Antimicrobial activity assay of the *Argemone mexicana* extracts

In the present study, alcoholic extract of *Argemone mexicana* leaves showed significant antimicrobial activity against some bacteria and fungi species, which is supported by another study which carried out previously. The results of this study suggest that alcoholic extract of *Argemone mexicana* may serve as an alternative to synthetic antimicrobial which might have significant applications in pharmaceutical or other industries for controlling microorganisms infections.

A recent study reported the potentials of the antimicrobial activity of *Argemone mexicana* leaf extracts against two bacterial strains (*Staphylococcus aureus* and *Pseudomonas aeruginosa*) and against two fungal strains (*Candida albicans* and *Asperigellus flavus*). The results of antimicrobial assay of both extracts shown in table (2).

Table (2) shows the results of antimicrobial assay of *Argemone mexicana* as methanolic and ethanolic extract:

Conc. of the extract	Zone of inhibition (in mm)							
	Bacteria				Fungi			
	<i>S. aureus</i>		<i>P. aeruginosa</i>		<i>C. albicans</i>		<i>A. flavus</i>	
	MeOH	EtOH	MeOH	EtOH	MeOH	EtOH	MeOH	EtOH

50 mg/ml	15	16	15	16	16	15	26	18
25 mg/ml	14	14	13	14	15	14	20	15
12.5 mg/ml	14	13	11	12	14	13	14	13
6.25 mg/ml	13	12	10	11	12	9	11	10
3.12 mg/ml	10	10	-	-	11	8	-	-
1.56 mg/ml	8	10	-	-	9	8	-	-
0.8 mg/ml	8	9	-	-	-	-	-	-
0.4 mg/ml	-	-	-	-	-	-	-	-
Ciprofloxacin	35	35	14	14	-	-	-	-
Fluconazole	-	-	-	-	18	20	15	16
DMSO	-	-	-	-	-	-	-	-

The antibacterial activity of ethanolic extract of *Argemone mexicana* leaf had more effective than the methanolic extract, but the antifungal activity of methanolic extract of *Argemone mexicana* leaf had shown more effective than the ethanolic extract.

The minimum inhibitory concentration (MIC) was (0.8mg/ml) with inhibition diameters (8 and 9mm) were observed in *S. aureus* in methanolic and ethanolic extracts respectively. For *P. aeruginosa* the MIC (6.25mg/ml) with zone of inhibition (10 and 11mm) was observed in methanolic and ethanolic extracts respectively. From the antibacterial results ethanolic extract more potent on bacteria than the methanolic extract. About fungi the MIC was 1.56mg/ml with inhibition diameters (8 and 9mm) were observed in *C. albicans* in methanolic and ethanolic extracts respectively. *A. flavus* the MIC was 6.25mg/ml with zone if inhibition (11 and 10mm) for methanolic and ethanolic extracts respectively. From these results that methanolic extract has more potent antifungal activity than ethanolic extract.

Results of quality assessment of cream and ointment *Argemone mexicana* formulations

Results of *Argemone mexicana* formulations

Table (3) shows the materials and their quantities used in the best *Argemone mexicana* Linn formulations for each dosage form:

Ingredient	Cream F1	Ointment F2
<i>A. mexicana</i> extract	0.62g	0.62g
Liquid paraffin	20ml	11ml
Non-ionic emulsifying wax	30gm	5gm
White soft paraffin	50gm	90gm
Tween 80	0.5ml	-
Glycerin	10ml	-
Distilled water	60ml	-

Cream formula was creamy greenish white in color with good appearance and smooth feel on application, and homogeneous with good texture. Spreadability and viscosity upon preparation

were accepted, with no phase separation or creaming. The pH measures were suitable to skin pH and conform to pharmacopeia requirements.

Also ointment formula showed good physical properties, homogeneous, smooth, greenish translucent in color, and elegant. Spreadability and homogeneity of this formula upon formulation were accepted with suitable pH.

Results of accelerated stability study

The Argemone mexicana cream and ointment were prepared and subjected to evaluation by various parameters. The results shown that cream (F1) and ointment (F2) maintained have the same appearance and good feeling on application after the accelerated stability study performed. The initial viscosity of formulated *Argemone mexicana* formulas showed constant stability in comparison with after accelerated stability study performed. Further 1 months stability was carried out at normal room conditions (25°C, 65% relative humidity) but results were unchanged. The drug antimicrobial assay of formulas were assessed and found to be uniform and unchanged significantly with the both formulas cream F1 and ointment F2.

The pH of human skin typically ranges from 4.0 to 6.0 [34]. Therefore, the formulas intended for application to skin should have pH closer to this range [35]. In this study, the pH values of samples kept at different conditions were shown weak alkalinity to neutral with no significant difference with both formulas (F1, F2) as shown in table (4).

Results of quality tests of cream formula F1

Table (4) illustrates the results of Argemone mexicana cream under normal and stress conditions at different time points:

Time period	Conditions	Parameters						
		PH	Color	Spread-ability	Washability	Homogeneity	Odor	
0 day	Room Conditions	5.60	Greenish white	Good	Easy	Good	No Change	
		28600pc	Viscosity					
1 st week	Room Conditions	5.56	Greenish white	Good	Easy	Good	No Change	
	Stress Conditions	5.70	Greenish white	Good	Easy	Good	No Change	
		28600pc						

2nd week	Room Conditions	5.55	28800pc	Greenish white	Good	Easy	Good	No Change
	Stress Conditions	5.65	28700pc	Greenish white	Good	Easy	Good	No Change
3rd week	Room Conditions	5.68	28800pc	Greenish white	Good	Easy	Good	No Change
	Stress Conditions	5.67	28900pc	Greenish white	Good	Easy	Good	No Change
4th week	Room Conditions	5.60	28800pc	Greenish white	Good	Easy	Good	No Change
	Stress Conditions	5.70	29000pc	Greenish white	Good	Easy	Good	No Change

**Room conditions (25°C, 65% relative humidity) *Stress conditions (40°C, 75±5% relative humidity)*

The formulated *Argemone mexicana* cream as shown in table (4) revealed good physical stability including easy to wash, non-greasy with good stable spreadability and smooth texture with no creaming, coalescence occur through analysis period of accelerated stability study. No liquefaction was observed in the sample of formula kept at 25°C and 40°C with 75% relative humidity during whole study period of one month. This showed that the emulsions were stable at different storage conditions i.e. 25°C and 40°C with 75% relative humidity throughout the period of analysis, i.e. one month.

The pH is a significant parameter insofar as the effectiveness of the cream F1 is concerned. The results were revealed there are no significant difference with the formulated cream F1 as shown in table (4). Finally, the formulated cream F1 was easy to wash, non-greasy with good stable spreadability and texture throughout analysis period i.e. one month.

Results of quality tests of ointment formula

Table (5) shows the results of *Argemone mexicana* ointment preparations under normal and stress conditions at different time points:

Time period	Conditions	Parameters						
		PH	Viscosity	Color	Spreadability	Homogeneity	Odor	

0 day	Room Conditions	5.19	30000 pc	Translucent Green	Good	Good	No Change
1st week	Room Conditions	5.15	29980pc	Translucent Green	Good	Good	No Change
	Stress Conditions	5.22	30000pc	Translucent Green	Good	Good	No Change
2nd week	Room Conditions	5.3	30000pc	Translucent Green	Good	Good	No Change
	Stress Conditions	5.25	29990pc	Translucent Green	Good	Good	No Change
3rd week	Room Conditions	5.1	29990pc	Translucent Green	Good	Good	No Change
	Stress Conditions	5.23	29980pc	Translucent Green	Good	Good	No Change
4th week	Room Conditions	5.18	29900pc	Translucent Green	Good	Good	No Change
	Stress Conditions	5.24	30000	Translucent Green	Good	Good	No Change

*Room conditions (25°C,65% relative humidity) *Stress conditions (40°C, 75±5% relative humidity)

The ointment formula F2 maintained good homogeneity, smoothness and texture, translucent green in color, elegant, good viscosity with no changes in odor throughout analysis period of accelerated stability study. Spreadability and homogeneity were accepted. Also suitable and stable pH values throughout the analysis period as shown in table (5).

Results of antimicrobial assay of finished product

The cream formula (F1) and the ointment (F2) were tested against microbes that used in this study by well diffusion method and the results showed in table (6).

Table (6) shows the antimicrobial assay of *Argemone mexicana* formulations F1, F2:

MICROBE	Cream (F1)		Ointment (F2)	
	Methanolic	Ethanolic	Methanolic	Ethanolic
<i>S. aureus</i>	12mm	13mm	8mm	9mm
<i>P. euorgenosa</i>	8mm	9mm	8mm	9mm
<i>C. albicans</i>	12mm	12mm	13mm	11mm
<i>A. flavus</i>	12mm	11mm	11mm	10mm

The table above shows that antimicrobial assay of the ethanolic extract was more potent against bacteria than the methanolic extract, and the methanolic extract has antifungal activity more potent than the ethanolic extract. Also the cream showed a little higher activity than ointment because the cream has more diffusion ability than ointment.

Microbial test for finished products

All batches for each dosage form conformed to the pharmacopeial requirements of microbial test (not more than 10^2 CFU/ml for fungi and not more than 10^3 CFU/ml for bacteria) under suitable aerobic and anaerobic conditions.

Conclusion

Argemone mexicana is one species of *Papaveraceae* family, it has antibacterial and antifungal activities against many species of these microorganisms. Different concentrations of *Argemone mexicana* extracts (methanolic and ethanolic) were prepared to measure the antimicrobial activity against some bacteria and fungi. The results showed the MIC was 6.25mg/ml for *A. flavus* and *P. aeruginosa* and MIC 0.8mg/ml, 1.56mg/ml for *S. aureus* and *C. albicans* respectively, all formulas prepared with concentration 6.25mg/ml that is active against bacteria and fungi. Two best formulas were selected F1 cream and F2 ointment then evaluated under different conditions (normal and stress conditions) i.e. at 25°C and 40°C with 75±5% RH for one month. The evaluation tests were physical tests including viscosity, pH, spreadability, homogeneity, color, odor, and the antimicrobial assay.

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