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Evaluation of antimicrobial and cytotoxic potential of *Argemone mexicana* L.

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ABSTRACT

This study is an experimental in vitro research, aimed to evaluate antimicrobial potential and cytotoxicity of plant species Argemone mexicana L. (Papaveraceae) with a treatment of infected wounds perspective. Crude ethanolic extract was obtained after the solution concentration in a rotary evaporator and submitted to a fractionation by classical liquid chromatography column, resulting in four fractions: hexane, chloroform, ethyl acetate and methanol. A phytochemical screening was performed in crude ethanolic extract. Antibacterial activity was determined by the Minimum Inhibitory Concentration (MIC), where fractions were tested against major infection-causing bacteria in wound and to evaluate the samples cytotoxicity was conducted the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay. In the studied samples, it was observed the presence of steroids, coumarins, anthrones and alkaloids, which is the majority metabolite. The cytotoxicity of the samples was observed at concentrations of 1000 µg and 100 µg. Among these, chloroform fraction at a concentration of 1000 µg and methanol fraction at a concentration of 100 µg showed no cytotoxicity with cell viability of 87 % and 90, 2 %, respectively. The antibacterial activity was observed in fractions against S. epidermidis and in methanol fraction front S. aureus, which showed a MIC of 166,66 µg/mL. This study may be useful to guide new investigations, depth and systematic, about the chemical compounds of this species and serves as subsidies for further herbal medicine development with perspective in treatment of infected wound.

Keywords: *Argemone mexicana*; Medicinal plants; Nursing; Antimicrobials.

INTRODUCTION

Wounds are result of the disruption of skin integrity [1]. It is a physical injury, which can achieve superficial and underlying skin structures [2].

With skin rupture, healing process is initiated, which comprise a sequence of complex molecular events involving cell organization, chemical signals and extracellular matrix aiming the injured tissue recovery [3].

The process of wound healing may be compromised by presence of local and systemic factors. Local factors directly influence the wound's own characteristics, while the systemic factors are related to health or disease that affect patient general condition and his healing ability [4].

Among factors related to skin compromising wound healing process, the presence of pathogenic microorganisms that cause damage to entire length of tissue reaching the aesthetics and functionality of the injured area, may be associated.

Treatment of infected wounds continues to be an important issue in health care, in a period where indiscriminate use of antibiotics has been hindering hospital treatment of patients and contributed to increasing the appearance of resistant microorganisms to antimicrobial agents [5].

The cost of pathologies treatment related to healing process delayed, as presence of infection in wound, increase the importance of studies related to search of new drugs and bandages capable of interacting with injured tissue and accelerating the repair phase [3].

Due to ineffectiveness of some drugs, strong side effects and high cost of available medications, it is observed in recent years, an increase in the use of medicinal plants as treatment for human health and a strong interest to pursue therapies that are less aggressive to the human body [6].

Medicinal plants continues to be studied as alternatives to treatment of several dermatological diseases, with emphasis on cutaneous lesions that have a complex healing process resolution [7].

Papaveraceae family, commonly known as Poppy family, has a big ethnopharmacological importance. It is represented by 44 genus and 760 species of flowering plants, among them, *Argemone mexicana* L., is used in several places in the world for treatment of diseases [8].

Argemone Mexicana L. belongs to the Magnoliopsida class. It is a plant that can be found in Central America, but with large distribution in many tropical and sub-tropical countries, including West Africa [9].

It is usually known as "Mexican prickly poppy," "cardo santo" or "chicalote". Its different parts are used for chronic skin diseases and as an emetic, expectorant, emollient and diuretic, seed and oil seeds are used as remedy for dysentery, ulcers, asthma and other intestinal affections [10].

In Brazil, it is traditionally used against numerous diseases [11, 12]. Its infusion is applied against hypertension [12], and its latex is used against conjunctivitis [11].

Biological activities of crude extracts and chemical isolated constituents of this plant species, were studied in several studies, among: antiviral, antibacterial, anti-inflammatory, wound healing, anti-allergic, anti-stress, vasoconstriction and vasorelaxant, larvicide, antioxidant, antidiabetic, anticancer [8].

Considering the research conducted by Araujo et al. [13], where crude ethanolic extract of this plant species proved active against 8 bacterial strains, with an inhibitory concentration ranged from 250-1000 μg/mL, this study aimed to perform a phytochemical screening in order to identify secondary metabolites present in *Argemone mexicana* L crude ethanolic extract and submit it to a fractionation by classical liquid chromatography column and evaluate the antimicrobial activity against strains of microorganisms that often cause skin wound infection and cytotoxic activity of these fractions.

EXPERIMENTAL SECTION

This study was an experimental in vitro research developed in Laboratory of Medicinal Chemistry - LQM (extract and fractions preparation), Research Laboratory in Treatment of Wounds (antibacterial activity) and Laboratory of Pharmacology and Immunology - Lafi - (cytotoxic activity) at Federal University of Alagoas.

Extract Preparation

Argemone mexicana Linn, known as “cardo santo”, was collected in a private property (geographic coordinates 9°35'38.2"S 35°44'57.3"W) in city of Maceio-AL, in August 2013 and identified by MAC at Institute for the Environment of Alagoas with number 57421.

In this study, leaves were used, and after drying at room temperature, were grinded. Crude ethanolic extract was obtained by ethanol (EtOH) 95 % maceration, concentrated on a rotary evaporator at maximum temperature of 40 °C and subsequent drying at room temperature.

Phytochemical Screening

Crude ethanolic extract was submitted to reactions of phytochemical characterization in order to identify the secondary metabolites present in this species. The phytochemical screening was performed according to methodology proposed by Matos (1997), where 30 mg of crude ethanolic extract was solubilized in 30 mL of absolute EtOH and placed in test tubes through reactions indicated presence or absence of secondary metabolites [14].

Qualitative and semi quantitative tests have identified the presence of secondary metabolites: anthocyanins, anthocyanins, flavonoids, leucoanthocyanidins, catechins, flavanones, flavonols, flavanones, and xanthones, steroids, triterpenes, saponins, alkaloids, anthraquinones, anthrones, coumarins, phenols and tannins.

Classical Liquid Chromatography Column

This technique was used for natural products isolation and products purification of chemical reactions. Crude ethanolic extract (2.5 g) of *Argemone mexicana* L. species was submitted to a Classical Liquid Chromatography Column performed according to methodology described by Degani, Cass and Vieira [15].

The stationary phase was composed of silica (60G) as adsorbent, was suspended in polar solvent, and mobile phase consisting of crude extract sample was placed on a rotary evaporator with silica, and submersed, in solvents obeyed polarity.

For fractions formation, were used four types of solvents at different volumes, and column initiated by more polar solvent, which was the Hydrocarbon Hexane (CH3 (CH2) 4CH3), through Chloroform (CHCl3) and Ethyl acetate, they are solvents of medium polarity but with different selectivity. The last solvent used was Methanol (CH 3 OH), for being the most polar.

Biological In vitro assays

Cell viability assay

For evaluation of samples cytotoxicity, was performed MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay, based on mitochondrial activity of cells by the MTT reduction by cleavage of tetrazolium salt (dark yellow) in formazan crystals (dark blue) by succinate dehydrogenase enzyme, present in active mitochondria. The darker the colour at the end of reaction, higher cell viability [16]. Resulting optical density of MTT assay was determined by spectrophotometer.

J774 macrophages lineage were placed in 96-well plates at a density of 2 x 10^5 cells per well, cultured in Dulbecco Mem (DMEM) supplemented with fetal bovine serum 10%. Each well received 200 µL of culture medium with cells.

Cells were treated with extract and fractions, submitted to antibacterial in vitro tests at concentrations of 1000 and 100 µg/mL for 48h, and maintained in an incubator at 5 % of CO_2. In the period of 1 hour before adding Metiltetrazolium (MTT), three wells containing cells were lysed by Triton 100X (2 µL), for comparison of cell death. Control wells consisted of dead cells as positive control (lysed cells - 3 wells) and cultured cells plus the diluent DMSO 0,1 % as negative control.

After the total period of incubation (48h), supernatant was discarded and to each well was added 100 µL of a MTT solution and reincubated for 1 hour in an oven at 37 °C. After this period, the supernatant was discarded and precipitate resuspended in 100 µL of DMSO.

To quantify the reduced formazan salt, plates were read with assistance of a microplate reader at a wavelength of 550 nm. This technique has the ability to analyse cell viability and metabolic state of cell, from reduction of tetrazolium salt (dark yellow colour) to formazan (dark blue colour) and is useful to evaluate in vitro cytotoxicity [16].

Data were expressed as absorbance mean ± SEM and statistical differences between the treated groups and control, and were analysed by ANOVA and Dunnett test, where significance levels compared to negative control group were identified by asterisk ($P < 0,05$). The percentage of cell viability was calculated according to absorbance of negative control and samples, using the formula:

$$\% \text{ Viability} = \frac{\text{Treatment Absorbance}}{\text{Negative Control Absorbance}} \; x \; 100$$

Broth Microdilution Method

The chromatographic fractions were solubilized in Dimethyl sulfoxide solution (DMSO) at 1 % and were tested against standardized bacteria by American Type Cell Collection - ATCC / Manassas - VA/USA: *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 14942, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus epidermidis* (ATCC 149990).

The antibacterial activity of crude ethanolic extract was determined by Minimum Inhibitory Concentration (MIC) method adapted from Clinical and Laboratory Standards Institute [CLSI] [17]. Dilutions of extracts were in triplicate, and prepared in microdilution plates of 96 wells, leaving a volume of 100 µL per well of compound. As positive control for antibacterial activity it was used Ciprofloxacin and for negative control was used DMSO 1 %.
To determine the MIC, bacteria samples were solubilized in a solution of 1,5 x 10^8 CFU/mL, with concentration according to standard of 0.5 in McFarland scale and subsequently diluted in 1:10 (v/v) to obtain the standard concentration (10^4 CFU/mL). Each well received 5 µl of bacterial inoculum, resulting in a concentration of 10^4 CFU/mL. After this period, 20 uL of 2,3,5-Triphenyltetrazolium chloride at 5 % was added in each well, and stored in bacteriological oven at 35 °C for 3 hours. The wells, which had red colour, indicated bacterial growth, while original colour indicated inhibition of bacterial growth.

RESULTS

Phytochemical Screening

Cruude ethanolic extract of *Argemone mexicana* L. species, was submitted to phytochemical analysis in order to identify in a qualitative way, secondary metabolites that were present in the samples. Table 1 shows the results obtained in phytochemical screening.

Table 1. Results of phytochemical screening performed in crude ethanolic extract of *Argemone mexicana* L.

Phytochemical Screening	
Secondary Metabolites	*Argemone mexicana* L.
Tannin	-
Phenol	-
Flavanone	-
Flavonols	-
Xanthones	-
Leucoanthocyanidins	-
Catechins	-
Flavonoids	-
Anthocyanidins	-
Anthocyanins	-
Chalcones and Aurones	-
Free steroids	+
Pentacyclic triterpenes	-
Saponins	-
Alkaloids	+
Anthraquinones	-
Anthrones	+
Coumarins	+

+: Presence of secondary metabolites, -: absence of secondary metabolites

Samples where submitted to the Dragendorff reagent on a thin layer chromatography for analytical identification of the alkaloid, demonstrated that this secondary metabolite as the major component of plant species.

Cytotoxicity by MTT reduction assay

Cytotoxicity of ethanolic extract and fractions of *Argemone mexicana* Linn was evaluated to obtain the absorbance mean, standard error and significance level of cytotoxic activity of samples compared to negative control groups (Table 2), where it was observed that activity varied in concentrations of 100 µg and 1000 µg in which they were tested.

At concentration of 1000 µg and 100 µg, crude extract sample, hexane fraction and ethyl acetate fraction showed a significant cytotoxic activity ($p < 0,05$), whereas cytotoxicity shown by chloroform and methanol fraction varied according to concentration.

Table 2: Cytotoxicity evaluation of the crude ethanolic extract and fractions through absorbance mean compared to negative control.

Cell Viability by Colorimetry		
Samples	Concentrations	
	1000 µg	100 µg
DMSO (-)	1,263 ± 0,03	1,242± 0,02
Lysed cells (+)	0,066 ±0,06	0,066 ±0,06
Crude ethanolic extract	0,376 ± 0,03*	0,687 ± 0,05*
Hexane fraction	0,986 ± 0,06*	0,956 ± 0,05*
Ethyl acetate fraction	0,066 ± 0,06*	0,572 ± 0,05*
Chloroform fraction	1,099 ± 0,01	0,714 ± 0,07*
Methanol fraction	0,783 ± 0,04*	1,121 ± 0,02

**p<0,05*

In MTT assay, at concentration of 1000 µg, chloroform fraction showed no significant cytotoxic activity on treated cells, showing percentage of 87 % viable cells, whereas concentration of 100 µg the methanol fraction showed no toxicity on cells in the end of treatment, with 90,26 % of cells remained viable.

Determination of Minimum Inhibitory Concentration

Results obtained for MIC by Broth Microdilution Method, the chromatographic fractions of leaves and crude ethanolic extract from *Argemone mexicana* Linn species, showed antibacterial activity.

The four fractions were tested against four bacterial strains, of which there was no inhibition for *Pseudomonas aeruginosa* and *Escherichia coli* (Table 3). Against *Staphylococcus aureus,* methanol fraction was relevant, with a MIC of 166, 66 µg/mL, while front *Staphylococcus epidermidis* showed a MIC of 416, 66 µg/mL.

Hexane and ethyl acetate fractions exhibit activity against two bacterial strains, highlighting against *S. epidermidis* with a MIC from 208.33 µg/mL.to 416.66 µg/mL.

Table 3: Minimum Inhibitory Concentration (MIC) of crude ethanolic extract, fractions and positive control

	Minimum Inhibitory Concentration (MIC) (µg/mL)			
Bacterial strains	Hexane fraction	Chloroform fraction	Ethyl acetate fraction	Methanol fraction
S. aureus	NI	NI	500	166,66
S. epidermidis	416,66	500	250	416,66
P. aeruginosa	NI	NI	NI	NI
E. coli	NI	NI	NI	NI

NI: No Inhibition

DISCUSSION

Medicinal plants are important sources of chemicals with a huge therapeutic benefits. Several studies have been performed over the years in order to elucidate pharmacological potential of plant species in wound healing process. Crude ethanolic extract of the *Argemone mexicana* L. leaves was submitted to a phytochemical screening, which identified presence of steroids, anthrones, coumarins and alkaloids, which is the major secondary metabolite of studied species.

Alkaloids are components that aroused great interest for present several biological activities, such as antiplatelet aggregation and antitumor activity [18], cytotoxicity [19], antibacterial and antispasmodic activity [20].

Toxicity is one of factors, which limited release, and consumption of drugs, their evaluation is performed in order to determine the potential of new substances causing harm to health and to establish its applicability and therapeutic index [21].

In this study it was used colorimetric in vitro method of Methyl Tetrazolium (MTT) assay, this test can define intrinsic ability of extract to cause cell death through damage the basic functions of its [22].

In MTT assay, crude extract and fractions of ethyl acetate and hexane fraction showed cytotoxicity front treated cells in both tested concentrations. This result confirms other research that shows cytotoxicity of *Argemone mexicana* Linn species, through activities such as antitumor, anti larvicide and sterilizing.

The study conducted by Varun and Sellappa [23], aqueous extract of *Argemone mexicana* Linn at concentration of 100 µg was evaluated against cell lines responsible for breast cancer and could inhibit efficiently the growth of more

than 97 % of cancer cells, as well as in another study, isolated alkaloids of this plant species were cytotoxic on cells responsible for nasopharyngeal and gastric cancer [24].

In another study, acetone fraction obtained from petroleum ether extract of *Argemone mexicana* Linn seeds, exhibited inhibitory activity against larvae of *Aedes aegypti* and sterilizing activity promoting 100 % sterilization of first-generation eggs of this mosquito [25].

In this study, chloroform fraction at concentration of 1000 µg and methanol fraction at concentration of 100 µg showed no significant cytotoxicity, and held 87 % and 90,2 % of viable cells, respectively, showing promise for a future applicability as herbal medicine without causing damage to health.

Regarding antimicrobial activity, crude extract of plant species present several compounds, which never developed resistance against pathogens responsible for infections. These compounds act synergistically to inhibit pathogen, justifying why the popular use of certain plants is so successful around the world [26].

The antibacterial activity of *Argemone mexicana* Linn chromatographic fractions was evidenced front of two bacterial strains used in this study. This corroborate with the researches that reveal the inhibitory action of different parts of this plant species.

Crude ethanolic extract of the leaves of this species, when was evaluated for antibacterial activity proved to be active against 8 bacterial strains with a MIC ranged from 250 – 1000 µg/mL, and *S. epidermidis* strain was the most sensitive sample in the test [13].

Recent research evaluates the antibacterial activity of the crude extracts of this species against a number of Gram-positive and Gram-negative bacteria. The organic extracts demonstrated a strong antibacterial activity, with MIC values ranging from 62,5 to 500 µg/mL, indicating the existence of some bactericidal chemical constituents in the plant, which may be useful in several applications [27]

In another study, the *Argemone mexicana* bacterial activity was confirmed by aqueous methanol extract at 50 %, which was tested against *Klebsiella oxytoca*, *Vibrio damsela*, *Enterobacter aerogenes* and *Escherichia coli* with higher efficacy against Gram-negative bacteria [28].

With these same procedures of studies presented above, another group of researchers studied antibacterial activity of leaves, stem and roots extract of same plant species with water based, acetone, ethanol and chloroform solvent in front of *Escherichia coli, Klebsiella pneumoniae, Bacillus cereus* and *Staphylococcus aureus,* showing that the stem extract has high inhibitory activity [29].

Hexane and ethyl acetate fractions evaluated in present study showed moderate inhibitory activity front of *S. epidermidis*, with MIC ranging from 208,33 to 416,66 µg/mL, and ethyl acetate fraction showed to be active against *S. aureus* (Table 2), this activity may be a result of cytotoxic ability present in these samples.

Although toxicity has been a factor that limits the production of some drugs, results expressed by MIC's against these strains are favourable for discovery of new drugs that are able to combat infections, and at same time have an adequate therapeutic index and not cause damage to health, since chemical constituents of natural products represent one of the successful alternative key for treatment of infections.

S. epidermidis strain has capacity to hold the polymer surfaces, resulting in biofilms that are important pathogenicity factors, which cause a reduced in immune response, and in the host defence capacity [30]. Due to biofilm production and its virulence power, microorganisms of this species are often associated with a reduction of sensitivity to antibiotics used in treatment of infection [31].

In a study done by Bhattacharjee et al. [32], *S. aureus* was sensitive front of leaves and seeds methanol extracts of *Argemone mexicana* Linn, and showed a maximum antibacterial activity against this pathogen. This result was also demonstrated in this study, where methanol fraction proved to be active against *S. aureus*, with MIC of 166,66 µg/mL [32].

This bacterial strain is considered one of the most important pathogens that causes infectious processes, and it is able to cover superficial lesions to severe systemic infections. Its virulence is due to existence of numerous toxins, enzymes and proteins associated with cell wall, which combine can cause tissue invasion and survival in infectious site; besides having strong acquired resistance to many antimicrobials [33].

Patients colonized by microorganisms are submitted to isolation during the hospital stay. Among these microorganisms, *S. aureus* is highlighted for being virulence to patient, especially to immunocompromised, given that contamination with this pathogen can cause several infectious processes [34].

Assays aimed evaluation of antibacterial potential front of *S. aureus* with organic substances and which had promising results, such as methanol fraction proved to be moderately active against this pathogen and non-cytotoxic, need a deeper understanding. Therefore, possible strategies for treatment of infections caused by resistant microorganisms can be developed.

Regard to inactivity results presented in MICs front *P. aeruginosa* and *E. coli*, it is possible to highlight the physical characteristics of crude ethanolic extract, where the presence of its primary and secondary metabolites may interfere with concentration of these substance, which is active against these microorganisms species, as well as the characteristics of these microorganisms.

Pseudomonas aeruginosa is an opportunistic pathogen, quite versatile and able to grow a wide of variety environments. It is responsible for a range of infections in immunocompromised hosts. Among the most significant infections are those located in soft tissue, as the case in chronic wounds and burns [35].

The absence of antibacterial action front to this strain was also confirmed in a study by Sahu, Debtae and Padhy [26], where ethanol extract of *Argemone mexicana* did not act against *P. aeruginosa* because presented flavonoids, tannins, sterols, terpenes, alkaloids and some reducing sugars, substances that are not shown to be effective against this bacterial species.

In another study, inhibition of *E. coli* was evidenced by methanol extract of this plant seeds. Besides inhibiting the growth of this bacterial strain, this extract was able to inhibit growth of other strains such as *P. aeruginosa*, *S. aureus* and *B. subtilis* [32].

With these results, it is clear that bacterial inactivity can be attributed to several factors, as affinity absence of active compounds with solvents, used in extract and fractions preparing as well as structure of the plant, where their compounds vary from local.

Moreover, the structural differences between Gram-positive and Gram-negative bacteria may prevent activity of chemical compounds present in crude plant extract and its fractions and thus can become active in higher concentrations than those made in MIC in this research or even can not be active on tested strains.

Previous knowledge of chemical components classes present in plants, is important because provides a list of active principles and once detected presence of certain chemical groups, the phytochemical and biological studies is guided [36]. Thus, in this type of in vitro tests, possibility of interference of active substance against tested microorganisms may be decreased and antimicrobial potential of plant species may be determined.

CONCLUSION

Faced with mentioned results, it is seen that crude ethanolic extract and hexane, chloroform, ethyl acetate and methanol fractions demonstrated antibacterial activity in view of the results presented in MIC as active moderately against *S. epidermidis* and *S. aureus*.

The cytotoxicity of plant species varies according to tested concentrations and may be considered as promising in samples of crude ethanolic extract and in ethyl acetate fraction.

Studies such as these may be useful to guide researchers in new investigations about this plant. More depth and systematic studies about chemical compounds of this species should be performed. This study serve as subsidies for further development of herbal medicine with perspective in treatment of infected wound, as well as for other activities such as antitumor and anti larvicide.

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