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Isolation and identification of antibacterial components in seed extracts of *Argemone mexicana* L. (Papaveraceae)

Indranil Bhattacharjee, Soroj Kumar Chatterjee, Goutam Chandra*

Mosquito & Microbiology Research Units, Parasitology Laboratory, Department of Zoology, The University of Burdwan, Burdwan-713104, West Bengal, India

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ABSTRACT

Objective: The column chromatographic fractions of chloroform (CH1, CH2, CH3) seed extracts of *Argemone mexicana(A. mexicana)* were screened for antibacterial activity and phytochemical analysis. **Methods:** CH3 fraction was isolated and identified by column chromatography, thin layer chromatography, spectral data analysis and phytochemical screening were used for analysis. **Results:** CH3 fraction was significantly active at 4 to 64 mg/mL against *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* with minimum inhibitory concentration of 1.5625 to 3.125 mg/mL. The active fraction (CH3) revealed presence of alkaloid with retention factor value of 0.44. The active antibacterial agent in the most potent fraction (CH3) was isolated and identified as N-demethyloxysanguinarine by thin layer chromatography (TLC) and phytochemical screening. The CH1 and CH2 fractions did not show inhibitory activity. **Conclusions:** The results support the ethnomedicinal use of seeds of *A. mexicana* for treatment of bacterial diseases.

1. Introduction

Natural plants are extensively used as primary health remedies due to their pharmacological properties[1]. The investigation of the efficacy of plant-based drugs has been paid great attention because of their fewer side effects, lower cost and easier availability comparing with chemical medicine[2-4]. 80% of the world population still relies mainly on plant drugs[5]. Resistance to antibiotics has been the reasons of research for newer drugs to treat microbial infections[6].

Argemone mexicana (A. Mexicana) L. (Papaveraceae), commonly known as prickly poppy, is used as a medicinal plant in several countries. In Mexico, the seeds are considered as an antidote to snake venom. In India, the smokes of the seeds are used to relieve toothache. The fresh yellow, milky seed extract contains protein-dissolving substances, effective in the treatment of warts, cold sores, cutaneous infections, skin diseases, itches and also dropsy and jaundice[7].

Natural antimicrobial components in plants can inhibit the growth of bacteria by unknown mechanisms other than that of known antibiotics[8]. Resistance to almost all the known antibiotics has developed. For these reasons, we suggest the continuation of the search for newer antibiotics. In an earlier work we reported the methanolic extract of seeds of *A. mexicana* having higher activity than the aqueous extract on some selected bacterial isolates[7]. In present study, the *in vitro* antibacterial activity of the column chromatographic fractions of seed extracts of *A. mexicana* was confirmed. Subsequently, the chloroform extract of the seed was fractionated using column chromatography and only the fraction (CH3) which tested positively for antibacterial activity was further isolated and identified. This study would contribute to the development of plant based antibacterial drugs.

2. Materials and methods

2.1. Plant materials

The plant material used in this study consists of seeds of *A. mexicana*, collected from a village named Lakudi of Burdwan district (23° 16′ N, 87° 54′ E), WB, India during spring (mid-March to mid-April 2008) and taxonomically authenticated by Dr. Ambarish Mukherjee Department of Botany, The University of Burdwan. Burdwan, West Bengal, India. Voucher specimen (voucher no. 110) was deposited in the herbarium of the Department of Botany, The University of Burdwan, West Bengal, India. The seeds

*Corresponding author: Goutam Chandra, Ph.D., D. SC, Professor, Department of Zoology, Mosquito & Microbiology Research Units, The University of Burdwan, Burdwan-713104, West Bengal, India
Tel: +91- 9434573881
E-mail: goutamchandra63@yahoo.co.in

were initially rinsed with distilled water and dried on paper towels in laboratory at (37+1) ℃ for 24 h.

2.2. Test bacterial strains

Four bacterial strains were used during the study. Out of the four, three of the strains namely *Staphylococcus aureus* MTCC 2940, *Escherichia coli* MTCC 739 and *Pseudomonas aeruginosa* MTCC 2453 were obtained from Microbiology Laboratory of Burdwan Medical College, Burdwan, West Bengal, India and the remaining one, *Klebsiella pneumoniae* MTCC 432 was obtained from Institute of Microbial Technology, Chandigarh, India. The bacteria were grown in nutrient broth Hi-Media, M002 (Hi-Media Laboratories Limited Mumbai, India) at 37 ℃ and maintained on nutrient agar Hi-Media, M012 (Hi-Media Laboratories Limited Mumbai, India) slants at 4 ℃.

2.3. Other materials and chemicals

Most of the chemicals used were of analytical grade. Silica gel column (60 g, Si 60, 40-63 μm) was from Merck, Germany and commercially prepared TLC plate (Silica gel 60 F_{254} (Merck, Germany) plates, 20 × 20 cm, 1 mm thick) were used.

2.4. Extraction and fractionation procedure

The plant extract was fractionated using activity-guided fractionation with ethanol: water (1:1) and chloroform as described by Springfield and Weitz[9]. The powdered seed (120 g) extract was extracted with ethanol: water (1:1, 1500 mL) and separated at room temperature over night. The extract was filtered and partitioned in chloroform separately (750 mL) and clarified by further filtration. Evaporation of chloroform to dryness in an oven at 45 ℃ yielded 7.5% (w/w) residue. The procedure was repeated to obtain more residues. Residues obtained were reconstituted in ethanol: water and chromatographed over silica gel column. The fractions obtained were evaporated and the residues were screened for antibacterial activity.

2.5. Column chromatographic separation

The chloroform extracts were chromatographed over silica gel column and eluted with ethanol: water (1:1) as described by Brain and Turner[10]. The residues obtained were: CH1 (1.0 g), CH2 (1.6 g) and CH3 (2.7 g). The column fractions were tested for antibacterial activity against four bacterial isolates. The fraction(s) that exhibited significant activity was selected for minimum inhibitory concentration (MIC) and the bioactive compound(s) in the most potent fraction (CH3) was further isolated and identified through thin-layer chromatography (TLC) and phytochemical screening.

2.6. Isolation of alkaloids

This was done according to standard procedures of Trease and Evans[11], Nuhu *et al*, [12] with some modifications. One gram of the sample was extracted with 30 mL of methanol: water (1:1, v/v) mixture. It was filtered through Whatmann No. 1 filter paper and the filtrate evaporated. The resultant residue was mixed with 10 mL of 0.0025 M H_2SO_4 and partitioned with ether to remove unwanted materials. The aqueous fraction was basified with strong NH3 solution and then extracted with excess chloroform to obtain the alkaloidal fraction or separated by filtration. The chloroform extraction was repeated several times and the bulk extract concentrated to dryness. The purity of the substance was determines by means of the TLC [13].

2.7. TLC of isolated alkaloids

Ten microliters of the bioactive alkaloids were further fractioned by means of commercially prepared thin layer chromatography plates (Silica gel 60 F_{254} (Merck, Germany) plates, 20 × 20 cm, 1 mm thick) using acetone: water: 25% ammonium solution (90: 7: 3; v: v: v) as a mobile phase. Fifty milligrams of standard sanguinarine (98% pure) obtained from Sigma Aldrich Chemical Co., St Louis, MO, USA was dissolved in 45 mL of 50% ethanol and 10 μL was applied as a band beside the sample solution. After the plate was developed, the positions of the compounds were detected by spraying first with Dragendorffs reagent and then with 0.1 M H_2SO_4. It was inspected immediately in day light. The R_f values of the bioactive fractions in the sample and the standard were determined[14].

2.8. Analysis of the active ingredient

The bioactive fraction was subjected to spectral analysis by UV-Vis, IR and Mass spectroscopy. The UV-Vis analysis was carried out using (UV-1601 PC, Shimadzu spectrophotometer) with medium scan speed and sampling interval of 0.5 sec. The IR spectroscopy analysis of the active fraction was done using KBr plates (JASCO FT-IR Model- 420) with a scanning speed of 2 mm sec^{-1}. Mass spectroscopy was done using GCMS - Shimadzu -QP5050A with RT-1.3. All solvents and reagents used were of analytical grade and purchased from Merck, Mumbai, India.

2.9. Phytochemical screening

The phytochemical analysis of chloroform column chromatographic fractions of *A. mexicana* seed was done using standard procedures of Trease and Evans[11] and El-Olemyl *et al*, [14]. The extract was dissolved in absolute alcohol and chromatographed using pre-coated and pre-heated (100 ℃ for 30 minutes) glass plates (eight glass plates), which were prepared with silica gel G using Unpoplan coating apparatus (Shadon, London). After 5 minutes of drying, each of the plates was placed in the separate glass chamber for TLC analysis, with different solvent systems as the mobile phase. After the movement of solvent at the top of the plates, each plate was removed from the glass chamber and separately air-dried. After 10 minutes each of plates was sprayed with a different spraying reagent for the identification of appropriate phytochemical. The phytochemicals included in the study were sapogenins, steroid, terpenoids, flavonoids, alkaloid, essential oils and phenolics Qualitative test was carried out to indicate the presence of saponins (frothing test); whereas remaining phytochemicals were determined using TLC analysis by the application of suitable solvents and spray reagents and, in each case, R_f values were recorded.

2.10. Antibacterial activity

The antibacterial activity was done against test bacterial strains using hole-in-plate bioassay procedures as reported by Vlietinck *et al*, [15]. Pure cultures of the organisms were inoculated onto Muller-Hinton nutrient broth (Becton Dickinson, Cockeysville, Md), incubated for 24 h at 37 ℃, diluted with sterile nutrient broth to a density of 9×10^8 cfu/mL equivalent to Mc-

Table 1
Antibacterial activity of column chromatographic fractions of chloroform extracts of *A. mexicana* seed.

Fraction	Fractions concentrations (mg/mL)	Diameters of the inhibitory zones (mm)			
		Staphylococcus aureus	*Escherichia coli*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*
CH1	4	–	–	–	–
	8	–	–	–	–
	16	–	–	–	–
	32	–	–	–	–
	64	–	–	–	–
CH2	4	–	–	–	–
	8	–	–	–	–
	16	–	–	–	–
	32	–	–	–	–
	64	–	–	–	–
CH3	4	15.30±0.05	13.11±0.12	17.35±0.32	16.20±0.13
	8	22.08±0.13	21.20±0.17	23.10±0.08	25.10±0.08
	16	31.50±0.33	25.10±0.06	28.60±0.14	30.12±0.10
	32	38.32±0.12	32.40±0.32	33.14±0.17	35.40±0.15
	64	42.03±0.03	35.27±0.01	39.70±0.20	40.10±0.16
Water	–	–	–	–	–
Tetracycline	10	12.00 ± 0.17	13.03 ± 0.12	11.16± 0.18	15.40 ± 0.21

CH1 = Chloroform fraction 1, CH2 = Chloroform fraction 2, CH3 = Chloroform fraction 3.

Farland test tube number 3. The suspension was used to streak for confluent growth on the surface of Muller-Hinton agar plates with sterile swab. Using a sterile cork-borer of 6 mm diameter, four holes were made in to the set agar in petri-dishes containing the bacterial culture. Concentrations 4 to 64 mg/mL of the extracts were poured in to the wells. Tetracycline (10 mg/mL) was used as positive control. Antibacterial activity of N-demethyloxysanguinarine was also tested similarly. The plates were placed in the incubator at 37 ℃ overnight. Antibacterial activity was recorded if the zone of inhibition was greater than 9 mm. The significance of the difference of the antibacterial activities of the extracts was tested by one-way analysis variance (ANOVA).

2.11. Determination of MIC

The CH3 fraction of chloroform extract that showed significant activity ($P<0.05$) was chosen to assay for MIC. This was determined by the standard method of Wariso and Ebong[16]. Nutrient broth was prepared and sterilized using autoclave. One mL of the prepared broth was dispensed in to the test tubes numbered 2-12 using sterile pipette. A stock solution containing 1.0 g of the extract in 10 mL of de-ionized water was prepared. Then 1 mL of the solution was dispensed into each of the tubes numbered 1 and 2. Subsequently, from tube 2, serial dilution was carried out and 1 mL from tube 2 was transferred up to tube number 10 and 1 mL from tube 10 was discarded. Tube 11 was control for sterility of the medium and tube 12 for viability of the organisms. An overnight culture (inoculums) of each of the test isolates was prepared in sterile nutrient broth 1: 100 (102 dilution of the broth). From this dilution, 1 mL of the inoculum was transferred into each tube from tube 2 to tube 12 with exception of tube 11, to which another sterile nutrient broth was added. The final concentration of the sample in each of the test tubes numbered 1-10 after dilution were 100; 50; 25; 12.5; 6.25; 3.125; 1.5625; 0.78125; 0.390625 and 0.1953125 mg/mL, respectively. Tetracycline was used as positive control. All tubes were incubated at 37 ℃ for 24-48 h and examined for growth. The last tube in which growth failed to occur was the MIC tube.

2.12. Statistical analysis

The data of the study were subjected to one way ANOVA. Statistical analyses were done using SPSS ver. 11.0 software [17,18].

2.13. N-demethyloxysanguinarine

UV (EtOH), λ max (log ε), 222 (4.02), 283 (3.36), and 327 (4.13) nm. IR (KBr) ν max, 1665 (carbonyl group), 943, 1032 (methylenedioxyl group) cm^{-1}. ^{1}H-NMR (400 MHz, $CDCl_3$), δ 6.11 (2H, s, $C_{2\&3}$ -OCH_2O-), 6.30 (2H, s, $C_{9\&10}$-OCH_2O-), 7.14 (1H, s, H-4), 7.29 (1H, d, J = 8.8 Hz, H-11), 7.55 (1H, d, J = 8.8 Hz, H-5) , 7.65 (1H, d, J = 8.8 Hz, H-12), 7.82 (1H, d, J = 8.8 Hz, H-6), 7.86 (1H, s, H-1); EI-MS, *m/z* (rel. int. %): 333 (57) $[M]^+$, 322 (13), 248 (10), 161 (21), 69 (70), 55 (100), $C_{19}H_{11}NO_5$.

3. Results

The *in vitro* antibacterial activities of chloroform column chromatographic fractions of seeds of *A. mexicana* against four pathogenic bacteria were presented in Table 1. The chloroform extract fraction (CH3) of the seed has shown significant ($F=224.39$, $P<0.05$) inhibitory activity against the bacterial isolates tested at 4 to 64 mg/mL. Minimum inhibitory concentration (MIC) value of bioactive fraction (CH3) was presented in Table 2. The fraction indicated MIC of 1.5625 to 3.125 mg/mL against the isolates used. CH1 and CH2 did not show any inhibitory activity within that range of concentration on all the isolates used.

Isolation and identification of the active constituents (alkaloids) in CH3 fraction on TLC indicated R_f value of 0.44 for CH3 fraction and 0.42 for the reference standard. Fraction CH3 gave an orange color with Dragendorff's reagent indicating its alkaloidal nature and its Mass revealed a molecular ion peak $[M]^+$ at *m/z* 333 corresponding to the molecular formula $C_{19}H_{11}NO_5$.

The double bond equivalence was calculated as 15. The IR spectrum showed an absorption band at 1665 cm^{-1} indicating the presence of amide carbonyl[19]. The ^{1}H NMR

Table 2
Minimum inhibitory concentration of bioactive chloroform column chromatographic fraction (CH3) of *A. mexicana* seed.

Fractions concentrations (mg/mL)	*Staphylococcus aureus*		*Escherichia coli*		*Klebsiella pneumoniae*		*Pseudomonas aeruginosa*	
	A. mexicana	TC	*A. mexicana*	TC	*A. mexicana*	TC	*A. mexicana*	TC
100	−	−	−	−	−	−	−	+
50	−	−	−	−	−	−	−	+
25	−	−	−	−	−	−	−	+
12.5	−	−	−	−	−	+	−	−
6.25	−	−	−	+	−	−	−	+
3.125	−	+	−	+	−	+	−	+
1.5625	−	+	+	+	−	+	−	+
0.78125	+	+	+	+	+	+	+	+
0.390625	+	+	+	+	+	+	+	+
0.1953125	+	+	+	+	+	+	+	+
Water (Negative control)	−		−		−		−	

− = No growth of test organism, + = Growth of test organism, TC = Tetracycline

Table 3
Phytochemical analysis of chloroform column chromatographic fractions of *A. mexicana* seed.

Extract Fractions	Phytochemicals	Solvent used	Spraying reagent	R_f values	Conclusions
CH1				−	Absent
CH2	Sapogenins	Acetone–hexane (4:1)	Antimony chloride in concentrated hydrochloric acid	−	Absent
CH3				−	Absent
CH1				−	Absent
CH2	Alkaloids	Methanol–concentrated ammonium hydroxide(200:3)	Dragendroff	−	Absent
CH3				0.440	Present (+++)
CH1				0.800	Present (+)
CH2	Steroids	Chloroform	Libermann–buchard	0.720	Present (+)
CH3				0.640	Present (+)
CH1				0.974	Absent
CH2	Essential oil	Chloroform–benzene (1:1)	Vanillin–sulphuric acid	−	Absent
CH3				−	Absent
CH1				−	Absent
CH2	Flavonoids	Chloroform–acetic acid–water (90:45:6)	Saturated alcoholic sodium acetate	−	Absent
CH3				−	Absent
CH1				−	Absent
CH2	Phenolics	Ethyl acetate–benzene (1:1)	Folin reagent	−	Absent
CH3				−	Absent
CH1				−	Absent
CH2	Terpenoids	Impregnation with chloroform treated with silver nitrate	Antimony chloride in chloroform	0.910	Present (+)
CH3				−	Absent

showed six aromatic protons and two methylenedioxy groups at δ 6.11 and 6.30 (each 2H, s), suggesting the presence of benzophenanthridine nucleus[20]. The spectrum showed two peaks between δ 7.14 (1H, s, H–4) and δ 7.55 (1H, d, J = 8.8 Hz, H–5) and between δ 7.65 (1H, d, J = 8.8 Hz, H–12) and δ 7.82 (1H, d, J = 8.8 Hz, H–6), indicating that the two methylenedioxy groups were attached to C–2/C–3 and C–9/C–10. Comparing the NMR data of CH3 with those of oxysanguinarine[21], it was found that they are similar except for the disappearance of the signal attributed to N methyl group. Based on the results mentioned above, it was concluded that CH3 is N–demethyloxysanguinarine.

The *in vitro* antibacterial activity (diameters of the inhibitory zones) of N–demethyloxysanguinarine (4 g/mL) against *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* were (17.30±0.03) mm, (15.8±0.12) mm, (18.72±0.18) mm, (17.32±0.06) mm, separately. N–demethyloxysanguinarine also has antibacterial potentiality which is slightly higher than our active fraction (CH3) in the same concentration (4 mg/mL). Phytochemical analysis of chloroform column chromatographic fractions of *A. mexicana* seeds were presented in Table 3. Presence of alkaloids in the active fraction (CH3) indicates that it is the principal antibacterial agent in the seed extract of *A. mexicana*.

4. Discussion

The increasing use of antibiotics and indiscriminate use by over–prescribing and poor patient compliance have led to the development of bacterial resistance to antibiotics [22]. This condition has forced scientists to search for new antimicrobial substances from various sources[3, 23–31]. The antibacterial properties of seed extracts of *A. mexicana* were shown to be an interesting field for application in pharmaceutical

industry[7]. The results support the view that *A. mexicana* is a potent antibacterial agent.

The results of the present study showing significant (P<0.05) inhibition of the bacterial isolates by the CH3 fraction only have really contradicted assertion. It is probable that the bioassay guided fractionation employed in the extraction, is responsible for the inactivity of the CH1 and CH2 chloroform fractions. The MIC studies show that *A. mexicana* chloroform extract (CH3) fraction was the most potent against the pathogenic bacteria employed at very low doses. Hence, this re-validates its pharmacological and therapeutic potentials. This underscores the ethnobotanical evidence for the selection of *A. mexicana* in the discovery of new array of bioactive compounds.

Present findings of low MIC values are comparable to those of Subramanian *et al*, [31] who indicated ethanolic extract of Aloe vera leaf, although a different family, with antibacterial activity against pathogenic bacteria at very low doses.

The mechanism of action of the active components (CH3) may be due to the pore formation in the cell wall and the leakage of cytoplasmic constituents by the active components (alkaloids) present in the seed extract[32]. It could also be by inhibition of nucleic acid, protein and membrane phospholipids biosynthesis[33]. It can be concluded that seed extract of *A. mexicana* have compounds with antibacterial potentials that could be harnessed for drug development.

Conflict of interest statement

We declare that we have no conflict of interest

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