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Antibacterial activity of *Argemone mexicana* L. against multidrug resistant *Pseudomonas aeruginosa*, isolated from clinical samples

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ABSTRACT

Objective: To monitor the antipseudomonad activity of the weed *Argemone mexicana* (*A. mexicana*), with multidrug strains isolated from clinical samples. **Methods:** Antibiogram of isolated strains were done with disc diffusion method and antipseudomonad activity was monitored with the agar well diffusion method. **Results:** Twenty seven strains of *Pseudomonas aeruginosa* (*P. aeruginosa*) were isolated from clinical samples from a hospital; among them, 22 were resistant to antibiotics (μg/disc): cefotaxime-30, 16 to amoxyclav-30, 15 to ofloxacin-5, 13 to gentamicin-10, 10 to piperacillin-100/tazobactam-10, 8 to amikacin-30, 7 to gatifloxacin-30, 6 to netilmicin-30, 4 to piperacillin100, 3 to imipenem-10 and 3 strains to nitrofurantoin-300. Each strain was resistant to several antibiotics at specified levels. Of these 27 clinical strains, 15 antibiotic-resistant strains and a antibiotic-sensitive standard strain were used in monitoring antimicrobial activity of leaf-extracts using 3 organic solvents (acetone, methanol and ethanol) and water of the weed, prickly poppy (*A. mexicana* L.). The methanol-extract had the highest level of antipseudomonad activity both with cold and hot extracts, confirmed by separate Kruskal-Wallis *H* tests. With the Student's *t*-test it was ascertained that the hot extraction concentrate yielded promising antipseudomonad activity than the cold extraction with methanol. Values of minimum inhibitory concentration (MIC) of extracts of *A. mexicana* using acetone, methanol and ethanol as solvents were 10, 8 and 8 mg/mL, respectively; corresponding values of minimum bactericidal concentration (MBC) were 32, 28 and 24 mg/mL for these solvents, respectively. **Conclusions:** This study suggests that *A. mexicana* leaf can be used as complementary medicine in treating diseases caused by multidrug resistant strains of *P. aeruginosa*.

1. Introduction

In recent years, it is found that some drug-resistant pathogenic bacteria are causative organisms of premature death or a long-standing infection with a disproportionately large number of cases[1]. As it is known, the emergence of multidrug resistant (MDR) strains of pathogens is a natural process and indiscriminate uses of antibiotics (the broad-spectrum ones, particularly), induce the development of MDR bacterial strains covertly at a frenetic pace[2]. Furthermore, apart from the use for human, antibiotics are equally used in agriculture and the management of livestock, which cause the emergence of an avalanche of antibiotic resistant pathogens. For example, resistant strains of *Staphylococcus intermedius* (*S. intermedius*), *Campylobacter* sp., *Salmonella* sp. and *Escherichia coli* (*E. coli*) have been found in pet- and food- animals, as well as their owners[3]. Indeed, older/earlier susceptibility ranges of pathogens to antibiotics get changed/evolved, due to continual updating of genomes of pathogens due to natural process of mutation/genetic recombination, insinuating appalling incarnations of MDR pathogens to the health domain[4], probably for the fact that microbial evolution is a fast process and the presence of a new antibiotic in an microbial mini-cosmos acts as the major factor for the 'positive selection pressure'. Moreover, microbes do not get eliminated completely from any ecological niche[5], not surprisingly perhaps, a fraction of population always survives with some specific survival mechanism[6]. If the surviving fraction of cells escapes to the environment, re-infection by a pathogen is usual, and favourable situations for nosocomial infection for a pathogenic bacterium are

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myriad[6]. In succinct, the mechanism of arrival of resistant pathogens is as follows: once a population of a bacterium reaches 10^7 cfu, chance mutation yields at least a single cfu that is resistant to any antimicrobial drug[4], resulting in a smattering proportion of the population that has probably undergone the first-step mutation, indicating a low-level resistance. The infection might be controlled; nevertheless surviving (resistant) isolates face less competition and can have unimpeded proliferation[8,9]. In healthy individuals in whom infection has been controlled, the emergence of these first-step mutants may not be an issue. But in moribund patients with immune-suppression and unhealed injuries, however, mutants emerge as a new population.

A mutant preventive concentration (MPC) or the highest point in minimum inhibitory concentration (MIC)-range of the isolates, of the drug necessary to prevent (or inhibit) the emergence of the first-step mutants is not prescribed, normally to evade non-target host toxicity[9]. However, synergistic treatment of two drugs helps prevent emergence of resistant mutants, notwithstanding, however the antagonistic situation wherein one of the drugs would partially inhibit the other, resulting in the rapid emergence of resistant mutants, at times[10]. As new MDR strains of pathogens arriving at the community cause hazards in managements of infectious diseases, there are searches for alternate antimicrobial substances to control MDR pathogens from several popular sources including medicinal plants. An overview on work published on antimicrobial activity of plants[11] indicated that a limited work[12] is reported against MDR pathogenic bacteria, especially isolated from clinical samples.

Particularly, the poisonous-weed, prickly poppy or *Argemone mexicana* (*A. mexicana*) L. (Papaveraceae), an analgesic, antispasmodic, possibly hallucinogenic and sedative weed-plant that is known to lend itself as a traditional healing agent in treatments of malaria, warts, cold sores, skin diseases, itches and a few more[12]. Scientifically, leaf-extracts of *A. mexicana* were used to examine antibacterial potentiality against several pathogenic bacteria: National Collection of Type Cultures (NCTC) strains (drug-sensitive strains) of *Staphylococcus aureus* (*S. aureus*), *E. coli*, *Proteus* sp., *Klebsiella pneumoniae* (*K. pneumoniae*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) [13]. Antimicrobial activities of extracts of *A. mexicana* with solvents, petroleum ether, benzene, chloroform, methanol and ethanol on NTCC cultures (wild strains) of *E. coli*, *K. pneumoniae*, *Proteus mirabilis* (*P. mirabilis*), *P. aeruginosa*, *Salmonella typhi* (*S. typhi*), *S. paratyphi*, *Shigella sp.* and *S. aureus* were reported[14]. Till date, pathogenic bacteria on which antimicrobial activity of plants have been described were not used for any antibiotic profiling. Therefore, it could not be confirmed whether those studies were done on MDR strains of pathogens. Several other reports on the antibacterial activity of *A. mexicana* using against pathogenic and non-pathogenic bacteria are described[15]. None of these reports record any antibiotic-susceptibility test of used bacteria. Further, the literature on plant extracts including *A. mexicana* against *P. aeruginosa* does not record any work on multidrug resistant strain. Obviously, the accumulated literature on plants as sources of anti-microbial agents record a lot of work on wild or drug-sensitive strains of pathogens[16].

This work describes antimicrobial activity of leaf-extracts of *A. mexicana* on 27 clinically isolated strains of MDR *P. aeruginosa* and a standard strain, which is sensitive to all antibiotics. Further, it is recognized as a dangerous pathogen owing to its resistance to many antibiotics and its capacity to acquire further resistance against progressively newly introduced antimicrobial agents[17,18]. This organism is reported to be responsible for a local nosocomial infection[19]. Paradigmatically, this work is a study of antimicrobial activity of a poisonous plant against a clinically isolated pathogen.

2. Materials and methods

2.1. Preparations of plant extracts

For cold extracts, 3 solvents (non-polar to polar solvent, in the order) namely, acetone, methanol and ethanol were used individually for 3 lots of powder-mass of leaves of *A. mexicana*. A lot of 20 g of powder-mass in an aliquot of 200 mL of a solvent (cold) was dissolved and stored at 4 ℃ for 48 h. After filtration, extracts were concentrated by a rotary evaporator for sticky-mass that were 80, 100, 80, 60, 110, 160, 200 and 180 mg, using individually 8 solvents, chloroform, ethyl acetate, acetone, dichloromethane, petroleum ether, methanol, ethanol and water, respectively. Each concentrated sticky mass was stored in 10% dimethyl sulfoxide (DMSO) at −4 ℃ till use. Of these 8 extracts, only plant extracts with cold solvents using acetone, methanol and ethanol exhibited distinct zones of inhibition on Mueller-Hinton agar (MHA) against *P. aeruginosa*. Further, these 3 solvents only were used for hot extraction. A sample lot of 40 g of the shade-dried powder of *A. mexicana* leaves was extracted in a soxhlet extractor for 40 siphons or cycles, with an aliquot 400 mL acetone, until colorless extract was obtained on the top of the extractor. Repetitions of extraction were done successively with methanol (40 siphons) and ethanol (40 siphons) with the same sample lot. Individual extracts were filtered and were concentrated with a rotary evaporator, run at 40 ℃ to get sticky-masses that were weighed and found 0.2, 0.6 and 0.8 mg from extracts using acetone, methanol and ethanol, respectively. Those were stored with 10% DMSO solution.

2.2. Isolation and identification of the bacterium

Antibiotic-sensitive *P. aeruginosa* NCTC strain no. 10662

was used in the study. Little lots of different clinical samples were cultured in suitable nutrient agar, for 18-24 h for isolation and concomitant identification of *P. aeruginosa*. Non-lactose-fermenting colonies were formed on MacConkey-agar, which too formed green-pigmented colonies on further streaking on nutrient agar (Himedia, Mumbai) indicated presence of *P. aeruginosa*. To confirm the presence of *P. aeruginosa* in clinical samples only and growing colonies were not from any nosocomial infection, repeated clinical samples were plated. Biochemical identification tests for confirmation of isolated strains were done for *P. aeruginosa* as described previously[19].

2.3. Antibiotic sensitivity test

Fifteen isolated strains and the standard strain of *P. aeruginosa* were subjected to antibiotic sensitivity test by the disc diffusion method, following the standard antibiotic susceptibility chart of National Committee for Clinical Laboratory Standards (NCCLS) guidelines, as described[19].

2.4.1. Antibacterial activity tests

Antibacterial activity tests were performed both by agar-well diffusion and disc diffusion methods. For the agar-well diffusion method[20], bacterial lawn was prepared as described previously[19], but the agar was 6 mm thick. Wells (6 mm depth) were punched in 30 min old agar lawn and each well was based by 50 μL molten MHA medium. Further, wells were filled with 100 μL aliquots of 30 mg/mL solvent-extracts of *A. mexicana* (diluted from the original stock by 10% DMSO to 30 mg plant extract/mL). Plates were incubated at 37 ℃ for 24 h. Antibacterial activities were evaluated by measuring the diameter of zone of inhibition. It was confirmed that 10% DMSO had no inhibitory effect on the bacterium. Gentamicin 40 μg/mL was taken as the reference-control.

2.4.2. Disc diffusion method

An aliquot of 10 μL plant extract (50 mg/mL) were soaked by sterile filter paper discs (5 mm diameter). Sterile filter paper discs were impregnated with 10 μL of plant extract placed on the surface of the medium and incubated at 37 ℃ for 24 h. The assessment of antibacterial activity was based on the value of diameter of the inhibition zone formed around the disc[20]. Gentamicin 10μg/disc was taken as the reference control.

2.5. Determinations of MIC and MBC

Original stock solutions of plant extracts prepared with acetone, methanol and ethanol (hot extracts) were 150, 200 and 200 mg plant extract/ml, respectively in 10% DMSO solution. Separate experiment was conducted for each solvent-extract. An aliquot of 80 μL of a suitable dilution of a solvent-extract was released to a well on a 96 welled (12×8) micro-titer plate along with an aliquot of 100 μL nutrient broth, an aliquot of 20 μL bacterial inocula (10^9 cfu/mL) and a 5 μL-aliquot of 0.5% 2,3,5-triphenyl tetrazolium chloride (TTC). After pouring all the above to a well, the microplate was incubated at 37 ℃ for 18 h. A pink colouration due to TTC in a well indicated bacterial growth and the absence of any colour was taken as inhibition of bacterial growth. The MIC value was noted at the well, where no colour was manifested. Further, bacteria from each well of the microplate were sub-cultured on a nutrient agar plate; the level of dilution, where no bacterial growth on the nutrient agar plate was observed, was noted as the MBC value.

2.6. Phytochemical screening

Extracts of leaves of *A. mexicana* using ethanol, methanol and acetone were subjected to various chemical tests in order to determine the secondary plant constituents:

2.6.1. Test for reducing sugars

To an aliquot of 2 mL of any extract, an aliquot of 5 mL of a mixture (1:1) of Fehling's solution I and II was added and the mixture was boiled for 5min; a brick-red precipitate indicated the presence of free reducing sugars[21].

2.6.2. Test for the presence of anthraquinones

An aliquot of 0.5 mL of the extract was shaken with 10 mL of benzene, filtered and an aliquot 5 mL of 10% ammonia solution was added to the filtrate and the mixture was shaken, the presence of a pink, red or violet colour in the ammoniac (lower) phase indicated the presence of anthraquinones[22].

2.6.3. Test for saponins

An aliquot of 0.5 mL of an extract was dissolved in an aliquot of 10 mL of distilled water in a test-tube was shaken vigorously for 30 s and then allowed to stand for 45 min. The appearance of a frothing, which persists on warming indicated the presence of saponins[21].

2.6.4. Test for flavonoids

To a portion of the dissolved extract, a few drops of 10% ferric chloride solution were added. A green or blue colour indicated the presence of flavonoids[21].

2.6.5. Test for steroids/terpenes

A lot of 500 mg of the extract from the rotary evaporator was dissolved in an aliquot of 2 mL of acetic anhydride and cooled at 0 to 4 ℃, to which a few drops of 12 N sulphuric acid were carefully added. A colour change from violet to blue-green indicated the presence of a steroidal nucleus[23]

2.6.6. Test for tannins

A fraction of 0.5 g of the extract was dissolved in 5 mL of water followed by a few drops of 10% ferric chloride. A blue-black, green, or blue-green precipitate would indicate the presence of tannins[21].

2.6.7. Test for alkaloids

A lot of 0.5 g of ethanol extract (from rotary evaporator) was stirred with an aliquot of 5 mL of 1% HCl on a steam bath and filtrated; to an aliquot of 1 mL of the filtrate, a few drops of Mayer's reagent was added, and to another aliquot of 1 mL of the filtrate, a few drops of Dragendorff's reagent were added. Turbidity or precipitation in tubes due to either of these reagents indicated the presence of alkaloids in the extract[21].

2.6.8. Test for resins

To an aliquot of 10 mL of the extract an aliquot of 10 mL of cupper acetate solution 1% was added and shaken vigorously and, a separate green colour indicated the presence of resin[23].

2.6.9. Test for glycosides

An aliquot of 5 mL of each extract was mixed with an aliquot of 2 mL of glacial acetic acid (1.048-1.049 g/mL), one drop of ferric chloride solution (1%), and mixed thoroughly. To this mixture, an aliquot of 1 mL of 12 N H_2SO_4 was added. A brown ring at the interface indicated the presence of glycosides[21].

3. Results

3.1. Antibiotic sensitivity of clinical isolates of P. aeruginosa

Twenty seven strains of *P. aeruginosa* were obtained from clinical samples and each strain was resistant to 11 antibiotics at levels, monitored by disc diffusion

Table 1.
Resistant patterns of clinical isolates of *P. aeruginosa* collected in Sum hospital.

S.No	Antibiotic sensitivity pattern of 27 isolates			
	Antibiotic (μg/disc)	Number of sensitive isolates	Number of resistant isolates	Percentage of resistant isolates
1	Amoxyclav 30	11	16	59.2
2	Gentamicin 10	14	13	48.1
3	Amikacin 30	19	8	29.6
4	Netilmicin 30	21	6	22.2
5	Nitrofurantoin 300	24	3	11.1
6	Gatifloxacin 30	20	7	25.9
7	Cefotaxime 30	5	22	81.1
8	Piperacillin 100	23	4	18.5
9	Piperacillin 100 /Tazobactam 10	17	10	37.0
10	Imipenem 10	24	3	11.1
11	Ofloxacin 5	12	15	55.5

Note: Out of 27 clinical isolates, 13 were from urine, 9 from pus, 2 from wound swab, 2 from sputum and 1 from pleural fluid. Antibiotic discs were from HiMedia (Mumbai).

Table 2.
Detailed record of antibiotic sensitivities of 8 antibiotic discs against lawns of 15 clinical isolates (taken from 27 isolates) of *P. aeruginosa*.

Strain number	AK 30	P100/T10	I 10	NT 30	OF 5	GF 30	CE 30	AC 30
1	R	R	S	R	R	R	R	R
2	R	MS	S	R	R	MS	MS	R
3	R	MS	S	R	R	MS	MS	R
4	S	S	S	S	R	MS	MS	R
5	R	R	S	R	R	R	R	R
6	MS	MS	S	MS	MS	S	R	R
7	R	MS	S	S	R	R	R	S
8	S	MS	S	S	S	S	S	S
9	MS	S	S	MS	R	S	S	S
10	MS	MS	MS	MS	R	MS	R	R
11	R	MS	S	MS	R	R	R	R
12	R	MS	MS	MS	R	R	R	R
13	MS	R	MS	R	R	R	R	R
14	MS	R	R	R	R	R	R	R
15	S	R	MS	R	MS	S	R	R
NCTC	S	S	S	S	S	S	S	S

Note: Abbreviations: amikacin (AK), piperacillin /tazobactam (P/T), imipenem (I), netilmicin (NT), ofloxacin (OF), gatifloxacin (GF), cefotaxime (CE) and amoxyclav (AC). Values given against each antibiotic are in μg/disc. R = resistant (no inhibitory zone); S = sensitive; MS = moderately sensitive, according to NCCLS guidelines.

Table 3.
Antibacterial activities of cold extracts of *A. mexicana* using different solvents against *P. aeruginosa* monitored by the agar-cup method, presented as zone of inhibition in mm.

Strain number	Acetone	Methanol	Ethanol	Gentamicin 40 μg/mL
1	12.0	18.0	13.0	15.5
2	10.0	17.0	12.5	19.0
3	13.0	19.0	13.0	17.0
4	12.0	18.0	13.0	19.0
5	12.0	18.0	14.0	16.0
6	11.0	17.0	13.0	18.0
7	12.0	17.0	12.0	17.5
8	10.0	19.0	13.5	21.0
9	13.0	18.0	12.5	22.5
10	12.0	17.5	14.0	19.0
11	12.0	17.0	13.0	19.5
12	11.0	16.5	12.0	20.0
13	12.0	17.0	12.0	15.5
14	10.0	18.0	13.5	15.0
15	13.0	17.0	12.5	18.0
NCTC	14.0	19.0	15.0	23.0
Total rank signs*	166.5	747.0	341.5	800.0

Note: An aliquot of 100 μL of 30 mg plant extract/ml of each solvent individually was given in each agar cup. Gentamicin 40 μg/mL was the reference control. *Kruskal-Wallis *H* test was applied; the *H* value is 46.62; the effectivity was in the order: gentamicin>methanol> ethanol>acetone, as evident from 'total rank signs'. Data of the third repeated experiment are presented.

Table 4.
Antibacterial activities as size of zones of inhibition in mm by hot solvent extracts of *A. mexicana* against clinical isolates of *P. aeruginosa* monitored by the agar cup method and the disk diffusion method.

Strain number	Acetone	Methanol	Ethanol	Gentamicin 40 μg/mL (10 μg/disc)
1	14.0(9.0)	19.0(14.0)	15.0(11.0)	15.5(17.0)
2	13.0(10.0)	18.0(13.0)	13.0(11.0)	19.0(16.5)
3	11.0(8.0)	21.0(17.0)	14.0(10.0)	17.0(16.0)
4	12.0(8.0)	18.0(15.0)	15.0(12.5)	19.0(16.0)
5	10.0(8.5)	19.0(14.0)	14.0(10.0)	16.0(15.0)
6	11.0(9.0)	18.0(13.0)	13.0(9.0)	18.0(18.0)
7	10.0(10.0)	19.0(14.0)	12.0(9.5)	17.5(17.5)
8	13.0(8.0)	20.0(15.0)	13.0(9.0)	21.0(19.0)
9	12.0(9.0)	18.0(13.5)	12.5(10.0)	22.5(20.5)
10	12.0(11.0)	19.0(12.0)	13.0(9.0)	19.0(19.0)
11	12.0(10.0)	17.0(12.5)	12.5(10.0)	19.5(19.5)
12	12.0(10.0)	19.0(13.0)	13.0(9.0)	20.0(18.0)
13	13.0(8.5)	17.0(12.0)	14.0(10.0)	15.5(15.5)
14	11.0(10.0)	18.0(11.0)	14.0(10.0)	15.0(15.0)
15	10.0(10.0)	19.0(14.5)	13.0(9.0)	18.0(17.0)
NCTC	14.0(8.5)	19.0(12.0)	15.0(11.0)	23.0(21.0)
Total rank signs*	178.5(218.5)	784.5(664.0)	354.0(331.0)	764.5(896.0)

Note: see the foot note of Table 3. Numbers in parenthesis are values from the disk diffusion method for which 10 μg/disk gentamicin was used. *Kruskal-Wallis *H* test was applied; the *H* value is 49.7 for the data from the agar cup method; similarly the *H* value is 57.18 for the data from the disk diffusion method. The effectivity is in the order: methanol> gentamicin>ethanol>acetone for the agar cup method, while the order was gentamicin>methanol> ethanol>acetone for the disk diffusion method. Data of the third repeated experiment are presented.

method, detailed in Table 2. Among the isolated 27 strains, cefotaxime-resistant isolates of the bacterium were the maximum (81.5%) and imipenem-resistant and nitrofurantoin-resistant isolates were the minimum (11.1%); in other words, 22 strains were resistant to cefotaxime 30 μg/disc and 3 strains were resistant to imipenem 10 μg/disc and nitrofurantoin 300 μg/disc, individually (Table 2). In summary, the order of numbers of isolated strains is (μg/disc): cefotaxime-30> amoxyclav-30> ofloxacin-5> gentamicin-10> piperacillin-100/tazobactam-10> amikacin-30> gatifloxacin-30> netilmicin-30> piperacillin-100> imipenem-10 or nitrofurantoin-300. Maximum number of clinical isolates was from urine and minimum number from pleural fluid (Table 2).

A strain sensitive to all antibiotics, *P. aeruginosa* NCTC no. 10662 was used as the standard strain in parallel to 15 clinically isolated strains of originally obtained 27 strains, in the study of monitoring antipseudomonal activities of plant extracts; 8 types of antibiotic discs were tested against individual clinical isolates, *i.e.*, nitrofurantoin, gentamicin

Table 5.
Preliminary phytochemical analyses of 3 extracts of leaves of *A. mexicana*.

Properties	Acetone	Methanol	Ethanol
Colour, pH	Light green, 6.4	Brownish-red, 5.2	Greenish-black, 4.92
Reducing sugar	−	−	+
Anthraquinone	−	+	−
Flavonoids	+	+	+
Saponins	+	+	−
Tannins	+	+	+
Sterols/terpenes	+	+	+
Alkaloids	+	+	+
Resins	−	−	−
Glycosides	+	+	−

and singular piperacillin were not used (Tables 3). Resistance of clinical isolates to used antibiotics could be arranged in the decreasing order of numbers of resistant strains among 15 isolates (μg/disc): amoxyclav-30> ofloxacin-5> gentamicin-10> piperacillin-100/tazobactam-10> amikacin-30> gatifloxacin-30> netilmicin-30. Strains moderately sensitive (MS) to selected antibiotics were also observed (Table 2).

3.2. Antibacterial activity tests

Anti-pseudomonad activities of 8 solvent-extracts using chloroform, ethyl acetate, acetone, dichloromethane petroleum ether, methanol, ethanol and water (non-polar to polar solvent in the order) were used by the agar-well diffusion method on lawns of 15 bacterial isolates and the NCTC strain. It was found that cold solvent-extracts, when tested against 16 *Pseudomonas* strains, the following solvent-extracts: chloroform, ethyl acetate, dichloromethane, petroleum ether and water had no zone of inhibition. But, rest three cold-extracts using acetone, methanol, and ethanol had prominent antibacterial activities (Table 3). Kruskal-Wallis *H* test was applied to the dataset of antibacterial activity cold-extracts with acetone, methanol and ethanol. It was found that Kruskal-Wallis *H* value was computed as 46.62, when tabulated H values are 5.99 at *P*=0.05 and 9.21 at *P*=0.01, for degree of freedom (df), 4 extracts minus 1 = 3. Since tabulated values are far less than the computed H value, both at *P*=0.05 and 0.01, the null hypothesis that there is no difference between inhibitory zones due to 3 cold solvent-extracts is outright rejected. In other words, differences in values of zones of inhibition of individual 3 cold-solvent-extracts are highly significant (Table 3). Secondly, the 'total rank signs' recorded in Table 4 for the methanolic extract is 747, against similar values due to acetone and ethanol as 166.5 and 341.5, respectively, while that of gentamicin 40 μg/mL was 800. These clearly indicated that cold methanol was the most suitable solvent for *A. mexicana* in cold extraction (Table 3), next to gentamicin, the control. Similar result of the highest antimicrobial activity of methanol was also obtained in the hot extraction with Kruskal-Wallis *H* value as 49.7 by the agar-well diffusion method (Table 4); the differences in values of zones of inhibition among 3 hot solvent-extracts were statistically significant both at *P*=0.05 and 0.01, as computed *H* value is far more than tabulated values, 5.99 or 9.21 (as mentioned above for *df* =3). The 'total rank signs' are recorded in Table 4 of data of agar-well diffusion and disc diffusion methods for methanolic, acetone or ethanol extracts along with gentamicin 40 μg/mL, again confirming that gentamicin was better than methanolic extract. Thus, as with cold extracts in hot extraction also methanol-extract was the most effective against *P. aeruginosa* strains. Student's *t* test was performed between 'cold versus hot extraction data' of values of zones of inhibition. As the calculated *t*=2.192 is more than the tabulated *t*=2.05, at *df*=28 (15+15-2), at *P*=0.05, the difference between values of zones of inhibition of cold extract and hot extract with methanol was statistically significant, at *P*=0.05. Thus, the hot methanol extraction caused higher values of zones of inhibition of all strains than the cold methanol extraction with *A. mexicana*, monitored in the agar-cup method.

The data from the disc diffusion method with hot extracts simply confirmed the results of data of agar-well diffusion method given in Table 4. The Kruskal-Wallis *H* value was similarly computed as 57.18 for the data from the disc diffusion method. In conclusion, methanol-extract was the most effective against *P. aeruginosa* strains, when antipseudomonad activity was monitored in cold/hot methanol extraction, by agar-well diffusion/ disc diffusion method.

3.3. Determinations of MIC and MBC

The hot acetone extract yielded the MIC value of 10 mg/mL on the NCTC strain and 15 clinical isolates. But MIC values of these 16 strains due to methanol and ethanol extracts were found to be same, that is 8 mg/mL. MBC values (or lethal concentration 100) for all the 16 strains of *P. aeruginosa* were 32, 28 and 24 mg/mL in response to acetone, methanol and ethanol extracts (hot extraction), respectively.

3.4. Phytochemical analysis

During phytochemical analysis of acetone, methanol and ethanol extraction of *A. mexicana* was found that flavonoids, tannins, sterols/terpenes and alkaloids were present in all the 3 types of extract. The results of other tests for colour, pH, reducing sugar, anthraquinone, saponins, resins and glycosides are summarized in Table 5.

4. Discussion

P. aeruginosa is a well-known opportunistic bacterial pathogen contaminating inanimate articles of hospitals such as, sinks, drains and many medical equipments, thereby it is marked as the notorious one in nosocomial spread, and even it is found from soil, but is rare in clinical isolates of healthy individuals, as it is known[6,24]. Moreover, antibiotics used so for against any bacterial pathogen including *P. aeruginosa* are from microbial sources. Gradually, the target bacterium develops resistance to those antibiotics readily and survives. Moreover, crude drugs from eukaryotic systems (as plants) have an array of compounds against which resistance can never be developed in any pathogen. In fact, eukaryotic compounds in crude extracts when employed against a pathogen, a synergistic effect is achieved with the eventual control of the pathogen; that is how certain plants are reported to be highly successful and popular in managing infections in 'traditional health care systems', worldwide. By the by, the development of scientifically un-approved drugs has proliferated so much that the plant-based crude-medicine trade in local and international markets is popular, worldwide[11]. Studies on crude phyto-extracts in monitoring of antimicrobial activities of plants are of practical importance. Herein, the efficiency of *A. mexicana* in the control of several types of infections in the age-old 'low-cost health-care module' of marginalized and poverty-stricken aborigine-folklore of India in crude plant extract[11-13]; *A. mexicana* belongs to the plant-family, Papaveraceae to which another the most successful medicinal plant, *Papaver somniferum*, (yielding morphine and many more) belongs, in controlling in vitro MDR strains of *P. aeruginosa*. Reminded that it is the causative organism in bacteremic form of pneumonia of aged and immune-compromised patients in bringing 80% mortality[24]. As flavonoides, tannins, sterols/terpenes and alkaloids were found to be present in acetone, methanol and ethanol extraction of *A. mexicana*, the recorded antimicrobial results must be due to above cited phytochemicals.

Most antibiotics showing antipseudomonal activity belong to the β-lactam and aminoglycoside groups. In fact, *P. aeruginosa* is found to be resistant to antibiotics of β-lactam derivatives used for control, due to low outer membrane permeability affording intrinsic resistance; β-lactam molecule penetrates outer membrane of the bacterium at rates lower than that of *E. coli*[25]. However, several third generation cephalosporin and fluroquinolones derivatives such as, the ciprofloxacin have been proved to be useful[26]. Among the β-lactam derivatives with antipseudomonal activity, piperacillin (acylureido penicillin), imipenem (a semi-synthetic derivative of thienamycin, carbapenems), cefotaxime (cephems, first generation cephalosporin) were found ineffective for *P. aeruginosa*. Further, the transfer of resistances to both carbenicillin and gentamicin to a sensitive strain of *P. aeruginosa* was attributed to R-plasmid mediated transfer[27]. Moreover, protein synthesis inhibiting aminoglycosides are gentamicin, amikacin and tobramycin that are commonly used against *P. aeruginosa*[27]. In the present study, *P. aeruginosa* is resistant to gentamicin and amikacin. Records on *P. aeruginosa* offering resistances to many antibiotics, by production of antibiotic-inactivating enzymes and/or alteration of target sites are available[26]. It has been shown that in an acute toxicity test of exposing the ethanolic extract of *A. mexicana* to live mouse, the lethal concentration 50 (LC_{50}) was 400 mg/kg body weight, typically in a study[28].

It has been reported that in the ethanolic extract of *A. mexicana* flavonoids, tannins, sterols, terpenes, alkaloids and some reducing sugar were present, while anthraquinone, saponins and resins were absent[29]. But, this study records presence of flavonoids, tannins, sterols/terpenes and alkaloids in three types of plant extracts and these constituents are expected to be effective in the present anti-pseudomonad activity. The effectivity of ethanolic extract on antipseudomonal activity in the present study was found medium (compared to that of methanolic extract), probably due partial extraction of phytochemicals by ethanol in the successive extraction procedure followed. This study of antibiogram of clinical isolates of *P. aeruginosa* should be helpful to establish appropriate treatment regimen in a situation of changing epidemiology of the organism. More particularly, corneal infections are rapidly progressive and require immediate appropriate chemotherapy for control of the infection[29]. Furthermore, the basis of ethnotherapeutic use of this nearly poisonous plant against infectious diseases as reported often from various countries is found suitable for control of *P. aeruginosa*. This organism is found in infections of skin sometimes with bacteria laden fluid lesions, subcutaneous nodules, metastatic abscess, bacillary endocarditis, pneumoniae, cystic fibrosis, recurrent urinary tract infections, musculoskeletal infections and meningitis[6]. As discussed, of these, the mortality rate from bacteremic form of pneumonia is approximately 80%[24]. This study would possibly help development of some avant-garde drug for the control of *Pseudomonas*, especially for MDR strains, from *A. mexicana* with methanolic extracts.

Conflict of interest statement

We declare that we have no conflict of interest.

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