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Evaluation of the Antimicrobial Activity of Ammoniacum Gum from *Dorema ammoniacum*

M. Rajani[1], N. Saxena[2], M.N. Ravishankara[1], N. Desai[2] and H. Padh[2]

[1]Pharmacognosy and Phytochemistry Department and [2]Microbiology Department, BV Patel Pharmaceutical Education & Research Development (PERD) Centre, Thaltej, Ahmedabad, India

Abstract

Antimicrobial activity of the dichloromethane-methanol (1 : 1) extract of ammoniacum gum (from *Dorema ammoniacum* D. Don) was evaluated against 14 microorganisms which included seven Gram-positive bacteria (*Bacillus cereus*, *Bacillus pumilus*, *Bacillus subtilis*, *Micrococcus luteus*, *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Streptococcus faecalis*), four Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aereuginosa*, *Klebsiella pneumoniae* and *Bordetella bronchiseptica*), one yeast (*Saccharomyces cereviseae*) and two fungi (*Aspergillus niger* and *Candida albicans*). The extract of ammoniacum gum exhibited a of broad spectrum antimicrobial activity by inhibiting all the seven Gram-positive bacterium, one Gram-negative bacterium, one yeast and one fungus, with a minimum inhibitory concentration (MIC) of 40 µg/ml. To overcome the solubility problem often faced when herbal extracts are added to aqueous medium, we employed a modified broth method where the broth cultures were agitated at 150 rpm in an orbital shaking incubator. This method reduced the MIC of the extract considerably, to 5–20 µg/ml, against *B. bronchiseptica*, *S. aureus* and *S. epidermidis*.

Keywords: Ammoniacum gum, Antimicrobial activity, *Dorema ammoniacum* D. Don, *Ushaq*.

Introduction

Infectious diseases are responsible for approximately one half of all deaths in tropical countries and the morbidity is increasing even in developed countries. This is attributed to the development of resistance to existing antibiotics and also to the opportunistic infections, especially in AIDS cases (Pinner et al., 1996). Plants, with their wide variety of chemical constituents, offer a promising source of new antimicrobial agents with general as well as specific antimicrobial activity (Evans, 1996). Evaluation of the plants used in traditional medicine for different afflictions involving infection is expected to provide new antimicrobial agents. In preliminary screening, several plant extracts (*e.g., Allium sativum*, *Hypericum*, *Garcinia*, *Hydrastis*) have been shown to have good antimicrobial activity (Murray, 1995; Evans, 1996). In the present study, ammoniacum gum was evaluated for its antimicrobial activity against a battery of microorganisms.

Ammoniacum gum, commonly known as *ushaq* in the Unani System of Medicine, is an oleogum resin obtained as an exudate from the stem and leaf of flowering and fruiting plants of *Dorema ammoniacum* D. Don (Umbelliferae) (British Herbal Pharmacopoeia (BHP), 1983; Rajani et al., 2001). The plant is native to Central and Eastern Iran (BHP, 1983). Traditionally, ammoniacum gum is used as an expectorant, stimulant and antispasmodic in Unani System of Medicine. It is also used to treat catarrh, asthma, chronic bronchitis and enlargement of liver and spleen (The Wealth of India, 1952). The oleogum resin contains a small amount of volatile oil (0.1–0.4%), resin and gum. Some of the chemical constituents include free salicylic acid, ammoresinol, doremin, doremine A and ammodoremin (BHP, 1983; Appendino et al., 1991; Arnone et al., 1991). We employed an *in vitro* agar streak method and a modified broth method to evaluate the antimicrobial properties of ammoniacum gum.

Materials and methods

All chemicals and solvents used in the experiments were of analytical grade.

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Address correspondence to: M. Rajani, Pharmacognosy and Phytochemistry Department, BV Patel Pharmaceutical Education & Research Development (PERD) Centre, Thaltej, Ahmedabad-380 054, India. E-mail: rajanivenkat@hotmail.com

Plant material

Ammoniacum gum was a gift sample from Dr. G. Nasini, CNR, Centro di studio per le sonstanze Organiche Naturali, Milan, Italy.

Extraction of ammoniacum gum

Powdered ammoniacum gum (50 g) was extracted with a 1 : 1 mixture of dichloromethane : methanol (3 × 50 ml) by cold maceration for 24 h. The extract was filtered and the solvent was removed under vacuum. The residue (10 g) obtained was stored in an air-tight glass bottle at 20 °C. The extract was dissolved in DMSO and further dilutions were made to obtain the required concentrations.

Characterization of the extract by TLC fingerprinting
(Rajani et al., 2001)

A fingerprint profile of the extract was established using HPTLC (CAMAG, Switzerland). Stock solution of the extract (1 mg/ml) was prepared by dissolving the extract in a 1 : 1 mixture of dichloromethane : methanol and 10 µl were applied on a precoated silica gel G60 F254 plate using a Linomat Automatic TLC Spotter and developed in a solvent system comprised of *n*-hexane : ethylacetate : methanol (8 : 2 : 1 v/v). The plate was dried at room temperature and scanned with a CAMAG TLC Scanner 3 at 254 and 366 nm, and chromatograms and absorption spectra were recorded. Further, the plate was derivatised by spraying with anisaldehyde-sulphuric acid reagent (Wagner et al., 1984). The colour, Rf values and spectra of the resolved bands, λ_{max} and peak areas were recorded. The relative percentage of each of the band was calculated from peak areas.

Microorganisms and media

Eleven bacterial strains (Gram-positive: *Bacillus cereus* ATCC 11778, *Bacillus pumilus* ATCC-14884, *Bacillus subtilis* ATCC-6633, *Micrococcus luteus* ATCC-9341, *Staphylococcus epidermidis* ATCC-6538, *Staphylococcus aureus* ATCC-29737, *Streptococcus faecalis* ATCC-8043; Gram-negative: *Bordetella bronchiseptica* ATCC-4617, *Escherichia coli* ATCC-10536, *Klebsiella pneumoniae* ATCC-10031 and *Pseudomonas aeruginosa* ATCC-9027), one yeast (*Saccharomyces cerevisiae* ATCC-9763) and two fungal strains (*Candida albicans* ATCC-10231 and *Aspergillus niger* ATCC-16404) were selected from the microorganisms given in United States Pharmacopoeia (2000), British Pharmacopoeia (1993) and Indian Pharmacopoeia (1996) for antimicrobial assays. Bacteria were cultured in Antibiotic Assay medium No. 1 (HiMedia), the composition of which is as per the above three Pharmacopoeias. Fungi and yeast were cultured in Sabouraud's Dextrose medium.

Preparation of innoculum

In order to maintain approximately uniform growth rate of each microorganism, a fresh microbial seed was prepared by subculturing microorganisms into respective broth media and incubated untill the optical density (OD, turbidity at 600 nm) of the culture reached 0.2, which is indicative of a bacterial density of 10^7–10^8 CFU/ml.

For the broth method, 1 ml of the above microbial suspension was added per 100 ml of medium, which gave approximately 10^5–10^6 organisms/ml.

Optimization of DMSO concentration

Different concentrations of DMSO were added to the respective media containing 1.5% agar. Microbial suspensions were streaked on the medium and incubated at 37 °C. The plates were observed for the growth of the microorganisms for 24 h.

Testing for antimicrobial activity

A. Agar streak method (Mitscher et al., 1972)

A stock of 40 mg/ml of extract was prepared in DMSO. Different concentrations of the extract (a broad range of 100 to 400 µg/ml and a narrow range of 0.1 µg to 100 µg/ml) were taken in plates and 25 ml of the medium (maintained at 40 °C) was poured into the plates aseptically by following standard protocols. (Final concentration of DMSO in the medium was maintained at 1%). After the medium was solidified, the bacterial suspension (with an optical density of 0.2) was taken in a loop and streaked on the medium. Suitable controls were maintained with the extract and the microorganisms. Ciprofloxacin (2 µg/ml) served as positive control. The plates were incubated at 37 °C for bacterial cultures and at 27 °C for fungal and yeast cultures. The cultures were observed for microbial growth after 24 and 48 h of incubation and MIC was noted.

B. Broth method

Antibacterial activity of the extract was tested against three bacteria (*B. bronchiseptica, S. aureus* and *S. epidermidis*) by the broth method. Antibiotic assay medium No. 1 (Hi-Media) was prepared and 25 ml were dispensed into each of the 250 ml Erlenmeyer flasks and sterilized in an autoclave. To this, different concentrations of the extract (1–40 µg/ml) were added, maintaining the DMSO concentration at 1% of the culture medium in each flask. After adding the extract, 0.25 ml of bacterial suspension (*B. bronchiseptica, S. aureus* and *S. epidermidis*) of 0.2 OD was added to each flask. Suitable controls were maintained with the extract and the organisms. Ciprofloxacin (2 µg/ml) served as positive control. The cultures were incubated at 37 °C in an orbital shaking incubator at 150 rpm. Optical density of the aliquots was recorded at 600 nm from the incubated cultures at

different time intervals up to 24 h of incubation, and visually observed for growth after 48 h of incubation. A graph of time *vs* absorbance (OD at 600 nm) was plotted for the growth curve, and the effect of the different concentrations of the extract on the growth of the bacteria was recorded.

Total viable count

To determine the number of viable bacteria left after treatment with the extract, a fixed amount (0.1 ml) of the suspension of *B. bronchiseptica* from 0 hr and after 3 hr of incubation (in log phase) was plated. For plating the suspension from the control experiment, suitable dilutions were made by serial dilution technique and inoculated. The plates were incubated at 37 °C for 24 h, colonies were counted, and total viable count (TVC) per ml was recorded.

All the experiments were run in duplicate and the MIC was confirmed by performing the experiments twice.

Results and discussion

Dichloromethane : methanol extract of ammoniacum gum was evaluated for its antimicrobial properties against a battery of microorganisms including several Gram-negative and Gram-positive bacteria, yeast and fungi with agar plate methods and modified broth methods. The organisms selected were from the lists given in the pharmacopoeias USP (2000), BP (1993) and IP (1996), since information is available on the potency of clinically-used antibiotics against these organisms.

The extract used in the experiments was characterized by TLC fingerprinting, the details of which are given in Table 1 and Figure 1. When scanned at UV 254 nm, the extract showed 9 bands. At UV 366 nm, the extract showed 7 bands, all of them having a λ_{max} around 315 nm and showed blue fluorescence, except the band at Rf 0.6 that had yellow fluorescence. When the TLC plate was derivatized with anisaldehyde sulphuric acid reagent (Wagner et al., 1984), it gave 9 bands of grey-blue colours.

Different concentrations of DMSO (1–5%) were tested for checking the toxic/inhibitory effect of DMSO on the bacterial growth (Table 2). We found that DMSO at concentrations beyond 2% was inhibitory for most organisms, while up to 2% it has no effect on the growth. Hence, for all the experiments, final concentration of 1% DMSO was used as solvent carrier.

In general, the extract exhibited a broad spectrum antimicrobial activity against ten out of the 14 microorganisms tested (Tables 2 and 3). It inhibited the growth of all the seven Gram-positive bacteria tested while growth of only one Gram-negative bacterium (*B. bronchiseptica*) was inhibited. In agar plate method, with the broad range of concentrations of 100–400 µg/ml of the extract, the growth of all the microorganisms, except *E. coli, K. pneumoniae, P. aeruginosa* and *C. albicans*, was inhibited. In the subsequent experiment using a narrow range of 1–100 µg/ml of the extract, the MIC for all the ten microorganisms was found to be between 20 and 40 µg/ml.

During the experiment it was observed that the extract, while preparing the dilutions and/or when added to the medium, formed a fine emulsion. Though a clear precipitate was not formed, the formation of emulsion indicated separation of nonpolar components of the extract in the aqueous medium. Since this may interfere with the availability of such components to the microorganisms and hence from exerting the activity, we modified the broth method, in which the medium containing the extract and the bacterial suspension was agitated in an orbital shaking incubator at 150 rpm. This is expected to affect an efficient mass transfer and bring the

Table 1. TLC fingerprint profile of dichloromethane : methanol (1 : 1) extract of ammoniacum gum.

Scanned at 254 nm[1]			Scanned at 366 nm[2]			Derivatized[3]	
Rf	λ_{max} nm	Relative %	Rf	λ_{max} nm	Relative %	Rf	Colour
0.23	315	6.44	0.21	315	7.38	0.11	blue
0.25	316	14.93	0.28	315	14.49	0.16	dark blue
0.30	315	8.77	0.30	316	15.39	0.23	dark blue
0.36	244, 315	3.72	0.39	316	3.92	0.27	grey
0.54	316	30.42	0.48	317	26.56	0.34	grey
0.58	244, 318	5.02	0.50	316	29.14	0.41	dark blue
0.67	310, 239	22.83	0.60	244, 318	3.11	0.45	greenish grey
0.78	249, 296	3.94				0.48	greenish grey
0.96	288, 312	3.93				0.66	pink

[1] Fluorescent-quenched bands.
[2] All bands have blue fluorescence except the one at Rf 0.6 which has yellow fluorescence.
[3] Derivatized with anisaldehyde sulphuric acid reagent.

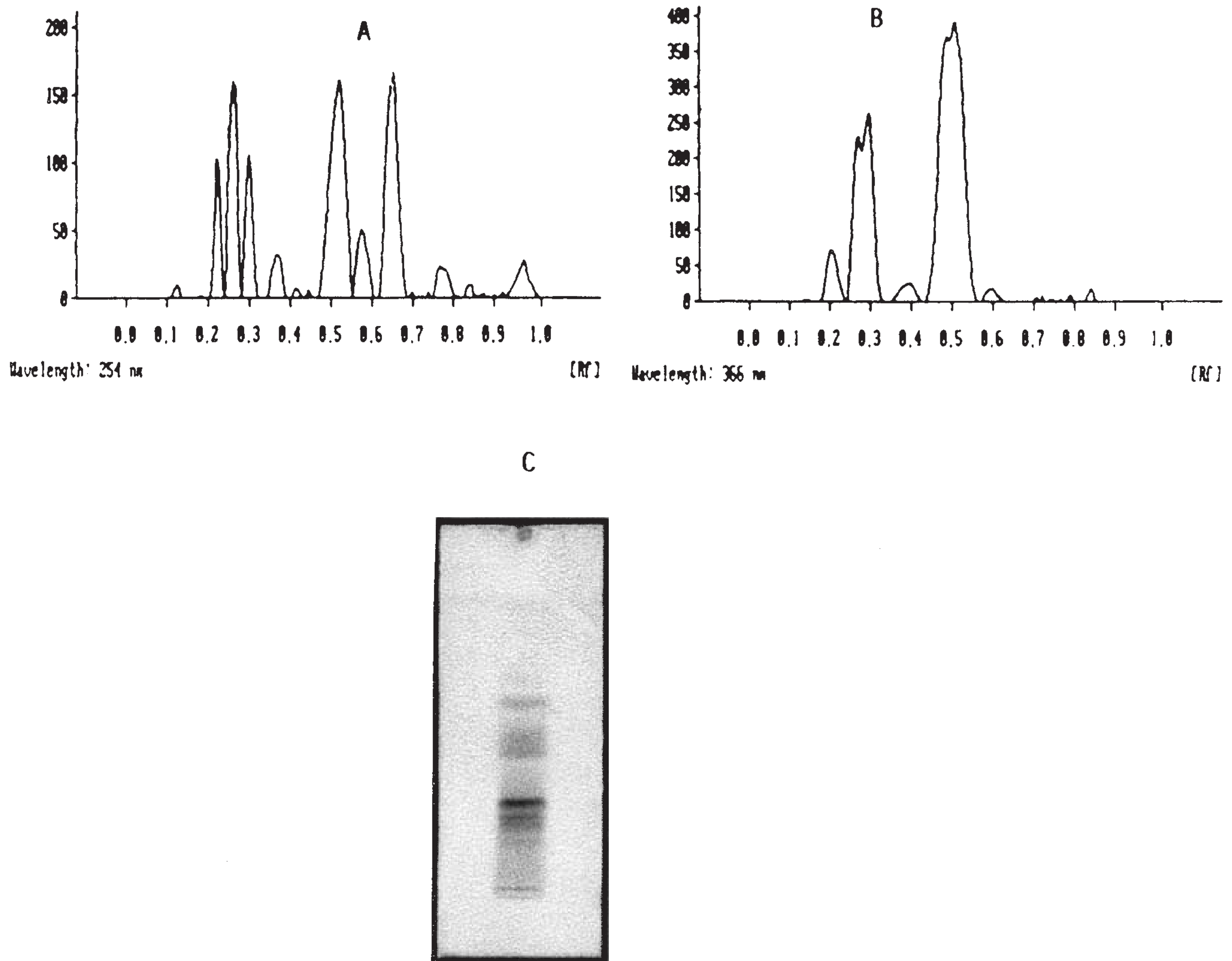

Figure 1. TLC of Dichloromethane : methanol (1 : 1) extract of *ammoniacum gum*. TLC chromatogram scanned at UV 254 nm (A), scanned at UV 366 nm (B), and TLC profile after derivatization with anisaldehyde sulphuric acid reagent (C).

components of the extract in constant contact with the bacteria. This reduced the MIC considerably for the three bacteria tested, *viz., B. bronchiseptica*, *S. aureus* and *S. epidermidis*. The results are discussed below.

Broth cultures and the effect of the extract on the growth kinetics of three bacteria

Since a minimum inhibitory concentration of 40 µg/ml of the extract was obtained in the agar streak method for five organisms, this concentration was taken as a starting point for testing the activity of the extract in broth cultures. Growth kinetics of the bacteria and the effect of the extract was studied by recording the turbidity (OD at 600 nm) of the cultures as an indication of bacterial density.

B. bronchiseptica

Growth kinetics of *B. bronchiseptica* showed a short lag phase of 1.5 h followed by 2.5 h of log phase (up to the 4th h) of rapid growth, followed by stationary phase. Addition of 1% DMSO did not influence the growth kinetics at all (Fig. 2). After establishing the growth kinetics, 40, 30 and 20 µg/ml of the extract was tested for antimicrobial activity. The extract inhibited the bacterial growth at all these concentrations (Fig. 3). Subsequently, 1, 5 and 10 µg/ml of the extract was tested. With 1 µg/ml of the extract, the bacteria started growing gradually after 4 h and by 24 h of incubation the bacterial density of the culture was same as that of the control. However, the lag phase was extended to 4 h and in log phase also the growth rate was slow. 5 and 10 µg/ml of the extract inhibited the bacterial growth completely (MIC 5 µg/ml) (Fig. 4).

To establish the bactericidal nature of the activity of the extract, viable count of *B. bronchiseptica* was recorded (Table 4) by plating a bacterial suspension treated with 5 µg/ml which is the MIC for *B. bronchiseptica*, and also 40 µg/ml, the highest concentration tested in the broth method. At 0 h all the cultures recorded around 9.0×10^4 TVC/ml. After 3 h of incubation with 5 µg/ml there were only

Table 2. Antimicrobial activity of the extract of ammoniacum gum tested with the agar plate method (broad range of concentrations).

Organism	Blank	1% DMSO	Extract 100 μg/ml	Extract 200 μg/ml	Ciprofloxacin (2 μg/ml)
B. cereus	+++	+++	–	–	–
B. plumilus	+++	+++	–	–	–
B. subtilis	+++	+++	–	–	–
M. luteus	+++	+++	–	–	–
S. epidermidis	+++	+++	–	–	–
S. aureus	+++	+++	–	–	–
S. faecalis	+++	+++	–	–	–
B. bronchiseptica	+++	+++	–	–	–
E. coli	+++	+++	+++	+++	–
K. pneumoniae	+++	+++	+++	+++	–
P. aeruginosa	+++	+++	+++	+++	–
S. cerevisiae	+++	+++	–	–	–
C. albicans	+++	+++	+++	+++	–
A. niger	+++	+++	–	–	–

–, no growth; +, growth; +++, abundant growth.

Table 3. Antimicrobial activity of the extract of ammoniacum gum tested with the agar plate method (narrow range of concentrations).

Organism	Blank	1% DMSO	Extract 10 μg/ml	Extract 20 μg/ml	Extract 40 μg/ml	Extract 60 μg/ml	Ciprofloxacin (2 μg /ml)
B. cereus	+++	+++	+++	+++	–	–	–
B. pumilus	+++	+++	+++	+++	–	–	–
B. subtilis	+++	+++	+++	+++	–	–	–
M. luteus	+++	+++	+++	+++	–	–	–
S. epidermidis	+++	+++	+++	+++	–	–	–
S. aureus	+++	+++	+++	+++	–	–	–
S. faecalis	+++	+++	+++	+++	–	–	–
B. bronchiseptica	+++	+++	+++	+++	–	–	–
E. coli	+++	+++	+++	+++	+++	+++	–
K. pneumoniae	+++	+++	+++	+++	+++	+++	–
P. aeruginosa	+++	+++	+++	+++	+++	+++	–
S. cerevisiae	+++	+++	+++	+++	–	–	–
C. albicans	+++	+++	+++	+++	+++	+++	–
A. niger	+++	+++	+++	+++	–	–	–

–, no growth; +, growth; +++, abundant growth.

43 TVC/ml and with 40 μg/ml there were no viable counts at all, while control recorded 1.73×10^7 TVC/ml. This suggests a bactericidal activity of the extract against *B. bronchiseptica*. More detailed experiments are underway to ascertain the bactericidal activity of the extract in *B. bronchiseptica* and also other bacteria.

In an interesting experiment to check the efficiency of the extract at a stage when the bacterial load is high and the bacteria are in exponential growth phase, 40 μg/ml of the extract were added in the middle of the log phase of bacterial growth (after 2.5 h of incubation; OD = 0.68), and growth was monitored by recording the OD at different intervals. The bacterial growth was inhibited completely (Fig. 5). There was a slight increase in OD (0.74) up to the 3rd h of incubation, after which it dropped to around 0.4 and remained at that level up to 11 h and further dropped to 0.3 by 24 h of incubation. When 1 ml of this suspension was inoculated into 25 ml of fresh broth and incubated at 37 °C, there was no growth of bacteria for 24 h. This indicates a bactericidal activity of the extract and the reduction in OD could be due to lysis of the bacteria.

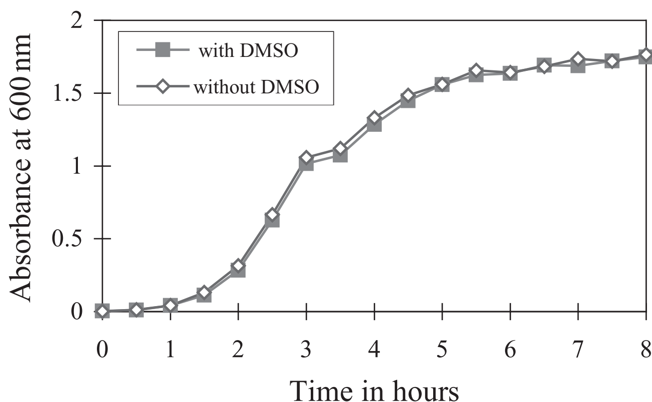

Figure 2. Effect of 1% DMSO on growth kinetics of *B. bronchiseptica*.

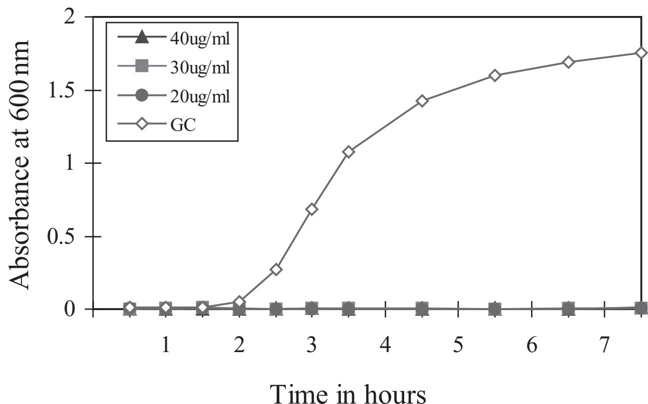

Figure 3. Antibacterial activity of ammoniacum gum extract (20 μg, 30 μg and 40 μg/ml) against *B. bronchiseptica* tested in modified broth method (GC – growth curve of *B. bronchiseptica*).

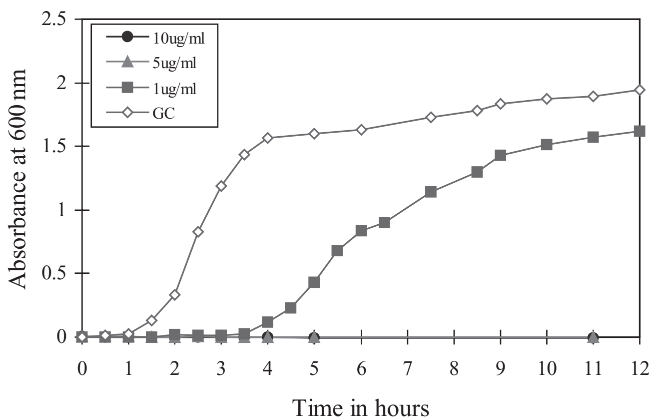

Figure 4. Antibacterial activity of ammoniacum gum extract (1 μg, 5 μg and 10 μg/ml) against *B. bronchiseptica* tested with the modified broth method (GC – growth curve of *B. bronchiseptica*).

Table 4. Total viable count of *B. bronchiseptica* (TVC/ml) after treatment with 5 μg/ml and 40 μg/ml of the extract of ammoniacum gum.

	TVC/ml		
Incubation	Control	5 μg/ml	40 μg/ml
0 hr	9.5×10^4	9.0×10^4	9.0×10^4
3 hr	1.73×10^7	43	0

n = 3.

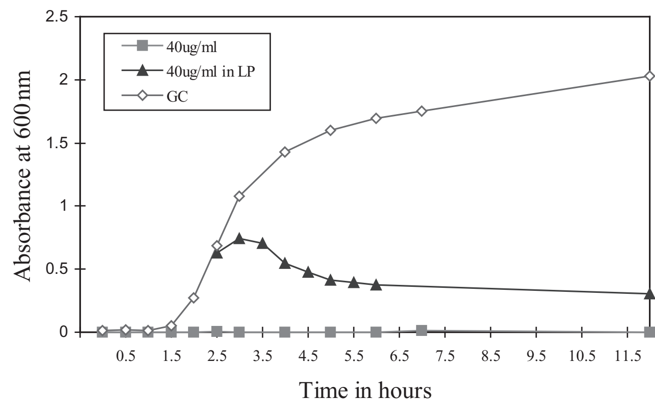

Figure 5. Bactericidal activity of 40 μg/ml of ammoniacum gum extract added in the middle of log phase of *B. bronchiseptica* (40 μg/ml in LP) with the modified broth method (GC – growth curve of *B. bronchiseptica*).

S. aureus

Growth kinetics of *S. aureus* showed a short lag phase of 3 h and a log phase of 6 h (3rd–9th h). DMSO (1%) did not influence the growth kinetics of the bacterium, as in the case of *B. bronchiseptica* (Fig. 2). The extract exerted a concentration dependent antibacterial activity against *S. aureus* (Fig. 6); 40 μg/ml of the extract inhibited bacterial growth completely. There was a very slight increase in the density from 0.007 at 0 h to 0.035 up to 17 h and by 24 h of incubation the density dropped to 0.01, while that of the control experiment reached 2.9. A subsequent experiment was carried out with 1, 5, 10 and 20 μg/ml of the extract; 20 μg/ml of the extract completely inhibited the growth of *S. aureus*, while the effect of 1 μg/ml was almost insignificant. With 5 and 10 μg/ml, the lag phase was extended up to the 8th and 14th h, respectively (MIC of the extract: 20 μg/ml).

S. epidermidis

Growth kinetics of *S. epidermidis* revealed a lag phase of 5 h and a log phase of 5 h (6th to 10th hour). DMSO (1%) did not have any effect on the growth kinetics of *S. epidermidis*, as in the case of *B. bronchiseptica* (Fig. 2). The extract

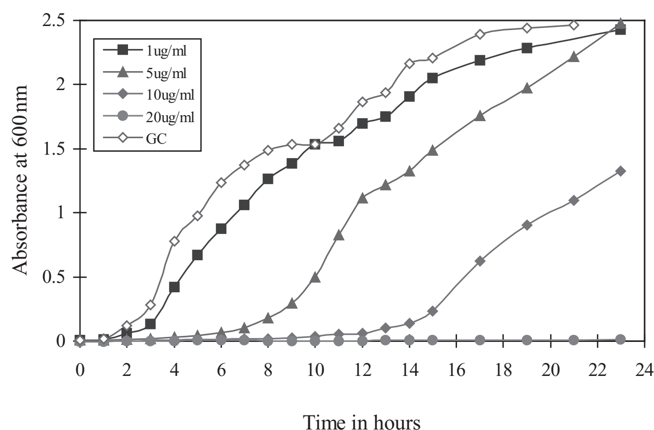

Figure 6. Antibacterial activity of ammoniacum gum extract (20, 10, 5, 1 µg/ml) against *S. aureus* tested with the modified broth method (GC – growth curve of *S. aureus*).

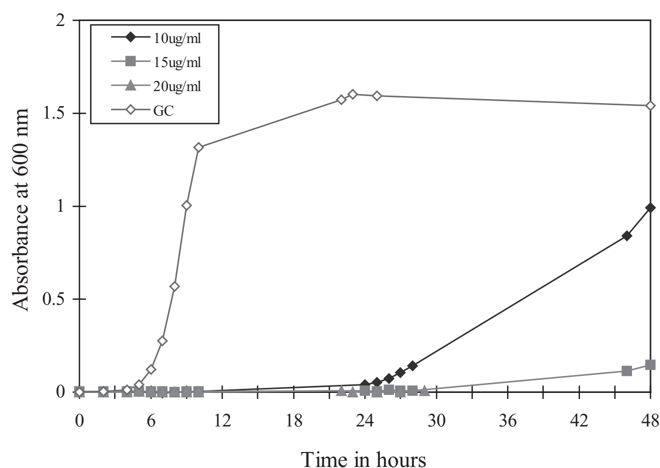

Figure 7. Antibacterial activity of ammoniacum gum extract (10, 15, 20 µg/ml) against *S. epidermidis* with the modified broth method (GC–growth curve of *S. epidermidis*).

exerted a concentration-dependent antibacterial activity against *S. epidermidis* (Fig. 7); 20 and 40 µg/ml of the extract completely inhibited the growth of the bacteria. With 10 and 15 µg/ml there was no growth up to 24 h, but by 48 h there was a raise in OD to 1 and 0.15, respectively. For *S. epidermidis* 20 µg/ml can be taken as MIC.

In preliminary screening, Sabahi et al. (1987) reported antibacterial activity of a methanol extract of aerial parts of *Dorema ammoniacum* against *Bacillus subtilis, Sarcina subflava* and *S. aureus*. In the present study, ammoniacum gum from *D. ammoniacum* exhibited broad spectrum antimicrobial activity with a possible bactericidal activity. The very low MIC indicates the potential of the drug as a promising antimicrobial agent. Further studies on identifying the compound(s) responsible for the activity are underway.

Conclusion

The above investigation indicates that the extract of ammoniacum gum has excellent antimicrobial activity, and provides a scientific basis for the traditional use of ammoniacum gum for bronchitis, respiratory infections, fever, cold and flu (BHP, 1983). Further, the modified broth method used for tackling the problem of solubility of the extract of ammoniacum gum has potential for wide applicability for evaluating herbal extracts for antimicrobial activity. It was clearly established that with effective mass transfer facilitated by agitating the medium at 150 rpm in an orbital shaking incubator, the MIC was reduced from 40 µg/ml in the experiment with the conventional solid agar medium to 5–20 µg/ml in the broth for various bacteria. Work is in progress to identify active antimicrobial compound(s) from ammoniacum gum.

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