ORIGINAL ARTICLE

In vitro antibacterial activity of *Artemisia annua* Linn. growing in India

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The crude extracts obtained from the aerial parts of *Artemisia annua* Linn. (Asteraceae) were investigated for their antibacterial activity by using agar well diffusion assays against five Gram-positive bacteria (*Staphylococcus aureus, Bacillus subtilis, Bacillus pumilus, Bacillus cereus*, and *Micrococcus luteus*) and three Gram-negative bacteria (*Escherichia coli, Salmonella typhi*, and *Pseudomonas aeruginosa*). Of the various extracts, the methanol extract showed the strongest activity against most bacteria used in this study. The most sensitive organism to the extracts was *M. luteus*. The minimum inhibitory concentration (MIC) values were determined by the tube dilution method. The results showed that *S. aureus* required ~0.25 mg/mL of the methanol extract for inhibition. The HPTLC fingerprint of the methanol extract after derivatization with anisaldehyde sulphuric acid reagent showed a maximum number of separated components. TLC bioautography of the methanol extract showed that the area of inhibition around compounds differentiated at R_f = 0.32, R_f = 0.42, R_f = 0.46, R_f = 0.77, and R_f = 0.87 against *S. aureus*. This is the first report of the antibacterial activity of *A. annua* against food-borne bacteria. The results indicated that aerial parts of *A. annua* might be potential sources of new antibacterial agents.

Key words: Antibacterial activity, *Artemisia annua*, MIC, TLC bioautography

INTRODUCTION

Medicinal plants have been used since long for mankind against various infectious and non-infectious diseases because they contain natural bioactive components for therapeutic value. According to the World Health Organization, medicinal plants are the best source to obtain a variety of drugs and ~80% of population from developed countries uses traditional medicines, which have bioactive compounds derived from medicinal plants for their primary health care needs.[1-2] Many approaches were made to search the antimicrobial compounds with a novel chemical structure from the medicinal plants. The development of new antimicrobial compounds against different microorganisms is becoming critically important, as infectious diseases are still one of the main causes of death in the world.[3] Currently, there has been an increased interest in antimicrobial agents from the plants origin due to the resistance that microorganisms have developed against traditional antibiotics.[4-5] Therefore, there is a need for the investigation of new source of potential antibacterial agents.

Artemisia annua Linn. (Asteraceae) (known as 'quinghao') is used as a source for antimalarial, antibacterial, anti-inflammatory, angiotensin-converting enzyme inhibitory, plant growth regulatory, cytokinin-like, and antitumor substances.[6] In India, it is being cultivated in Gujarat, Uttar Pradesh, Himachal Pradesh, and Karnataka. However, on a large scale it is cultivated in China, Vietnam, Turkey, Iran, Afghanistan, and Australia.[7] Presently, there is a lack of data in the available literature reference on antibacterial activity against food-borne bacteria. The aim of this study was to investigate the antibacterial activity of the aerial parts of *A. annua* against food-borne bacteria. Bioautography is a technique that combines chromatography with bioassay in situ. It allows the localization of the active constituents. It is hoped that these active constituents will provide useful information for discovering new compounds with better activity against food-borne bacteria than agents currently available.

MATERIALS AND METHODS

Plant Material

The whole plant was purchased from M/S International Traders in Herbs, New Delhi, India, in August 2007. Voucher specimen (MB-005) was deposited at the Microbiology Laboratory and identified by Dr. Prakash Joshi, Homoeopathic Pharmacopeia Laboratory, Ghaziabad, Uttar Pradesh, India. The plant material was air dried under shade at room temperature and made into a powder.

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Preparation of Extracts

The extracts were prepared by the cold extraction method,[8] in which 20 g of dry powdered material was soaked in the solvents (5 × 200 mL) viz., methanol, chloroform, hexane, and petroleum ether (Qualigens, AR grade) for 24 hours at room temperature and shaken from time to time. Each extracts were filtered through the Whatmann filter paper no. 1. The extracts were concentrated to complete dryness, using rotatory evaporator at 50°C. Dried extracts were collected and stored in labeled sterile screw-capped bottles in a refrigerator until further use.

Microorganisms

Following test organisms were used: *Escherichia coli* (ATCC: 8739), *Salmonella typhi* (ATCC: 23564), *Pseudomonas aeruginosa* (ATCC: 25668), *Staphylococcus aureus* (ATCC: 9144), *Bacillus pumilus* (ATCC: 14884), *Bacillus subtilis* (ATCC: 6633), *Bacillus cereus* (ATCC: 11778), and *Micrococcus luteus* (ATCC: 9341). The bacterial strains were maintained and stored at 5 - 8ºC on brain heart infusion agar (HI-Media). For determing antibacterial activity, 24-hour old cultures were used. An inoculum size of 10^6 CFU/mL of bacteria, compared with 0.5 McFarland turbidity standards, was used.

Screening for Antibacterial Activity Test

The antibacterial assays were performed by the agar well diffusion method.[9] Petri dishes (100 mm) were poured with nutrient agar (HI-Media) and allowed to solidify to make base layers. The seed layers were prepared by inoculating 100 µL of test organism suspension in 25 mL nutrient agar and wells, 10 mm in diameter, were made in the agar medium with the help of a sterile steel borer. About 100 µL of each extract (stock 200 mg/mL) was added aseptically in wells. All the plates were incubated at 37 ± 1°C for 24 hours in the upright position. At the end of the incubation times, the diameters of the inhibition zones were measured in millimeters. Antibiotics such as ciprofloxacin (20 µg/mL) and solvent (methanol, chloroform, hexane, and petroleum ether) without the test compound were used as positive and negative controls, respectively. The tests were conducted in triplicate.

Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) values were evaluated by the tube dilution method against *S. aureus*. The methanol extract MIC was determined by dilution of the extract to various concentrations range 0.125–8.0 mg/mL and compared with the standard (ciprofloxacin). All the tubes were incubated at 37 ± 1°C for 24 hours. The MIC values were taken as lowest concentration of the extract that showed no turbidity after incubation when compared with control tubes.[10]

HPTLC Fingerprinting

The HPTLC analysis was performed on aluminum sheet pre-coated with silica gel 60 F_{254} (E-Merck grade). Before use, plates were pre-washed with methanol, and dried in an oven at 105°C for 1 hour. The extracts (10 µL) were applied on the plates as bands of 7-mm width with the help of a linomat-5 sample applicator. The plate was developed in a Camag twin-trough chamber previously equilibrated with a mobile phase for 20 minutes. The solvent system containing toluene: ethyl acetate: formic acid (7:3:1)[7] was used. After development, plate was derivatized with freshly prepared anisaldehyde sulphuric acid reagent, heated in an oven at 70°C and observed under visible light. The color of resolved bands and R_f values were recorded (all solvents used were of Qualigens, AR grade).

TLC Bioautography

Bioautography was performed with a culture of *S. aureus* which showed a good sensitivity to the extracts. Developed TLC plates were carefully dried for complete removal of solvent, and overlaid with agar containing an aliquot of an overnight culture of *S. aureus*. The plate was incubated for 24 hours at 37 ± 1°C and sprayed with aqueous solution of 5 mg/mL of tetrazolium salts (Loba Chemie). The plates were run in duplicate; one set was used as the reference chromatogram and the other was used for bioautography.

RESULTS AND DISCUSSION

The *in vitro* antibacterial activities of the extracts of *A. annua* and standard antibiotic are shown in Table 1.

Table 1: Antibacterial activity of *Artemisia annua* aerial parts extracts

Organisms	Zone of inhibition (mm)				
	Methanol	Chloroform	Hexane	Petroleum ether	Ciprofloxacin
Escherichia coli	NZ	14	NZ	NZ	20
Salmonella typhi	NZ	14	NZ	NZ	30
Pseudomonas aeruginosa	17	14	NZ	NZ	23
Staphylococcus aureus	27	14	12	13	18
Bacillus pumilus	23	NZ	NZ	NZ	21
Bacillus subtilis	24	12	12	13	22
Bacillus cereus	20	NZ	12	12	22
Micrococcus luteus	21	12	16	14	22

NZ = no zone of inhibition. *Zone of inhibition (in mm) including the diameter of well. Ciprofloxacin used as a standard.

The solvent controls did not show any activity against the microorganisms used in this study. The methanol and chloroform extracts showed activity against Gram-positive and Gram-negative bacteria tested (inhibition diameters ranged from 12 to 27 mm). The methanol extract showed the maximum zone of inhibition against *S. aureus* (27 mm) which is greater than the standard antibiotic used, whereas the hexane and petroleum ether extracts showed activity against the Gram-positive bacteria (inhibition diameters ranged from 12 to 16 mm) but did not show any activity against the Gram-negative bacteria. The results are in concordance with the work of Juteau *et al*.[11]

The results indicated that the antimicrobial activity against Gram-positive was more pronounced than against Gram-negative bacteria. The results obtained are in agreement with the work of Nair *et al*.,[12] Parkeh and Chanda,[13] and Encarnacion *et al*.[14] The differences may be attributed to the fact that the cell wall in Gram-positive bacteria consists of a single layer, whereas the Gram-negative bacteria it is a multilayer structure and is quite complex.[5].

The MIC studies of the methanol extract were carried out because the zone of inhibition was prominent only in the methanol extract. The MIC value for the P. aeruginosa was 2.0 mg/mL. Furthermore, B. pumilus, B. subtilis, B. cereus, and M. luteus showed a MIC value of 0.5 mg/mL, whereas S. aureus required ~0.25 mg/mL of the methanol extract for inhibition [Table 2].

Table 2: Minimum inhibitory concentrations of methanol extract of *Artemisia annua* aerial parts

Organisms	Methanol extract MIC (mg/mL)
Pseudomonas aeruginosa	2.0
Staphylococcus aureus	0.25
Bacillus pumilus	0.5
Bacillus subtilis	0.5
Bacillus cereus	0.5
Micrococcus luteus	0.5

The plant extracts were further analyzed by HPTLC to fingerprint the active constituents in the extracts [Figure 1]. The HPTLC of the methanol and chloroform extracts showed the presence of 11 and 4 major substances, respectively [Table 3]. It is noted that the hexane and petroleum ether extracts showed six compounds with same R_f values. This might be one of the reasons that the hexane and petroleum ether showed same inhibition diameter in the agar-well diffusion method. The bioautography results were obtained using S. aureus as the test organism for the methanol extract. The clear zones were located in separate places on the TLC plate [Figure 2], suggesting that more than one compound possessed an antimicrobial effect. The result revealed that the compounds eluted at R_f = 0.32 (green spot), R_f = 0.42 (blue spot), R_f = 0.46 (blue spot), R_f = 0.77 (green spot), and R_f = 0.87 (green spot) exhibited strong antibacterial activity.

Based on the results obtained in the current study, it may be conclude that *A. annua* have a stronger and broader spectrum of antimicrobial activity against a number of food-borne bacteria, and the extracts may be used to discover

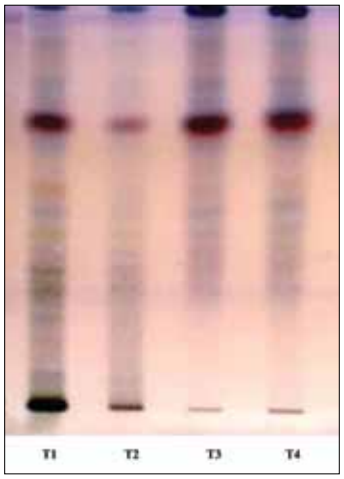

Figure 1: HPTLC profile of extracts of *Artemisia annua* after derivatization with anisaldehyde-sulphuric acid reagent. Legends T1 – methanol extract, T2 – chloroform extract T3 – hexane extract, T4 – petroleum ether extract

Table 3: R_f values and bands color of *Artemisia annua* aerial parts extracts after derivatization

Bands color	R_f Value			
	Methanol	Chloroform	Hexane	Petroleum ether
Green	0.87	0.87	0.87	0.87
Red	0.83	N/v	0.83	0.83
Green	0.77	0.77	0.77	0.77
Red	0.68	0.68	0.68	0.68
Blue	0.65	N/v	0.65	0.65
Orange	0.52	N/v	N/v	N/v
Blue	0.46	0.46	0.46	0.46
Blue	0.42	N/v	N/v	N/v
Green	0.32	N/v	N/v	N/v
Green	0.27	N/v	N/v	N/v
Blue	0.20	N/v	N/v	N/v

N/v: not visible.

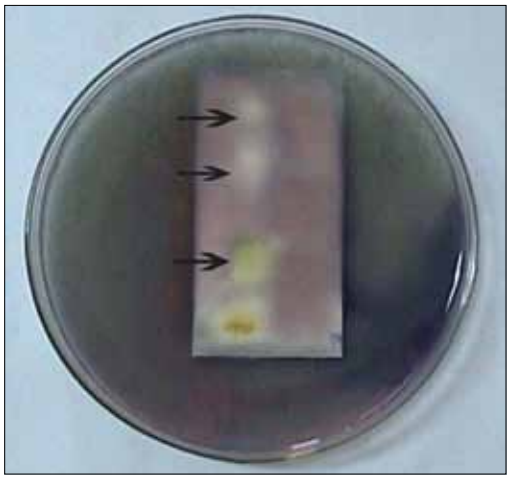

Figure 2: TLC bioautography overlay assay against *Staphylococcus aureus*. The inhibition spot is marked with arrow head

bioactive natural products that may serve as basic source for the development of new antimicrobial compounds to overcome the problem of increasing resistance to known traditional antibiotics.

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