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Evaluation of Root Extracts of *Asparagus racemosus* for Antibacterial Activity

^{1,2}Himanshu Aggarwal, ¹Gyanprakash, ¹Alka Rao and ¹Vinod Chhokar

¹Department of Bio and Nano Technology, Guru Jambheshwar University of Science and Technology, Hisar, 125001, Haryana, India

²Department of Biotechnology, Maharishi Markandeshwar University, Mullana, 133207, Haryana, India

Corresponding Author: Vinod Chhokar, Department of Bio and Nano Technology, Guru Jambheshwar University of Science and Technology, Hisar, 125001, Haryana, India

ABSTRACT

The root extract of *Asparagus racemosus* was studied *in vitro* against five strains of bacteria by using agar well/disc-diffusion method and growth inhibition in liquid culture. Among the three different extraction methods namely, cold maceration method, soxhlet extraction and reflux extraction that were employed for the extraction of bioactive components from the roots of the *A. racemosus*, the methanolic extract prepared by reflux extraction was found to have the maximum antibacterial activity against *Escherichia coli* DH5 α and *Pseudomonas aeruginosa* (MTCC1688). The MIC value of methanolic *A. racemosus* Root Extract (ARE) as determined by agar disc diffusion assay was found considerably significant for a crude extract at 0.5 mg mL⁻¹ against *P. aeruginosa* (MTCC1688) and as low as 0.25 mg mL⁻¹ in case of *E. coli* DH5 α in comparison with the synthetic antibiotic ampicillin.

Key words: *Asparagus racemosus*, Antibacterial, MIC

INTRODUCTION

In recent years, apart from increasing infections, antibiotic as well as multidrug, resistance has becomes a major therapeutic problem world-wide. This has led to an increased thrust towards identification of novel antimicrobial agents. On the other hand, globally there is a patient-driven trend towards “natural remedies”. The World Health Organization in 2003 has estimated that 80% of the populations of developing countries being unable to afford pharmaceutical drugs rely on traditional medicines, mainly plant based, to sustain their primary health care needs. India is one of the most medico-culturally diverse countries in the world where the medicinal plant sector is part of a time-honoured tradition that is respected even today.

The phyto-therapy and the use of herbal remedies in healthcare preparations have been well documented even in ancient texts like the Vedas and the Bible. In fact, plants produce a diverse range of bioactive molecules, making them a rich source of different types of medicines. Over 50% of all modern clinical drugs are of natural product origin and natural products play an important role in drug development programs in the pharmaceutical industry. The increased awareness of the limited ability of synthetic pharmaceutical products to control major diseases and the need to discover new molecular structures as lead compounds from the plant kingdom has further fuelled the interest in finding novel antimicrobials from higher plants like *T. cordifolia*, *A. racemosus*, *E. officinalis*, *A. vera* etc.

A. racemosus commonly termed as “Shatavari” is a woody climber of the family Liliaceae. It is common at low altitudes in shade and in tropical climates throughout India, Asia, Australia and

Africa. The medicinal usage of *A. racemosus* has been reported in the Indian and British Pharmacopoeias and in indigenous systems of medicine (Bopana and Saxena, 2007; Saxena *et al.*, 2010). The genus asparagus includes about 300 species around the world. Out of 22 species of asparagus recorded in India; *A. racemosus* is the one most commonly used in traditional medicine. *Asparagus* is considered to be medicinally important by virtue of the presence of components like steroidal saponins (known as Shatavarins I-IV. Shatavarin I is the major glycoside), isoflavones including 8-methoxy-5, 6, 4'-trihydroxyisoflavone 7-O-beta-D-glucopyranoside, asparagamine, a polycyclic alkaloid, racemosol, a cyclic hydrocarbon (9,10-dihydrophenanthrene) polysaccharides and mucilage in various parts of the plant (Sekine *et al.*, 1995; Sekine, 1997). Apart from a potent adaptogenic activity, hormonal activity, antioxytocic activity, immunological activity, diuretic activity and anti-lithic effect, asparagus has also been attributed a potent antibacterial activity (Mandal *et al.*, 2000; Rekhate *et al.*, 2010).

The studies on various ethno medicinal plants across the world has been suggestive of that the crude extracts from plants used in alternative medicine are potential sources of antiviral, antitumoral and antimicrobial agents (Valsaraj *et al.*, 1997; Kumar *et al.*, 2006). In the present investigation, the root extract of *A. racemosus* prepared by various extraction processes using different solvents was screened for potential antibacterial activity against both gram positive and gram negative strains of bacteria.

MATERIALS AND METHODS

Plant material: Roots of *A. racemosus* were collected from Medicinal Plant Nursery, Chaudhary Charan Singh Haryana Agricultural University, Hisar, India. Plant roots were washed thoroughly under running tap water followed by a wash with sterile water, dried in oven, powdered and used for extraction. The solvents used for the extraction of plants were water and methanol.

Bacterial strains: The microorganisms used as test organisms in this study includes four gram negative bacteria namely, *E. coli* DH5 α , *P. aeruginosa* (MTCC1688), clinical isolates of *Klebsiella pneumonia*, *Enterococcus cloacae* and clinical isolate of one gram positive bacteria *Staphylococcus aureus*. The strains were obtained from Institute of Microbial Technology, Chandigarh and Mangalam Laboratory, Hisar, respectively.

Preparation of extracts (Aqueous/Organic solvent): Three different methods employed for extraction were cold maceration method, soxhlet extraction and reflux extraction. In routine the root samples were dried in oven for 15 days at 45°C and powdered using pestle and mortar. For cold maceration method, 45 g of the root powder was placed in 450 mL of 80% ethanol (Qualigens Chemical, India)/ water in a conical flask, plugged with cotton and then kept on a rotary shaker at 180-200 rpm for 72h. After 72h, the extract was filtered. The final dried extract (600 mg) was dissolved in 500 μ L of Dimethylsulfoxide (DMSO, S. D. Fine Chemicals Ltd., India). For soxhlet extraction 200 g of plant root powder was extracted using solvent (750 mL methanol/water) at 65°C for 16 h. The extracted sample was dried using distillation or in water bath at 80°C. The final dried extract (2.5 g) was dissolved in 1 mL DMSO. During reflux extraction 60 g of root powder was refluxed in 300 mL methanol/water at 65°C for 8h. After 8h, the extract was filtered and the residue was refluxed again. The procedure was repeated 3 times. All the filtrates were collected and the solvent was evaporated in water bath at 80°C. The final dried extract (7670 mg) was dissolved in 9 mL DMSO. All the extracts were finally dissolved in DMSO, stored at 4°C and subjected to antibacterial activity assay.

Bacterial cultures: Nutrient broth (Hi Media) was used for liquid culture and nutrient agar (Hi Media) for solid. 13.5 g of nutrient broth was dissolved in 1000 mL distilled water (pH 7.4±0.2). All solutions were sterilised in an autoclave for 15 min at 121°C. A loopful of cultures were streaked on nutrient agar plates and incubated at 37°C for 24 h for appearance of discrete colonies for diffusion assays whereas for growth curve studies in liquid cultures single colonies were inoculated in 5 mL nutrient broth and allowed to grow for 18 h (overnight) at 37°C in a rotary shaker at 180 rpm.

Assays to determine antimicrobial activity and minimum inhibitory concentration (MIC): The screening for antimicrobial activity of methanolic *A. racemosus* Root Extract (ARE) was performed by three methods, agar disc diffusion method, agar well diffusion method as well as by studying effect of extracts on the growth of the bacterial strains in liquid cultures. In routine a suspension of (100 µL) bacterial culture grown in nutrient broth for 18 h was used for seeding the nutrient agar to form a lawn. For agar disc diffusion method, the discs (5 mm) were saturated with 50 µL of the methanolic ARE (test plant root extract) then allowed to dry and were finally placed on the upper layer of the seeded agar plate. For agar well diffusion method, a well was cut in to the plates with the help of a sterile 200 µL pipette tip. Fifty microliter of the methanolic ARE was introduced into the well. The plates were incubated overnight at 37°C. Antibacterial effect of the extract on microbial growth was determined by measuring the diameter of clear zone around the well/disc (zone of inhibition). For each bacterial strain, negative and positive controls were maintained where 12.5 µL of 200 µg µL⁻¹ ampicillin (as positive control) and pure solvent (DMSO) or empty well (as negative control) were used instead of the methanolic ARE in the well/disc on same seeded plate. To study the effect of methanolic ARE on growth of bacterial strains in liquid cultures, the growth curves (OD 600 nm was measured at an interval of 1-2 h after the addition of the test and control samples in the log phase bacterial cultures) for each individual cultures along with all control cultures were monitored. The MIC value of methanolic ARE against *E. coli* DH5α and *P. aeruginosa* MTCC1688 was measured using decreasing concentrations of methanolic ARE in the range of 2.50-0.125 mg mL⁻¹ with the help of agar disc diffusion assay. All the values reported in this study are the average of a minimum of three sets of experiments performed independently.

RESULTS AND DISCUSSION

Microorganisms tested during this study are important enteropathogenic bacteria causing considerable damage to public health. Considering that usually the plant antibiotic substances have been found less effective against gram negative bacteria than to gram positive four of the total five strains used in the study belong to gram negative bacteria. It may be remembered that penicillin and some of the other prominent antibiotic agents of fungal origin are also rather selective in their inhibitory action, most of them being inhibitory to gram positive bacteria only (Kumar *et al.*, 2006).

Among the three different extraction methods namely, cold maceration method, soxhlet extraction and reflux extraction that were employed for the extraction of bioactive components from the roots of the *A. racemosus*, the reflux extraction was found the best. The extracts prepared using reflux extraction method produced antibacterial effect, reproducibly against two of the five bacterial strains tested. The solvents used for the extraction of plants were water, ethanol and methanol. The antibacterial activity was more significant in methanol extracts as compared to aqueous extract indicating that the active principle responsible for antibacterial activity is more soluble in organic solvents as indicated previously (Boger *et al.*, 1985). The better results with reflux extraction can be attributed to the fact that the reflux extraction involves higher temperatures than cold

maceration method yielding better extraction in the presence of organic solvents like methanol for the extraction of bioactive compounds like cyclic hydrocarbons (9,10-dihydrophenanthrene). Soxhlet extraction usually is performed for the extraction of essential oils where as the principle component that has been predicted to be involved in antibacterial effect of asparagus root extract is a cyclic hydrocarbon-9,10-dihydrophenanthrene (Boger *et al.*, 1985; Prabuseenivasan *et al.*, 2006).

The preliminary screening for the antibacterial effect of *A. racemosus* root extracts prepared by various methods using different solvents was made by the agar well diffusion assay. The extracts obtained by cold maceration method and soxhlet method did not produce zone of inhibition against any of the five strains of bacteria tested. The methanolic extracts prepared using reflux extraction exhibited strong antibacterial activity in terms of appearance of a clear zone of inhibition on bacterial culture plates of *E. coli* and *P. aeruginosa* as shown in Fig. 1. None of the asparagus root

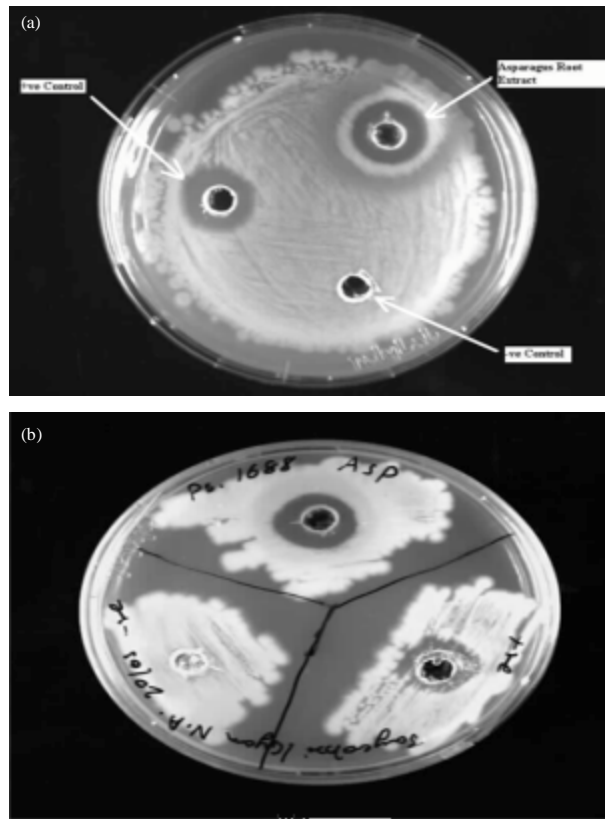


Fig. 1: Antibacterial effect of methanolic root extract of *Asparagus racemosus* wild using agar well diffusion assay. Fifty microliter well⁻¹ of the *Asparagus* root extract (0.50 mg mL⁻¹ in DMSO) was used as test sample Application (12.5 μL of 200 μL mL⁻¹ well⁻¹ served as positive control whereas 50 μL well of pure solvent (DMSO) as negative control. Antobacterial effect of the extract on different bacterial strains was determined by measuring the diameter of clear zone around the well after 18 h of incubation at 37°C, Plate A: Agar well diffusion assay against *E. coli* DH5α, Plate B: Agar well diffusion assay against *P. aeruginosa* MTCC 1688

Table 1: Minimum inhibitory concentration (MIC) of methanolic root extract of *Asparagus racemosus* W using agar disc diffusion assay. Inhibition zone includes the diameter of paper disc (5 mm) and all values are a mean of three set of independent experiments

Bacterial strain	<i>A. racemosus</i> root extract (mg mL ⁻¹)							Ampicillin (200 µg mL ⁻¹)
	2.50	1.80	1.25	0.625	0.50	0.25	0.125	
<i>E.coli</i> DH5α (Zone of inhibition mm ⁻¹)	16	13.5	13.0	12	10	9	--	13
<i>P.aeruginosa</i> MTCC1688 (Zone of inhibition mm ⁻¹)	14	13.0	12.5	12	9	--	--	11

extracts prepared using different methods and solvents were found inhibiting the growth of bacterial cultures reproducibly when tested against clinical isolates of *K. pneumonia*, *S. aureus* and *E. cloacae*. Taking into account that all the three strains growth of which were unaffected upon treatment with asparagus roots extracts were clinical isolates. It is possible that these strains could be the resistant/multi drug resistant ones. The strains that were found susceptible to methanolic ARE during this study, *E. coli* DH5α and *P. aeruginosa* MTCC1688 were further subjected to agar disc diffusion assay and the MIC value of the methanolic ARE (10 mg mL⁻¹) extract was determined by using dilutions of the methanolic ARE in DMSO in the range of 2.50-0.125 mg mL⁻¹ against *E. coli* DH5α and *P. aeruginosa* MTCC1688. The MIC of methanolic ARE was found to be as low as 0.250 mg mL⁻¹ for *E. coli* DH5α whereas it was slightly higher at 0.50 mg mL⁻¹ for *P. aeruginosa*. Table 1 indicates the values for the diameters of zones of inhibition produced by different dilutions of methanolic ARE in comparison with the zone of inhibition produced by ampicillin (as positive control) against *E. coli* DH5α and *P. aeruginosa*, respectively. The size of the inhibition zone for the same bacterium may be influenced by multiple factors like, diffusion capacity of bioactive components (present in the extracts) in the agar medium, antimicrobial activity of diffused substances, pH of substrates in plates, growth and metabolic activity of microorganisms in the medium etc. Therefore the inhibitory activity might not necessarily be proportional to the inhibition zone diameter, especially when comparing different extracts but it still can safely be considered as one of the best and efficient method of identifying bioactive components from crude plant extracts.

Finally the results obtained from agar disc/well diffusion assay were corroborated by the inhibitory effects of methanolic ARE extract (at concentration 10 mg mL⁻¹ and 5 mg mL⁻¹) as observed on the growth curves of the liquid cultures of susceptible bacterial strains *E. coli* and *P. aeruginosa*, respectively (Fig. 2). The growth inhibition patterns (Fig. 2) observed for bacteria in liquid culture also suggests that methanolic ARE at a concentration of 5-10 mg mL⁻¹ is primarily bacteriostatic in nature and not bacteriocidal in which case the OD 600 should have becomes static and never rise again. The reproducibility of the results in liquid culture proves the efficacy of the extracts. In case of *P. aeruginosa*, the 5 mg mL⁻¹ concentration of methanolic ARE was found more inhibitory to the growth of bacteria as compared to 10 mg mL⁻¹ concentration. This could be due to the fact that the extracts used were not pure and crude preparations were tested. So some compounds working antagonistically to the biologically active compounds might also be present. The reduction in concentration of such compounds may lead to better results at lower concentration.

Mandal *et al.* (2000) have also shown previously the *in vitro* antibacterial efficacy of the methanol extract of the roots of *A. racemosus* at a concentration of 50 mg mL⁻¹ against *E. coli*, *S. dysenteriae*, *S. sonnei*, *S. flexneri*, *V. cholerae*, *S. typhi*, *S.typhimurium*, *P. putida*, *B. subtilis* and *S. saureus*. Whereas in this study the methanolic extracts of roots of asparagus has been found to show antibacterial effect in concentration as low as 0.125 mg mL⁻¹ against *E. coli* which is quite significant when compared to the synthetic antibiotic like ampicillin.

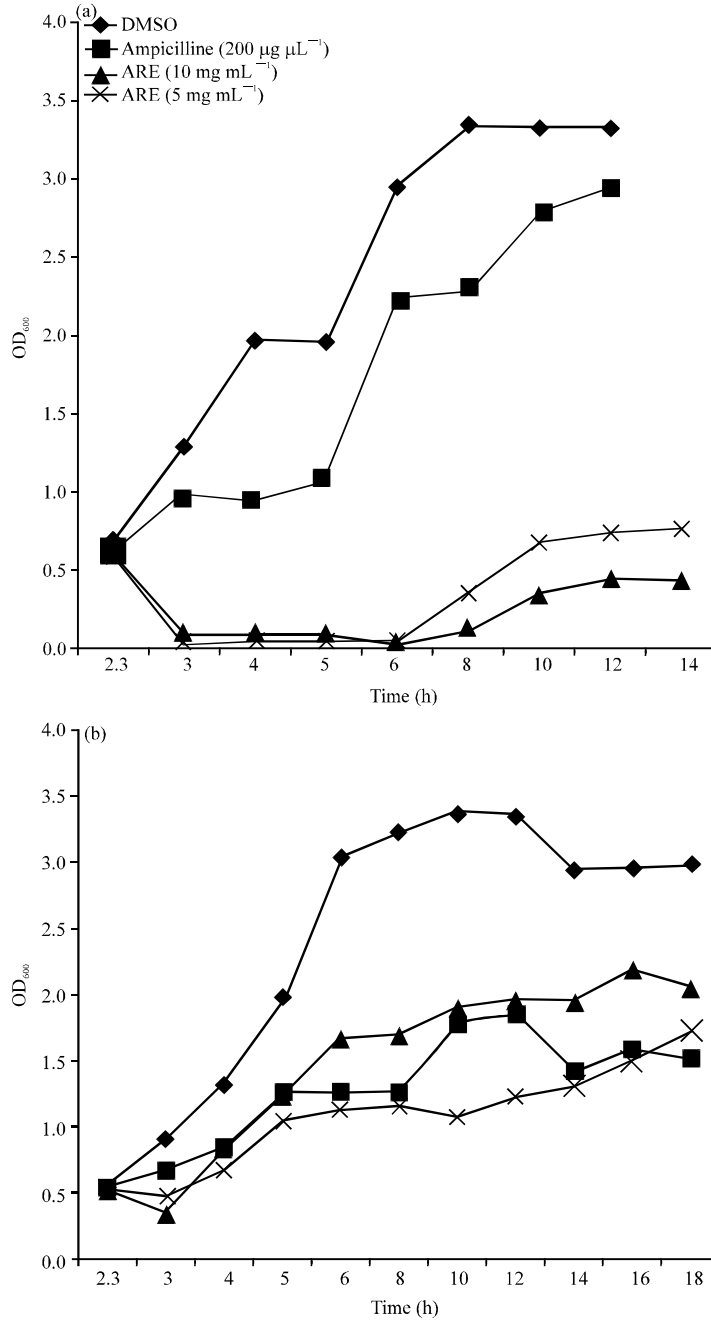


Fig. 2: Studies on antibacterial effect of methanolic root extract of *Asparagus racemosus* wild on liquid culture growth. Methanolic root extract *Asparagus* (ARE) was added at a concentration of 10 mg mL⁻¹ to 20 mL of log phase different bacterial cultures. O.D. 600_{nm} was measured after every 1 to 2 h till stable reading observed. Ampicillin (200 μg mL⁻¹) was used as positive control and plain solvent (DMSO) as negative control. Graph A: Growth curve of *E. coli* DH5α showing growth inhibition in the presence of methanolic root extract of *A. Racemosus* wild, Graph B: Growth curve of *P. aeruginosa* MTTC 1688 showing growth inhibition in the presence of methanolic root extract of *A. racemosus* wild

E. coli and *P. aeruginosa* both are important human pathogen with high resistance to several antimicrobials. Infections caused by *P. aeruginosa* are among the most difficult to treat with conventional antibiotics (Levison and Jawetz, 1992). The present study shows that growth of *E. coli* as well as *P. aeruginosa* was significantly inhibited by methanolic ARE extracts. In future, studies towards isolation and evaluation of bioactive potential of various components of crude extracts of *Asparagus*, in isolation as well as in different combinations with each other *in vivo* shall narrow down to the actual and most effective antimicrobial component of the extract. *Asparagus* as well as many other hitherto unexplored ethno medicinal plants may thus be a source which could yield lead drug molecules that could improve the treatment of infections caused by these and similar organisms.

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