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Evaluation of antimicrobial potential of *Anemone obtusiloba* D. Don.

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ABSTRACT

Medicinal plants have been used from centuries to treat infectious diseases as an alternative form of health care. In recent years there has been intensifying attention in the detection of new antimicrobial compounds; due to alarming raise in the rate of diseases with multi-drug resistant microorganisms. In a search for plant extracts with antimicrobial activity the stem extract of Anemone obtusiloba was evaluated against Staphylococcus aureus and Escherichia coli by Agar disc-diffusion and micro dilution methods. The ethanolic (90%) extract showed significant effects with inhibition zones against S.ureus (10.6±0.5 mm) and E. coli (11.3±0.5 mm), with MIC's agai nst S.ureus (0.625 mg/ml) and E. coli (0.425 mg/ml). Tetracycline was used as standard drug for antimicrobial activity. The results demonstrate that the ethanolic (90%) extract of the stem of A. obtusiloba has significant antimicrobial activity and suggest that it may be useful in the treatment of infections.

Keywords*:* Antimicrobial; Agar disc-diffusion method; Micro dilution Method; Tetracycline

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INTRODUCTION

Diseases caused by bacteria are widespread worldwide. The treatment of these infections is mainly based on the use of antibiotics. Plants used for traditional medicine contain a wide range of substances that can be used to treat chronic as well as communicable diseases [1]. In recent years there has been an increasing interest in the use of natural bioactive compounds and the emergence of multiple resistant strains of clinically important pathogens have led to the search for more effective antimicrobial agents of plant origin with the aim of discovering potentially active ingredients to serve as a basis for the synthesis of new antimicrobials.

A. obtusiloba D. Don. (Ranunculaceae) commonly know as Padar, Rattanjog, Kawashud. It is a densely tufted perennial herb occurring in the Alpine Himalaya from Kashmir to Skkim altitude, 2100 m-4200m and in the Nilgiri hills, altitude above 1800 m [2]. It bears blue coloured buttercup hermaphrodite flowers with short and tufted stems. It is used as purgative and in treatment of rheumatic joints, jaundice, kidney troubles, spleen disorders and also as an antidote of snakebite [3,4]. It contains saponins in the ethanolic extract viz Obtusilobinin, Obtusilobin [5] and Obtusilobicinin [6]. It also contains protoanemonin, an irritating acrid oil that is an enzymatic breakdown product of the glycoside ranunculin [7]. It shows antiinflammatory [8], antirheumatic [7] and Cytological activity [9]. The present study is designed to investigate the antimicrobial activity of the stem extracts of *A. obtusiloba* against selected bacteria with the aim to establish the claimed biological activities of this plant.

MATERIALS AND METHODS

Plant material

The stems of the plant were collected in the month of April from Solan, Himachel Pradesh and authenticated by Dr. Sunita Garg, Chief Scientist, NISCAIR, New Delhi, India (Ref. No.-NISCAIR/RHMD/Consult/2013/2311/91 dated13/09/2013). The stems were shade dried, coarsely powdered and stored in an air tight container till use.

Microorganisms used
The *Staphylococcus aureus* (NCTC 7447) as Gram +ve bacteria and *Escherichia coli* (NCTC 10418) as Gram –ve bacteria strains were employed for the present study. The microorganisms were maintained by sub-culturing and used at regular intervals in nutrient agar medium.

Extraction
The plant material was extracted by cold maceration with ethanol (90%) and distilled water till exhausted completely. The extracts so obtained were freed off solvent under vacuum and used for further studies.

Antimicrobial activity
The antimicrobial activity of *Anemone obtusiloba* (stems) was performed against *Staphylococcus aureus* (NCTC 7447) Gram +ve & *Escherichia coli* (NCTC 10418)–ve bacteria.

Preparation of Standard Bacterial
The strains of *E. coli* and *Staphylococcus aureus* were maintained on Muller Hinton agar (Beef, casein, acid hydrolysate, starch and agar) incubated at 37°C and stored in incubator till use.

Disc diffusion assay:
A modified disc-diffusion method was adapted used for antimicrobial susceptibility testing. The dried plant extracts were dissolved in sterile water, to reach a final concentration of 20 mg/ml and sterilized by filtration by 0.22 µm Millipore filters. The media used were Muller Hinton agar for the bacteria. The discs (6 mm in diameter) were impregnated with 10 µl of the extracts (200µg/disc) at a concentration of 20 mg/ml and placed on the inoculated agar. The antimicrobial (µg/disk) from tetracyclic (30 UI/disc) was used as positive reference standard to determine the sensitivity of the tested microbial strains. The inoculated plates were incubated at 37°C for 24 h for bacterial strains. Antimicrobial activity was evaluated by measuring the zone of inhibition against the tested organisms. All inhibition assays and controls were made in triplicate. [10].

Micro dilution method:
The 96-well plates were prepared by dispensing 50 µl of Mueller–Hinton broth for bacteria, into each well. A 50 µl from the stock solution of tested extracts (concentration of 200 mg/ml) was added into the first row of the plate. Then, twofold, serial dilutions were performed by using a micropipette. The obtained concentration range was from 100 to 0.1953 mg/ml, and then added 10 µl of inocula to each well except a positive control. Plant extract with media was used as a positive control and inoculum with media was used as a negative control. The test plates were incubated at 37^{o}C for 18 h. After 18 h 50 µl of a 0.01% solution of 2, 3, 5- triphenyl tetrazolium chloride (TTC) was added to the wells and the plate was incubated for another hour. Since the colourless tetrazolium salt is reduced to red colored product by biological active bacteria, the inhibition of growth can be detected when the solution in the well remains clear after incubation with TTC. MIC was defined as the lowest sample concentration showing no color change (clear) and exhibited complete the inhibition of growth [11].

RESULTS AND DISCUSSION

The antimicrobial activity of plant material ethanolic (90%) and Aqueous extracts were determined against two microorganisms i.e. *Staphylococcus aureus* as Gram +ve *and Escherichia coli* as Gram –ve determined by agar diffusion and micro dilution methods. The antimicrobial effect of the extracts was observed against the tested microorganisms by agar diffusion method was reported in Table 1 and graphical representation in Fig. 1.

Table 1: Inhibitory effect of samples in Agar diffusion method

Test Sample	Extracts	*Staphylococcus aureus*	*Escherichia coli*
		Zone of Inhibition	
A. obtusiloba (Stem Extract)	Ethanolic Extract (AOEE)	10.6±0.5 mm	11.3±0.5 mm
	Aqueous Extract (AOAE)	7.3±0.5 mm	6.3±0.5 mm
Tetracycline	Reference Standard	24.2±0.5 mm	26.3±0.5 mm

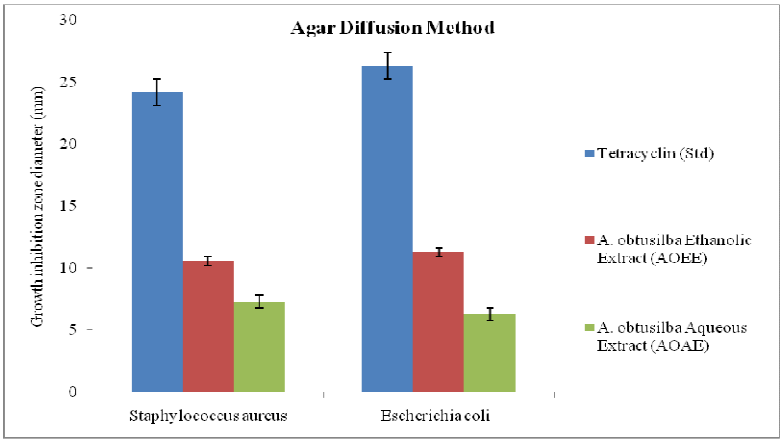

Fig.1: Zone of inhibition by Agar diffusion method

In this method, the bacterial inoculum is adjusted to certain concentration, inoculated onto the entire surface of a Mueller-Hinton agar (MHA) plate with a sterile cotton-tipped swab to form an even lawn. The paper disks impregnated with antibiotic solution was placed on the surface of each MHA plate using a sterile pair of forceps. Then the plates were incubated aerobically and the diameter of zone inhibition was measured by a ruler or caliper. Based on the diameter of the inhibiton zone and the results are then assigned to three categories, susceptible, intermediate, or resistant. The bigger the diameter of the inhibition zone, the more susceptible is the micro-organism to the antimicrobial [12]. In agar diffusion method, the minimum acceptable zone of inhibition was 7mm. The ethanolic (90%) extract of *A. obtusiloba* showed zone of inhibition against *Staphylococcus aureus* - 10.6±0.5mm & *Escherichia coli* - 11.3±0.5mm. In this present studies aqueous extract had showed mild antimicrobial activity as compared to the ethanolic (90%) extracts with respect to zone of inhibition of tetracycline as standard against *Staphylococcus aureus* - 24.2±0.5mm & *Escherichia coli* - 26.3±0.5mm.

Micro Dilution Method

The antimicrobial effect of the ethanolic (90%) and aqueous extracts was observed against the tested microorganisms by Micro dilution method was reported in Table 2 and graphical representation in Fig. 2.

Table 2: Inhibitory effect of Samples in Microdilution Method

Test Sample	Extracts	*Staphylococcus aureus*	*Escherichia coli*
		Minimum inhibitory concentration	
A. obtusiloba (Stem Extract)	Ethanolic Extract (AOEE)	0.625 mg/ml	0.425 mg/ml
	Aqueous Extract (AOAE)	1.15 mg/ml	1.35 mg/ml
Tetracycline	Reference Standard	0.025	0.01

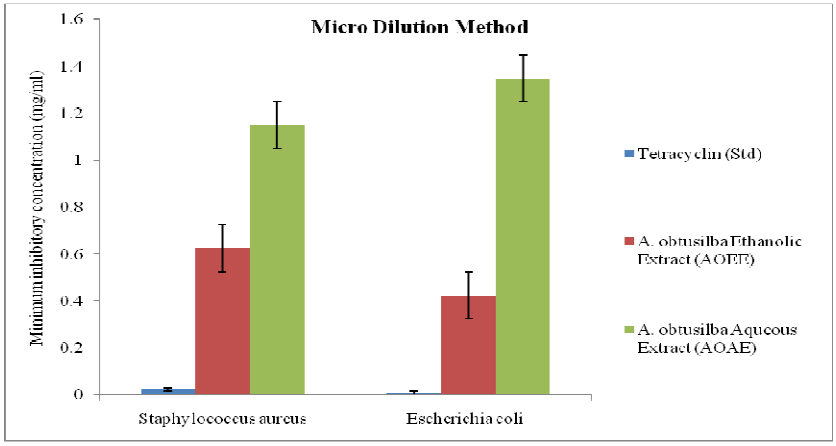

Fig.2: Minimum inhibitory concentration (mg/ml) by Micro dilution method

In micro dilution method, two-fold serial dilutions of an antibiotic made in Mueller-Hinton agar (MHA) medium and then bacterial suspensions were inoculated on the MHA using a Cathra replicator with 1 mm pins. The advantages of agar dilution include the ability to simultaneously test the susceptibility of a number of bacteria in one plate and the ability to test susceptibility of fastidious organisms since the agar with supplements is able to

adequately support the bacteria growth. Moreover, as mentioned above, the test results yield MIC values for testing bacteria [13]. The antimicrobial agents are expressed its potency as minimum inhibitory concentration (MIC) in this method. The method was carried out in a broth dilution test, in which a specific amount of bacteria was added to the serial dilution of antimicrobial agents in broth wells. After incubation, bacterial growth was indicated by turbidity and its lack was indicated as growth inhibited by the antimicrobial agent. The ethanolic (90%) extract of *A. obtusiloba* had minimum inhibitory concentration against *Staphylococcus aureus* - 0.625mg/dl & *Escherichia coli* - 0.425mg/dl. Tetracycline as standard had minimum inhibitory concentration against *Staphylococcus aureus* - 0.025mg/dl & *Escherichia coli* - 0.01mg/dl.

CONCLUSION

Microbial growth commonly persuades undesirable changes during the storage of food products. The avoidance of pathological growth in food products by using antimicrobial compound provides benefit to the shelf life. Antimicrobial substances are abundant in the nature but have encountered problems because of the diversity of criteria and techniques employed for evaluation. Nature has served as a rich source of medicinal plants for thousands of years and an impressive number of modern drugs has been isolated from natural antimicrobial agents with plant origin are not associated with side effects and have an enormous therapeutic potential to heal many infectious diseases. The research on plants as natural antimicrobial has been carried out by *in-vitro* method. The ethanolic (90%) and aqueous extracts of the stem of *A. obtusiloba* showed the significant antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli* in agar diffusion and micro dilution methods. The effect of plant on more pathogenic organisms, and toxicological investigations and further purification, however, need to be carried out.

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