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EXPLORING THE ANTIMICROBIAL PROPERTIES OF *ARTEMISIA ANNUA* DURING DIFFERENT FLOWERING STAGES

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ABSTRACT: Many of the plants used to treat certain diseases because they have shown antimicrobial activity. In this case, many studies have conducted on antimicrobial activity of *Artemisia annua*. The crude extracts obtained from *Artemisia annua* L. (Asteraceae) were investigated for their antibacterial activity by using agar well diffusion assays against three bacterial species (*Escherichia coli*, *Bacillus subtilis*, and *Salmonella typhi*). The purpose of this study is to determine the antimicrobial activity of ethanol, methanol, and hexane extracts of *Artemisia annua* during different flowering stages. The zone of inhibition was calculated. Results indicate that the different concentrations of various extracts under study exhibit antimicrobial activity among different microorganisms. When compared to three organic solvents ethanol extract showed maximum zone of inhibition. Results proved that the parts of *Artemisia annua* might be potential sources of new antibacterial agents. At post flowering stages of the plant, the maximum zone of inhibition was observed when compared to the pre-flowering stage of the plant.

INTRODUCTION: Plant extracts and photochemical compounds have been used for antimicrobial properties and have significant therapeutic properties. In recent years, various investigations have been conducted worldwide to confirm such efficiency of the compounds. Various plants showed antimicrobial properties, which were similar to that of synthetic standard antimicrobial agents. Medicinal plants have been used for mankind against various infectious and non-infectious diseases. Because medicinal plants contain natural bioactive components for therapeutic value.

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According to the World Health Organization, medicinal plants are the best source to obtain a variety of drugs. 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 [1]. Currently, there has been an increased interest in antimicrobial agents from the plant origin due to the resistance that microorganisms have developed against traditional antibiotics [2].

Research studies have been carried out to evaluate the antimicrobial potential of the essential oils obtained from *A. annua*. Although there are 400 species in the genus *Artemisia annua*, only *Artemisia annua* produces artemisinin [3]. Experiments revealed that essential oil showed antimicrobial potential against a wide range of

Gram-ve bacteria and Gram+ve bacteria. Studies based on chemical evaluation of plant extracts evidenced that phytoconstituents are responsible for conferring this antimicrobial potential. Extracts of medicinal herbs in particular essential oils have shown many potential applications in folk medicine, fragrance, cosmetic, phyto preparations, and food technology as reported by several researchers [4, 5].

Artemisinin is also lethal to cancer cell lines, fungi, and bacteria. It has also been used to remove necrotic materials from the body [6]. Today their role in regulating plant growth and coping with cancer has also been proved [7]. The last discovery made from this study was that artemisinin has the potential to be used as a drug for sepsis treatment [8]. Studies have shown that ethanol and chloroform extracts of this plant have been able to prevent the growth of *Bacillus subtilis* and *E. coli*.

Artemisia annua is one of the diverse genera of Asteraceae family with many important medicinally valuable essential oils and secondary metabolites. Artemisia species essential oils have been widely used for a variety of medicinal purposes for many years. Various species of *Artemisia* have been characterized for their biological activities. It is considered to produce secondary metabolites which have greater medicinal importance [9]. *Artemisia* species showed a series of antimicrobial and antioxidant activities [10-14]. By using techniques like HPLC, GC-MS, and NMR, the qualitative determination of various secondary metabolites like flavonoids, terpenoids, saponins, and polysaccharides of *Artemisia* species were detected [15, 16].

Few considerable secondary metabolites were successfully isolated and used in the food industry as an alternative to synthetic antimicrobials [17, 18]. Furthermore, extracts of Artemisia species were used as a natural pesticide and also in the treatment of few human diseases [19-22]. The determination of the potential antimicrobial activity of *Artemisia annua* extracts could be more informative for future use in controlling phytopathogens and also in clinical treatment as natural antimicrobial agents.

Recently, scientists have found that gram-positive bacteria have been developing resistance to antibiotics. Data shows that 12% of bloodstream infections from *E. coli* have developed resistance to antibacterial compounds. By these studies, scientists have worried about bacterial infections. Conclusively, there is now a growing need to find a new antibacterial. The organisms like *Escherichia coli, Bacillus subtilus,* and *Salmonella typhi,* are implicated in causing severe infections in human, as they are found in multiple environmental habitats [23].

In the present study, the antimicrobial potency of methanol, ethanol, and hexane extracts of *Artemisia annua* plant samples during the pre-flowering and post-flowering stages were investigated.

MATERIALS AND METHODS:

Plant Material: Thirty days old seedlings were collected from CIMAP Lucknow, and these were cultivated in pots and collected samples (Leaves, Stem, Roots, and Whole plant) separately before flowering and during flowering.

Preparation of Extracts: About five gms of *Artemisia annua* Leaf, Stem, Root and Whole plant (Before Flowering) and Leaf, Stem, Root and Whole plant (During Flowering) samples were shade dried, powdered and weighed accurately and subjected to extraction in a Soxhlet apparatus at room temperature for 48 h using solvent systems methanol, ethanol and hexane. Alcoholic extracts were concentrated to complete dryness, using rotary evaporator at 50 ºC. Dried extracts were collected and stored in labeled sterile screw capped bottles in a refrigerator for further antimicrobial activity study.

Test Organisms Used: The test microorganisms used for the antimicrobial activity screening were *Bacillus subtilis, Escherichia coli,* and *Salmonella typhi*. Each bacterial strain was suspended in Luria broth and incubated at 37 ºC for 18 h.

Culture Preparation: A loopful of each tested bacterial strains was aseptically transferred into 5 ml of maintained media and incubated at 37 ºC for 18-24 h before use. The optical density at 600 nm of each active culture was adjusted using fresh broth to obtain approximately 10^6 CFU/ml. Bacterial counts were confirmed by plating out on their suitable media and incubated for 48 h. The

stock cultures were maintained in nutrient agar slant at 4 ºC and subcultured monthly.

Antibacterial Bioassay:

Agar Well Diffusion Bioassay: For bioassays, a suspension of approximately 1.5×10^8 bacteria/ml in sterile normal saline was prepared. About 1.5ml was uniformly spread on nutrient agar media (Hi-media) in glass Petri dishes. Kept aside for 15 min and excess of suspension was drained and discarded properly. Wells of 6 mm in diameter and about 2 cm apart were punched in the agar culture medium using sterile cork borer. Respective concentrations were administered to fullness in each well. Culture plates were incubated at 37 ºC for 48 h. Bioactivity was determined by measuring the Diameter of the Inhibition Zone (DIZ) in nm. The plant extract concentrations were taken from 50, 100, 150, and 200 µg/ml were evaluated for a good method. Each experiment was done in triplicates, and the mean of the DIZ was calculated.

Determination of Zone of Inhibition:

Preparation of the Extracts: The extract was dissolved in 10 ml of DMSO to get the concentration of 20 mg/ml. Evaluation of the activity was carried out by cup-plate technique using nutrient agar medium, and the antimicrobial activity was measured in terms of zone of inhibition.

Procedure: The medium was prepared by dissolving all the ingredients in distilled water and subjected to sterilization in an autoclave at 121 ºC for 15 min. The Petri plates were washed thoroughly and sterilized in a hot air oven at 160 ºC for 1 h. 30 ml of sterile molten agar medium was seeded by organisms (about 2ml according to McFarland s standard), in semi-hot conditions (40 ºC) was poured aseptically. Petri plates were allowed to solidify at room temperature. Bores were made on the medium using sterile borer, and 0.1ml of the extract was added to respective bore. The Petri plates seeded with organisms, containing extract were kept in the refrigerator at 4 ºC for 1 h to facilitate the diffusion of the extract. After diffusion, the Petri plates were incubated at 37º + 10 ºC for 24 h in a BOD incubator, and zone of inhibition was observed and measured using a scale. The results of the antibacterial activity of *Artemisia annua* are tabulated in tables.

RESULTS: The antibacterial activity of *A. annua* at Pre & Post flowering stages were examined against three pathogens respectively. The extractions were carried out using ethanol, methanol, and hexane solvents. Compare to three organic solvents ethanol extracts showed the highest zone of inhibition with three microorganisms in comparison to other solvents, respectively. The increased artemisinin content was found in Post flowering stage of the plants. At Pre flowering stage the artemisinin was found in leaves. In the case of root and stemless artemisinin was observed. Highest zone of inhibition was observed in Post flowering stage samples when compared to Pre flowering stage samples. Moderate zone of inhibition was observed with hexane and methanol solvents. Hence, it is suggested that a low inhibition zone may be the cause of decreased activity in phytopathogens. The methanol extract showed the zone of inhibition even at low concentration with *E. coli* compared to *B. subtilis* and *S. typhi*. Our results are also in agreement with these studies suggesting the efficacy of ethanol extract of *A. annua* against different pathogens.

DISCUSSION: The plant extracts can also be used in combination with traditional antibiotics. In the literature, there are reports regarding the use of plant crude extracts [24, 25] in combination with fewer amounts of antibiotics for antibacterial activities, especially for antibiotic-resistant bacteria. The use of antibiotics has reduced the incidence of infectious diseases, but their extensive uses in therapy have led to the appearance of drug-resistant bacteria [26]. For this purpose, numerous plant extracts were screened for antimicrobial properties that could protect people from microbial infections [27]. Results obtained in this study proved that all the pre-flowering parts with ethanol extract (leaf, stem, root, and whole plant) contain a decreased amount of artemisinin. At the pre-flowering stage, artemisinin was found only in leaves. The zone of inhibition was absent in the root sample with a high concentration of the extract (*i.e.*, at 200µl). In the case of the post-flowering stage of the plant, the highest zone of inhibition was observed with all the four samples and with all the three microorganisms. Compare to stem and root, leaves and whole plant showed the highest zone of inhibition **Table 1.**

TABLE 1: ANTIBACTERIAL ACTIVITY OF *A. ANNUA* WITH ETHANOL EXTRACT

Type of Extract	Name of the Micro-Organisms											
	E. coli				*B. subtilis*				*S. typhi*			
	50	100	150	200	50	100	150	200	50	100	150	200
Before Flowering												
Leaf	0	11.3±0.8	14.7±0.6	18.0±1.0	0	11.2±1.0	14.3±1.3	18.0±2.0	0	11.2±1.3	15.7±0.8	20.2±0.8
Stem	0	0	11.7±1.5	15.3±1.5	0	0	11.7±1.5	13.7±1.5	0	9.2±0.8	12.2±1.0	14.7±1.5
Root	0	9.0±1.0	11.0±1.0	13.7±1.5	0	9.3±0.6	12.3±1.5	15.3±1.5	0	0	0	0
Whole Plant	0	9.7±1.5	12.0±2.0	15.7±2.1	0	10.8±1.0	13.8±0.8	16.3±1.5	0	0	0	11.8±1.3
During Flowering												
Leaf	6.7±1.5	12.3±1.5	16.0±1.0	20.0±1.0	0	12.0±1.5	16.2±1.3	20.0±2.0	0	11.8±1.8	17.2±0.8	21.2±1.0
Stem	0	7.7±1.5	16.7±1.5	17.5±1.8	0	7.2±1.0	13.8±1.8	16.0±2.0	0	9.3±1.3	13.0±0.5	16.7±1.5
Root	0	11.8±1.3	14.4±0.6	17.7±1.5	0	10.5±1.5	15.5±1.8	18.2±0.8	0	0	9.0±1.0	14.0±2.0
Whole Plant	0	11.2±1.0	15.0±1.0	20.2±1.3	9.0±1.0	12.0±2.0	13.8±1.3	20.3±1.5	0	11.8±1.3	18.3±1.0	21.2±1.0

Values are means of three replicates ± SD

Samples with hexane extract showed moderate zone of inhibition. When compared to all the four samples at pre-flowering stage, whole plant sample showed less zone of inhibition with all the three microorganisms. The remaining samples showed less zone of inhibition. Whereas in post-flowering stage, even in low concentration (*i.e.*, at 50µl) minimum zone of inhibition was observed in leaf and whole plant samples. Highest zone of inhibition was observed in root and whole plant samples at (*i.e.* at 200µl) concentration **Table 2.**

TABLE 2: ANTIBACTERIAL ACTIVITY OF *A. ANNUA* WITH HEXANE EXTRACT

Type of Extract	Name of the Micro-Organisms											
	E. coli				*B. subtilis*				*S. typhi*			
	50	100	150	200	50	100	150	200	50	100	150	200
Before Flowering												
Leaf	0	0	16.5±0.5	18.5±0.5	0	0	14.7±0.8	17.8±0.8	0	0	10.7±0.8	14.2±0.8
Stem	0	0	13.2±1.0	15.5±0.5	0	0	12.0±0.5	14.0±0.5	0	0	10.0±0.5	13.2±0.8
Root	0	0	14.2±1.0	17.2±1.0	0	0	10.8±0.8	13.0±0.5	0	0	11.0±0.5	13.5±1.0
Whole Plant	0	0	18.0±0.5	19.5±0.5	0	0	16.1±0.9	18.3±0.8	0	0	11.3±0.8	15.5±0.5
During Flowering												
Leaf	0	5.8±0.8	15.7±0.8	19.2±1.0	0	0	17.0±0.5	19.8±0.08	0	0	12.5±0.05	16.2±0.8
Stem	0	0	14.5±0.5	17.2±0.8	0	0	14.0±0.5	15.7±0.8	0	0	11.5±0.5	14.2±0.8
Root	0	0	15.3±1.0	18.0±0.5	0	0	16.2±1.0	19.0±0.5	0	0	12.5±0.5	17.4±0.7
Whole Plant	0	9.8±0.8	19.0±0.5	19.8±0.8	0	0	18.0±0.5	20.5±0.5	0	0	15.2±1.0	17.3±0.8

Values are means of three replicates ± SD

When compared to three solvents used, methanol extract showed less zone of inhibition at the pre-flowering stage. *E. coli* and *B. subtilis* showed minimum zone of inhibition with all the concentrations. Whereas *S. typhi* showed less zone of inhibition at 200µl concentration. In the case of the post-flowering stage, all the three microorganisms showed maximum zone of inhibition with all the four samples **Table 3.**

TABLE 3: ANTIBACTERIAL ACTIVITY OF *A. ANNUA* WITH METHANOL EXTRACT

Type of Extract	Name of the Micro-Organisms											
	E. coli				*B. subtilis*				*S. typhi*			
	50	100	150	200	50	100	150	200	50	100	150	200
Before Flowering												
Leaf	0	9.0±0.5	13.2±1.0	17.5±1.0	0	8.0±0.5	13.2±0.8	17.8±0.8	0	7.2±1.0	12.2±0.8	16.5±0.5
Stem	0	8.0±0.5	11.3±0.8	14.7±0.8	0	0	11.7±0.8	15.8±0.8	0	0	9.2±0.8	14.3±0.8
Root	0	6.3±0.8	10.0±0.5	15.8±0.8	0	9.2±0.8	10.3±0.8	13.2±0.8	0	0	10.0±0.5	12.2±0.8
Whole Plant	0	7.2±1.0	10.3±0.8	14.3±1.0	0	9.2 ±1.0	11.2±0.8	13.7±0.8	0	0	0	9.0 ±0.5
During Flowering												
Leaf	7.2±1.3	11.3±0.8	16.0±0.5	20.2±0.8	0	11.5±0.5	15.5±0.5	19.5±0.5	0	8.7±0.8	13.5±0.5	18.0±0.5
Stem	0	8.3±0.8	13.0±0.5	17.2±0.8	0	7.2±0.8	15.0±0.5	18.8±1.0	0	7.8±0.8	11.0±0.5	17.3±0.8
Root	0	8.7±0.8	12.2±0.8	17.8±0.8	0	9.3±1.0	13.2±1.0	17.2±1.0	0	5.0±0.1	10.8±0.8	13.0±0.5
Whole Plant	0	9.3±1.0	12.3±1.0	17.2±0.8	0	10.2±0.8	13.5±0.5	16.3±1.0	0	8.5±0.5	11.3±0.8	15.3±1.0

Values are means of three replicates ± SD

This study provides scientific validation for highly medicinal plants, in having the potential to be a good drug. Results revealed that *A. annua* is effective against pathogens. This plant is effective even at low concentration and requires further pharmacological screening for the isolation and identification of active compounds.

Studies have shown that phenolic compounds play an important role in the antimicrobial properties of plants. These compounds destroy microorganisms through destroying the cell walls and proteins, interfering in the work of membrane enzymes and affecting DNA and RNA replication. The antimicrobial activity of the extracts of the leaves of this species showed that the ethanol extracts of aerial parts cause repelling of insects [28]. Different antimicrobial activities for the essential oil of *Artemisia annua* was studied [29].

CONCLUSION: Based on the results, obtained from the current study, it is concluded that *A.annua* has a stronger and broader spectrum of antimicrobial activity against several bacteria. The extracts may be used to discover bioactive natural products that may serve as a basic source for the development of new antimicrobial compounds. So, the results overcome the problem of increasing resistance to the known traditional antibiotics. Further studies are going on to identify further uses in the field of medicine.

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