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RESEARCH ARTICLE

EVALUATION OF ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY OF ARTEMISIA ANNUA DURING PRE AND POST FLOWERING STAGES

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ABSTRACT

Artemisia annua L., a medicinal herb, produces secondary metabolites with antimicrobial properties. The purpose of this study is to determine the antimicrobial activity of ethanol, methanol and hexane extracts of *Artemisia annua* during different flowering stages against bacterial species (*Klebsiella pneumoniae, Shigella dysenteriae* and *Staphylococcus areus*) and fungal species (*Aspergillus niger* and *Aspergillus flavus*). The zone of inhibition was calculated. Results indicate that the different concentrations of various extracts under study exhibit antimicrobial activity among different microorganisms. When compare to bacterial and fungal species, bacterial species showed highest zone of inhibition. At post flowering stages of the plant, maximum zone of inhibition was observed when compare to pre flowering stage of the plant.

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INTRODUCTION

Medicinal plants have been used for mankind against various infectious and non-infectious diseases. According to 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 (Cerdeiras *et al.,* 2007). 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 (Essawi and Srour, 2000). Although hundreds of plant species have been tested for antimicrobial properties, the vast majority have not been adequately evaluated (Balandrin *et al.,* 1985). Considering the vast potentiality of plants as sources for antimicrobial drugs with reference to antibacterial and antifungal agents, a systematic investigation was undertaken to study the antibacterial and antifungal activity of *A. annua*. Many strategies are taken intending to prevent fungal growth and further mycotoxin production and food contamination, including chemical, physical or biological treatments which require sophisticated equipment and expensive chemicals or reagents (Reddy *et al.,* 2010a). The use of natural plant extracts provides an opportunity to avoid chemical preservation. Hence, the search for new antifungal agents from natural sources for food preservation has increased (Soliman and Badea, 2002; Irkin and Korukluoglu, 2007). Research studies have been carried out to evaluate 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 (Van de Berg Retrived from http://gordon. library.wpi.edu.) Experiments revealed that essential oil showed antimicrobial potential against 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.

Artemisia annua is one of the diverse genera of *Asteraceae* family with many important medicinally valuable essential oils and secondary metabolites. Essential oils from *Artemisia* species 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

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greater medicinal importance (Priscila *et al.,* 2007). *Artemisia* species showed a series of antimicrobial and antioxidant activities (Juteau *et al.,* 2002; Kordali *et al.,* 2005; Curini *et al.,* 2006; Cha *et al.,* 2007). 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 (Xie *et al.,* 2008; Avula *et al.,* 2009). Few considerable secondary metabolites were successfully isolated and used in food industry as an alternative to synthetic antimicrobials (Ng TB: 2004; Pattison *et al.,* 2004).

Furthermore, extracts of *Artemisia* species were used as natural pesticide and also in the treatment of few human diseases (Mueller *et al.,* 2000; Rahman and Saleh 2006; Saddi *et al.,* 2007; Meneses *et al.,* 2007). The determination of potential antimicrobial activity of *Artemisia annua* extracts could be more informative for the future use in controlling phytopathogens and also in clinical treatment as natural antimicrobial agents. The organisms like *Klebsiella pneumoniae, Shigella dysenteriae, Staphylococcus aureus, Aspergillus niger* and *Aspergillus flavus* are implicated to cause severe infections in human, as they are found in multiple environmental habitats (Maleki *et al*., 2008). In the present study, the antimicrobial potency of methanol, ethanol and hexane extracts of *Artemisia annua* plant samples during pre flowering and post flowering stages were investigated.

MATERIALS AND METHODS

Sample Preparation

About 60 day old seedlings were grown in pots and leaves, stem, roots and whole plants were collected separately before flowering and during flowering.

Preparation of Extracts

About 5gm of *Artemisia annua* leaf, stem, root and whole plant (collected before and during flowering) dry powder was macerated and extracted with 200ml of Methanol, Ethanol, Hexane and distilled water and kept on rotary shaker for 48 hours. The whole extract was then filtered using Whatman No.1 filter paper. The collected extract was then evaporated to dryness and converted into dry powderwith rotary vacuum evaporator at 40^{o}C. This dry powder was dissolved in DMSOto get20mg/ml concentration of extract.

Test Organisms Used

Klebsiella pneumoniae (ATCC 15380)*, Shigella dysenteriae* (ATCC 11835) and *Staphylococcus aureus* (ATCC 25923) are the bacterial *Aspergillus niger* (ATCC 16404) and *Aspergillus flavus* (ATCC 9643) are the fungal microorganisms used for the anti-microbial activity. Each bacterial strain was suspended in Luria broth (Himedia, India) and incubated at 37^{o}C for 18 h. Mueller-Hinton Agar (MHA, Difco, France) was used for testing antibacterial activity. PDA (Himedia, India) was used for testing antifungal activity.

Culture Preparation

A loopful of each bacterial strainwas aseptically transferred into 5ml of maintained media and incubated at 37^{o}C for 18-24 hours before use. The Optical Density at 600nm 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 24 hours.

Determination of Zone of Inhibition Preparation of the Extracts

All the extracts were dissolved in 10 ml of DMSO to get the concentration of 20mg/ml. Evaluation of the activity was carried out by cup-plate technique, using Luria agar medium and the antimicrobial activity was measured in terms of zone of inhibition.

Agar well diffusion bioassay

Antimicrobial Bioassay

For bacterial bioassays, approximately 1.5x10^{8}cells/ml suspension was prepared in sterile normal saline. About 1.5ml of this suspension was uniformly spread on Luria agar media (Hi-media) in glass petri dishes and kept aside for 15 min. The excess suspension was drained and discarded properly. By using sterile cork borer, wells of 6mm in diameter and about 2cm apart were punched in the agar culture medium and labelled for the respective plant extract. Respective concentrations of plant extract were administered to fullness in each well and the culture plates were incubated at 37^{o}C for 24hrs. Potato Dextrose Agar media (Hi-media) was used for fungal bioassay.

Bioactivity was determined by measuring Diameter of the Inhibition Zone (DIZ) in mm. The plant extract concentrations were taken from 50, 100, 150 and 200µg/ml were evaluated for well method. Tetracycline (10 µg/ml) was used as control for bacterial bioassay. Nystatin (10 µg/ml) was used as control for fungal bioassay. Each experiment was done in triplicates and mean of the DIZ was calculated.

RESULTS

Natural products are considered as an important source of new antibacterial agents. Medicinal plants continue to be used worldwide for the treatment of various diseases and have a great potential for providing novel drug leads with novel mechanism of action (Vatlak *et al*., 2014).

Klebsiella pneumoniae is a Gram–negative non motile, encapsulated, lactose fermenting, facultative anaerobic, rod shaped bacterium. *Klebsiella*causes different types of health care associated infections, including pneumonia, bloodstream infections, wound or surgical site infections and meningitis. Antibacterial activity of the extracts of *A. annua* was studied in different concentrations (50, 100, 150 and 200 µg/ml) against *Klebsiella pneumoniae.*

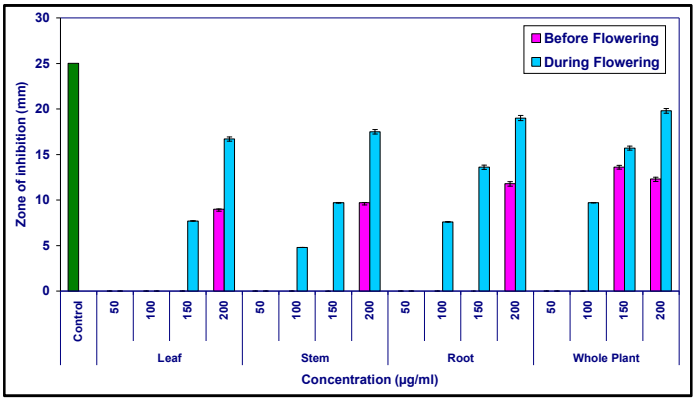

Fig. 1. Antibacterial activity of ethanol extract of different plant parts of *Artemisia annua* on *Klebsiella pneumoniae* before flowering stage and during flowering stage

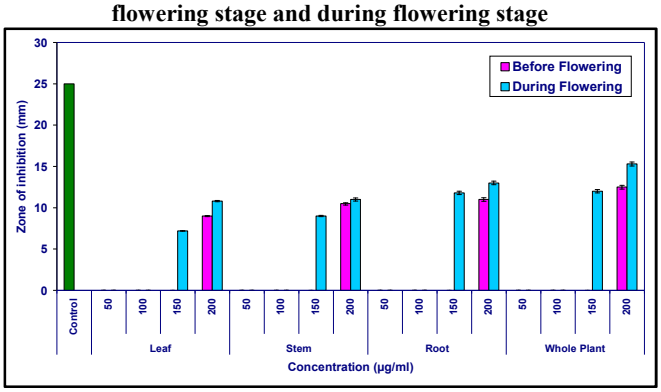

Fig. 2. Antibacterial activity of methanol extract of different plant parts of *Artemisia annua* on *Klebsiella pneumoniae* before flowering stage and during flowering stage

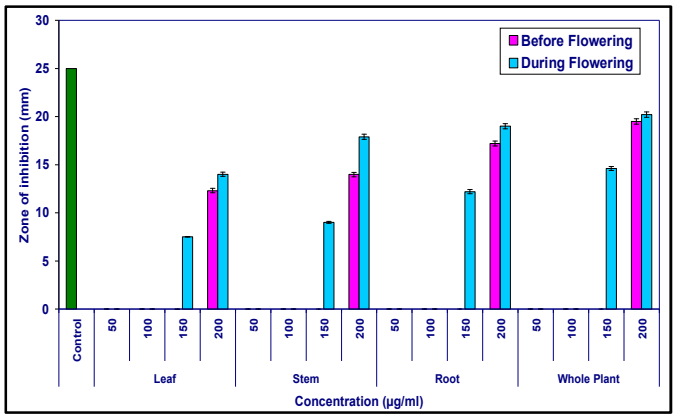

Fig. 3. Antibacterial activity of hexane extract of different plant parts of *Artemisia annua* on *Klebsiella pneumoniae* before flowering stage and during flowering stage

Whole plant of *A. annua* showed highest zone of inhibition (19.8 mm) with ethanol extract followed by root (19 mm) and stem (17.5 mm). Among all the samples, leaf showed lowest zone of inhibition (16.7 mm) with ethanol extract (Fig. 1). Methanol extract showed zone of inhibition with highest concentration i.e., 150 and 200 µl. Leaf sample showed lowest zone of inhibition (10.8 mm) followed by stem (11 mm) and root (13 mm). Highest zone of inhibition was observed in whole plant sample (15.3 mm) (Fig. 2). Hexane extract showed zone of inhibition in flowering stage samples of *A. annua* and absent in before flowering stage samples. Zone of inhibition was observed in before flowering stage with highest concentration i.e., 200µl. Highest zone of inhibition was observed in whole plant (20.2 mm) followed by root (19 mm) and stem (17.9 mm) respectively. Lowest zone of inhibition was observed in the leaf sample (14 mm) (Fig. 3). According to the results obtained, *Klebsiella pneumoniae* showed maximum zone of inhibition with ethanol extract compared to methanol and hexane.

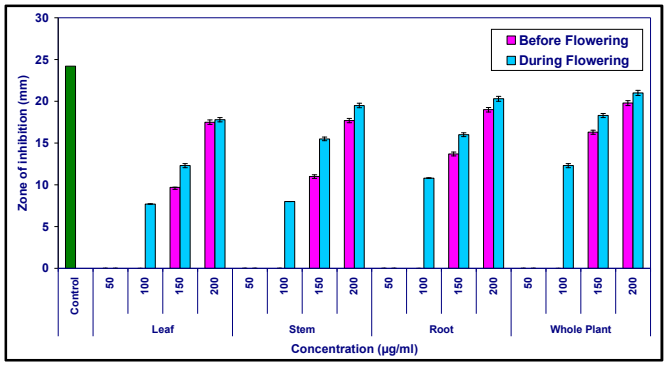

Fig. 4. Antibacterial activity of ethanol extract of different plant parts of *Artemisia annua* on *Shigella dysenteriae*before flowering stage and during flowering stage

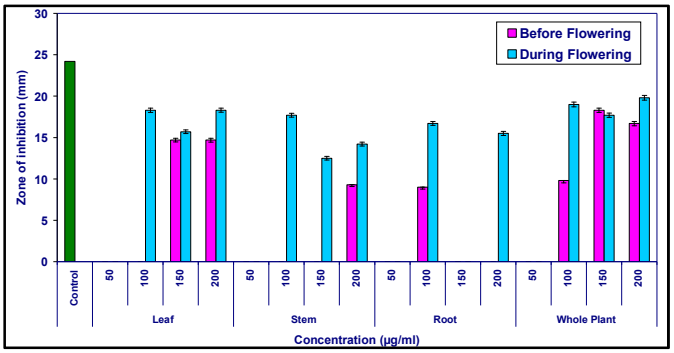

Fig. 5. Antibacterial activity of methanol extract of different plant parts of *Artemisia annua* on *Shigella dysenteriae* before flowering stage and during flowering stage

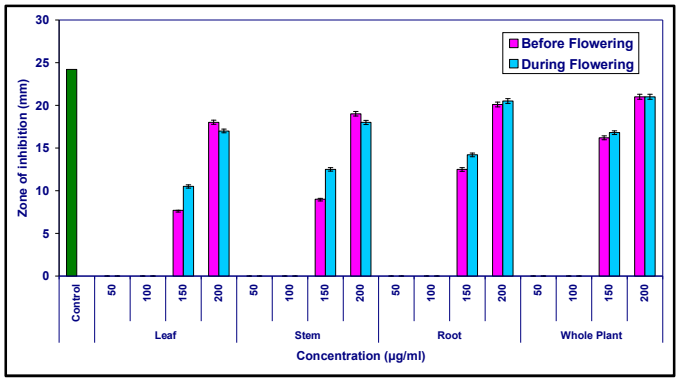

Fig. 6. Antibacterial activity of hexane extract of different plant parts of *Artemisia annua* on *Shigella dysenteriae* before flowering stage and during flowering stage

Shigella dysenteriae is a Gram–negative non spore forming, facultative anaerobic, non motile bacteria. Antibacterial activity of the extracts of *A. annua* were studied with different concentrations (50, 100, 150 and 200 µg/ml) against *Shigella dysenteriae.* Ethanol extract of leaf showed lowest zone of inhibition (17.8 mm) followed by stem (19.5 mm) and root

(20.3 mm). Highest zone of inhibition was observed in whole plant sample (21 mm) during flowering stage of *A. annua* (Fig. 4). Methanol extract of whole plant of *A. annua* showed highest zone of inhibition (19.8 mm) followed by leaf (18.3 mm) and root (15.5 mm). The zone of inhibition was decreased in stem (14.2 mm) (Fig. 5). Hexane extract showed maximum zone of inhibition with all the samples at before and after flowering stages. Whole plant of *A. annua* showed highest zone of inhibition (21 mm) followed by root (20.5 mm) and stem (19 mm). The leaf sample of *A. annua* showed lowest zone of inhibition (18 mm) (Fig.6). Among all the three extracts, ethanol and hexane extracts of *A. annua* showed maximum zone of inhibition. Moderate zone of inhibition was observed with methanol extract.

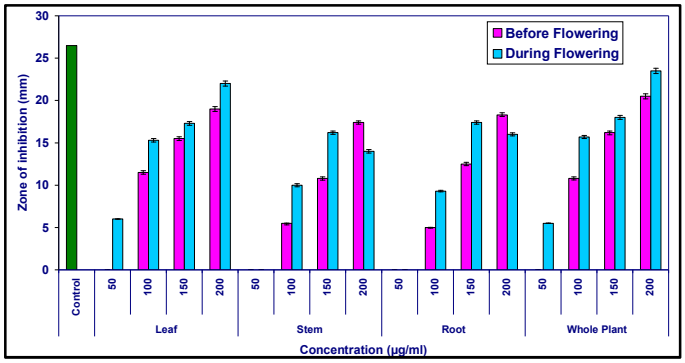

Fig. 7. Antibacterial activity of ethanol extract of different plant parts of *Artemisia annua* on *Staphylococcus aureus* before flowering stage and during flowering stage

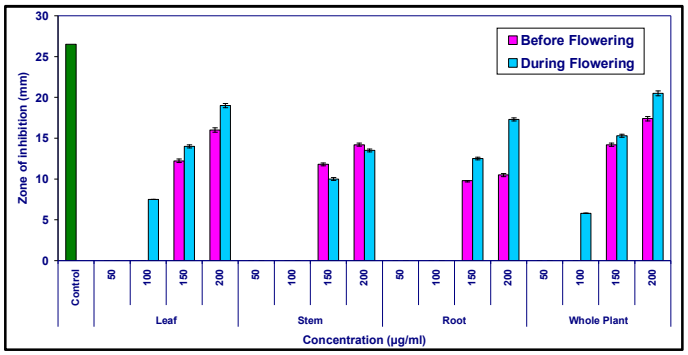

Fig. 8. Antibacterial activity of methanol extract of different plant parts of *Artemisia annua* on *Staphylococcus aureus* before flowering stage and during flowering stage

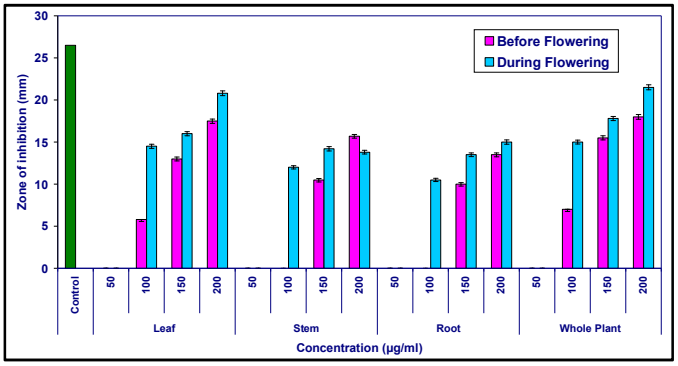

Fig. 9. Antibacterial activity of hexane extract of different plant parts of *Artemisia annua* on *Staphylococcus aureus* before flowering stage and during flowering stage

Staphylococcus aureus is a Gram positive coccal bacterium. It causes common skin infections, respiratory disease and food poisoning. The antibacterial activity of the extracts of *A. annua* were studied in different concentrations (50, 100, 150 and 200 µg/ml) against *S. aureus*. Ethanol extract showed highest zone of inhibition in whole plant (23.5 mm) of *A. annua* followed by leaf (22 mm), root (18.3 mm) and stem (17.4 mm). Samples of *A. annua* before flowering stage showed maximum zone of inhibition compared to during flowering stage with ethanol extract (Fig. 7). Methanol extract of whole plant showed (20.5 mm) followed by leaf (19 mm) and root (17.3 mm). The zone of inhibition was decreased in stem (14.2 mm) (Fig. 8). Hexane extract showed effective zone of inhibition in whole plant (21.5 mm) of *A. annua* followed by leaf (20.8 mm). Lowest zone of inhibition was seen in stem (15.7 mm) and root (15 mm) respectively (Fig. 9). Among all the three extracts, ethanol extract showed maximum zone of inhibition. Moderate zone of inhibition was observed with methanol and hexane.

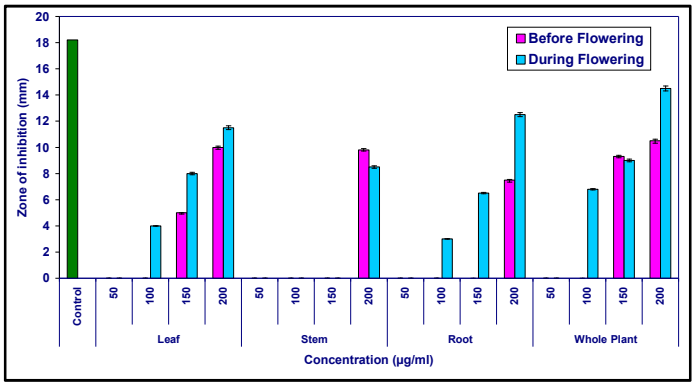

Fig.10. Antifungal activity of ethanol extract of different plant parts of *Artemisia annua* on *Aspergillus niger* before flowering stage and during flowering stage

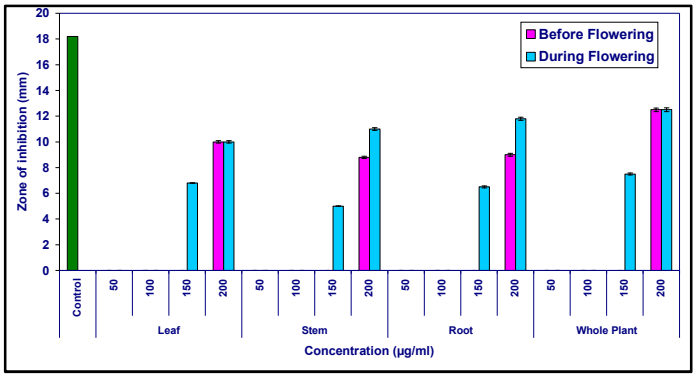

Fig.11:Antifungal activity of methanol extract of different plant parts of *Artemisia annua* on *Aspergillus niger* before flowering stage and during flowering stage

Aspergillus niger is a fungus and one of the most common species of the genus *Aspergillus*. It causes a disease called black mould on certain fruits and vegetables.Antifungal ctivity of the extracts of *A. annua* were studied in different concentrations (50, 100, 150 and 200 µg/ml) against *A. niger*. The highest zone of inhibition was observed in whole plant (14.5 mm) sample of ethanol extract. The second highest zone of inhibition was observed in root (12.5 mm) followed by leaf (11.5 mm) and stem (9.8 mm) (Fig. 10).

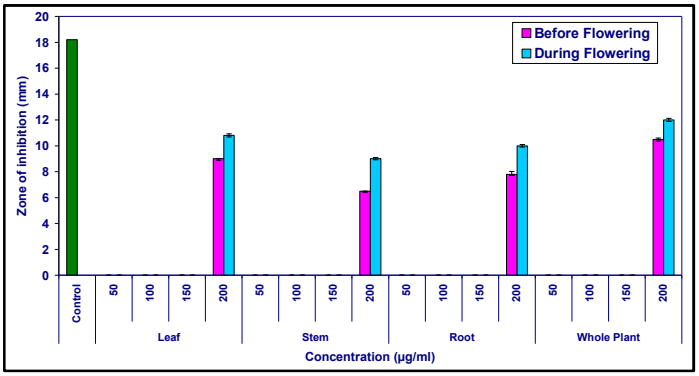

Fig.12:Antifungal activity of hexane extract of different plant parts of *Artemisia annua* on *Aspergillus niger* before flowering stage and during flowering stage

Methanol extract showed equal zone of inhibition at before flowering stage and during flowering stage. Whole plant sample of *A. annua* showed highest zone of inhibition (12.5 mm) followed by root (11.8 mm), stem (11 mm) and leaf (10.8 mm) (Fig. 11). Hexane extract showed highest zone of inhibition in whole plant (12 mm) followed by leaf (10 mm), root (10 mm) and stem (9 mm) (Fig. 12). Among all the three extracts maximum zone of inhibition was observed in ethanol extract. Moderate zone of inhibition was observed in methanol extract and lowest zone of inhibition was observed in hexane extract.

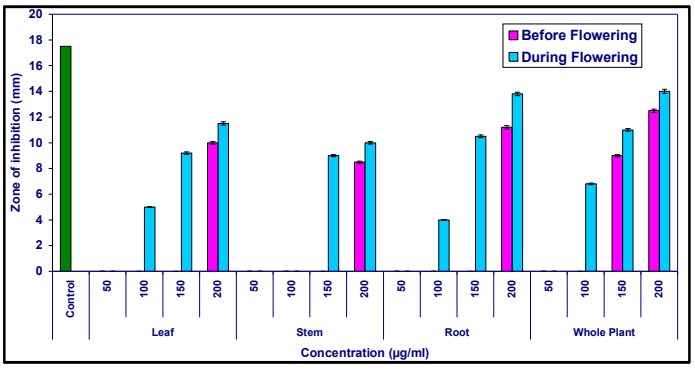

Fig. 13. Antifungal activity of ethanol extract of different plant parts of *Artemisia annua* on *Aspergillus flavus* before flowering stage and during flowering stage

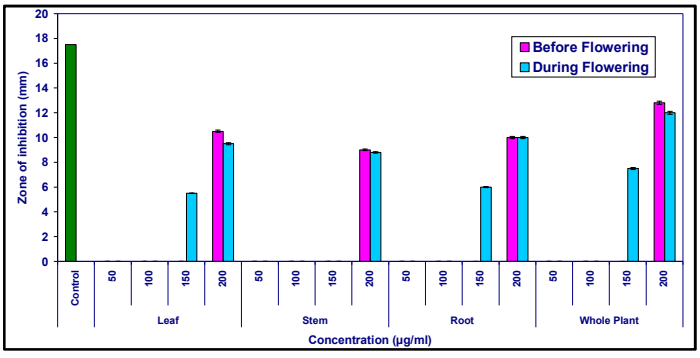

Fig. 14. Antifungal activity of methanol extract of different plant parts of *Artemisia annua* on *Aspergillus flavus* before flowering stage and during flowering stage

Aspergillus flavus is a fungus, able to contaminate many kinds of food including fruit, vegetables and cereals. *A. flavus* is the most common species mainly involved in food spoilage in particular by secretion of highly poisonous secondary metabolites- aflatoxin. It grows by producing thread like branching filaments known as hyphae. *A. flavus* is a saprophyte and grows on dead plant and animal tissue in the soil. Antifungal activity of the extracts of *A. annua* were studied in different concentrations (50, 100, 150 and 200 µg/ml) against *A. flavus.* Ethanol extract showed maximum zone of inhibition in whole plant (14 mm) followed by root (13.8 mm), leaf (11.5 mm) and stem (10 mm) (Fig. 13).

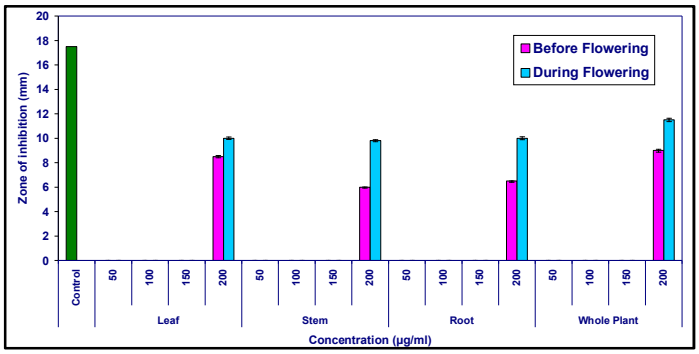

Fig. 15. Antifungal activity of hexane extract of different plant parts of *Artemisia annua* on *Aspergillus flavus* before flowering stage and during flowering stage

Compared to flowering stage samples, before flowering samples showed an effective zone of inhibition with methanol extract. Methanol extract showed highest zone of inhibition in whole plant sample (12.8 mm) followed by leaf (10.5 mm), root (10 mm) and stem (9 mm) (Fig. 14). Hexane extract showed an effective zone of inhibition with flowering stage samples. Whole plant of *A. annua* showed highest zone of inhibition (11.5 mm) compared to leaf (10 mm), root (10 mm) and stem (9.8 mm) (Fig. 15). Among all the three extracts ethanol extract showed maximum zone of inhibition *before* and *during* flowering stages. Moderate zone of inhibition was observed in methanol extract. Hexane extract showed lowest zone of inhibition in all the samples.

DISCUSSION

The use of antibiotics has reduced the incidence of infectious diseases but their extensive uses in therapy, has led to the appearance of drug resistant bacteria (Normanno *et al.,* 2007). For this purpose, numerous plant extracts were screened for antimicrobial properties that could protect people from microbial infections (Lou *et al.,* 2010). This study provides a scientific validation for medicinal plants having potential to be a good drug. This work is a base for the further isolation and identification of potent antimicrobial compounds. Results revealed that *A.annua*is effective against pathogens. This plant is effective even at low concentration. This work requires further pharmacological screening for the isolation and identification of active compounds. Out of the threebacterial isolates and two fungal isolates tested in the current survey, the highest activity was recorded with ethanol extract. While the least susceptible isolates were *A. niger and A. flavus,* compared

to fungal isolates, bacterial isolates showed the significant zone of inhibition. Antibacterial activity of *A. annua* on *S. aureus* and *E. coli* were assessed in France (Juteau *et al.,* 2002), Iran (Verdian-Rizi *et al.,* 2008) and China (Li *et al.,* 2011). While in the France the isolates used grew in the presence of the oil they were strongly inhibited in China, as observed during these works in Cameroon. The activity of the bacteria was influenced by local features like culture conditions, harvest, drying and storage, for instance (Abad *et al.,* 2012). The overall antifungal effect of the tested compounds was weak against the tested fungi, same conclusion was drawn by Galal *et al.,* (2005).

Meanwhile, seems having a free hydroxyl group in the molecules as in dihydroartemisinin favourable for enhancement of the antifungal activity, compared to the presence of carbonyl or ether and ester moieties as in the parent compound or in artemether and artesunate, respectively. The low effect of artesunate may be due to its high hydrophilicity compared to other tested compounds. While there are a number of literature reports on the antimicrobial activity of the essential oils and plant extracts from other *Artemisia* species (Graven *et al.,* 1991; Mehrotraand Rawat, 1993; Kalemba *et al.,* 2002; Stojanovic *et al.,* 2000; Yan *et al.,* 1993; Shafi *et al.,* 2004). To the best of our knowledge, this study was the first study to demonstrate antimicrobial activities of *A. annua* during *pre* and *post* flowering stages against bacteria and fungi.

Studies have shown that the 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. Maggi *et al*. (2005) who studied the antimicrobial activity of the extracts of the leaves of this species showed that the ethanol extracts of aerial parts causes repelling of insects. Friedman *et al.* (2002) showed different antimicrobial activities for the essential oil. It is hoped that this study would lead to the establishment of some extracts that could be used to formulate new and more potent antimicrobial drugs. Widespread public concerns for health and environmental effects of synthetic fungicides and the restriction of their use have led to an urgent push for the search for safer alternative natural preservatives to replace synthetic chemicals. The antimicrobial properties of plant products have been well known and used for food preservation and in medicine for centuries. Nowadays, interest in natural antimicrobial compounds as food preservation and medicine has been increasingly growing.

The results of present investigation clearly indicate that the antibacterial and antifungal activity vary with the species of the plant parts used. The plant studied here can be seen as a potential source of useful drugs. The ability to inhibit microbial growth suggested that *in vitro* cultured *A. annua* could be an alternative for the production of these potential antimicrobial drugs. Further studies must be in progress to further evaluate the mechanisms of action of these extracts on some organisms associated with human diseases and more uses in the field of medicine. 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 bacteria, and the extracts may be used to discover 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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