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Antibacterial and Anticancer activity of essential oil of *Artemisia amygdalina* from Kashmir india

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ABSTRACT

Artemisia amygdalina used as medicinal plant in folk medicine, traditionally used to cure high blood pressure, gastrointestinal ailments, other pharmacological actions such as protecting liver, eliminating fever, sedation and anti-inflammation. This study evaluates the anti-bacterial, and anticancer activity of Artemisia amygdalina essential oil, which is endemic to Himalayan region of Kashmir, India. The antibacterial activity was evaluated by agar well diffusion method and MIC was calculated through Agar dilution method while as anticancer activity was evaluated through MTT method. The zones of inhibition were measured at three different concentrations 5, 10 and 15µl respectively and highest zones of inhibition were found against bacillus subtilis and proteus vulgaris and MIC of five tested bacteria was found between 3.2-6.4 µg/mL. IC_{50} value was found to be at a conc. of 9.7µg/mL for lung and 7.05µg/mL for Hek cancer cell line tested.

Keywords: Artemisia amygdalina, essential oil, antibacterial and anticancer activity

I.INTRODUCTION

Artemisia species, widespread throughout the world, are one of the most popular plants in Chinese traditional preparations and are frequently used for the treatment of diseases such as malaria, hepatitis, cancer, inflammation and infections by fungi, bacteria and viruses. The genus *Artemisia* has been reported to possess a vast range of biological activities such as anti-hepatotoxic, antibacterial, antifungal anti-malarial, cyto-toxic, anti-inflammatory, and antioxidant. Terpenoids, flavonoids, coumarins and sterols are the main constituents of *Artemisia* species [1]. The genus is known to contain many bioactive compounds such as artemisinin which has been isolated from *Artemisia annua*, is not only the current drug of choice for malaria but also possesses profound cytotoxicity against tumour cells [2], *Artemisia amygdalina* Decne commonly known as "Veer Teeth-wan" (Kashmir) is a critically endangered-cum-endemic plant species of the Himalayan region of Pakistan and Kashmir [3]. Callus has been analysed for antioxidant activity via DPPH, riboflavin photoxidation and DNA damage assays. Methanol and aqueous extracts of the callus exhibit more scavenging in DPPH, deoxyribose assay and in contrast, petroleum ether and ethyl acetate extracts have higher activity in riboflavin photoxidation assay [4]. The antidiabetic and antihyperlipidemic effects of petroleum ether, ethyl acetate, methanol, and hydroethanolic extracts of *Artemisia amygdalina* has been reported [5]. Isolation, cytotoxic studies and

simultaneous quantification of the constituents from *Artemisia amygdalina* has been reported [6]. The chemical composition of leaf and stem essential oil of *Artemisia amygdalina* through a combination of capillary GC-FID and GC-MS techniques is reported [7]. Further, the comparison of chemical composition of essential oil of micropropagated plant and that of field grown plant of *Artemisia amygdalina* is also reported [8]. The indiscriminate use of antimicrobial agents has resulted in the emergence of a number of drug resistant bacteria. To overcome the increasing resistance of pathogenic microbes, more resistant antimicrobial agents with novel modes of action must be developed. Therefore there has been an increased interest in looking antimicrobial properties of extracts from aromatic plants particularly essential oils [9]. Cancer which is characterized by unlimited growth of the cells is now a major cause of death in the world. Most commonly used synthetic drugs for cancer chemotherapy not kill only the tumor cells but also normal cells are killed. Also these drugs have some severe side effects. Natural products from medicinal plants represent a fertile ground for the development of novel anticancer agents. Therefore, there is need for novel treatment options with improved features [10-11]. In continuation of our previous work, which has been published in the journal of phyto-pharmacology [12-13], we herein report the anti-bacterial and cytotoxic studies of the essential oil of afore mentioned plant.

II.MATERIALS AND METHODS

Plant Material

The aerial part of the plant was collected from the Botanical garden of Kashmir University, Srinagar, India in Nov-2013. The specimen was identified and authenticated by Akhter H. Malik, curator, Centre for Biodiversity and Taxonomy, University of Kashmir and voucher specimen was deposited in the herbarium (Voucher specimen no.1909 KASH).

Essential Oil Extraction

The aerial parts of plant material were subjected to hydro-distillation for 3 hours using clevenger-type apparatus for oil extraction. The oil sample was dried over anhydrous sodium sulphate and kept in glass vials at – 4^0C prior to analysis.

Chemical Composition

GC–MS Analysis

GC–MS analysis was carried on a Varian Gas Chromatograph series 3800(Ohio Valley, Marietta, U.S.A) fitted with a VF-5 or DB-1 MS fused silica capillary column (60 m × 0.25 mm, film thickness 0.25 µm) coupled with a 4000 series mass detector. Injection volume used was 0.20 µl with split ratio 1:60, Helium was used as carrier gas at 1.0 ml/min constant flow mode. Injector temperature was 230^0C, Oven temperature was programmed from 40 to 250^0C at 3^0C/min. Mass spectra: electron impact (EI+) mode, 70 ev and ion source temperature was 250^0C. Mass spectra were recorded over 50–500 a.m.u. range.

Evaluation of Antimicrobial Activity of Essential Oil

Antibacterial Assay

Bacterial Strains and Culture Media

The antibacterial activity of the essential oil of *Artemisia amygdalina* were tested against a panel of six bacterial strains obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH) Chandigarh, India. The bacterial strains used were *Bacillus subtilis* MTCC 441, *Pseudomonas aeruginosa* MTCC 1688, *Staphylococcus aureus* MTCC 96, *Kellisbie pneumonia* MTCC 19, *Escherchi coli* MTCC 443, and *Proteus vulgaris* MTCC 1771. Bacterial strains were grown in nutrient agar plates at 37 ^{0}C and maintained on nutrient agar slants at 4^{o}C throughout the study and used as stock cultures. Muller Hinton Agar obtained from Hi Media Laboratories, Mumbai, India were used for anti-bacterial activity evaluation.

Agar Well Diffusion Assay

The antibacterial susceptibility tests were carried out using agar well diffusion assay [14], with some modification. Briefly, the overnight cultures of the indicator strains of bacteria were added to 20 mL of liquid nutrient agar. The contents of tubes were transferred to petri plates. After 10 minutes of solidification of the agar petri plates at room temperature, the wells punched on the plates were filled with 20 µL of essential oil, using a capillary micropipette. The incubation was carried out for 18 hours at 37^{o}C for bacteria. After the incubation period, the antimicrobial activity was evaluated by measuring the width of zones of inhibition, using a vernier scale against indicator strains of test microorganisms. Streptomycin sulphate (1000mg/L) was used as a positive control for bacteria.

Determination of Minimum Inhibitory Concentration (MIC)

The agar dilution method recommended by the National Committee for Clinical Laboratory Standards [15], was used with the modification. Muller Hinton medium was prepared and 0.5% of tween-20 was dissolved per 100 mL of agar medium in order to facilitate proper diffusion of agar in the oil. 20 mL aliquot was transferred in to each boiling tube. Sterilization of boiling tubes was carried out in autoclave. Temperature of tubes was regulated up to 38^{0}C and oil samples were added in the concentration range of 0.2-25.6 µg/mL. The contents of the tube were transferred into petri plates which were kept under laminar flow and allowed to dry for 30 minutes. Bacteria were inoculated from fresh cultures into the broth and its turbidity was adjusted in the range of 0.08-0.13 at 625nm. 3µl of inoculums of each bacteria containing 10^5 CFU/mL was added into the plates. Inoculated plates were incubated at 37^{0}C for 18 hours and MIC was determined. Streptomycin sulphate (1000mg/L) was used as positive control for bacteria. Experiments were carried out in triplicate. Inhibition of bacterial growth in the plates containing test oil was judged by comparison with growth, in blank control plate. The MIC was determined as the lowest concentration of the essential oil inhibiting visible growth of each organism on the agar plate [16].

Cytotoxic Activity

Cell Lines and Culture

Cytotoxic assay was carried out by MTT (3-(4,5-Dimethylthiazol-2-yl)- 2,5 diphenyltetrazolium bromide, a tetrazole) protocol in order to evaluate the anti proliferative effect of oil. A sufficient number of exponentially growing cells were used to avoid confluence of the culture during the treatment. Lung (A-549) and Hek (human embryonic kidney) cancer cell lines were seeded at 10^4cells/well and allowed to adhere for 12 hours

Cytotoxicity Assay

In order to evaluate the optimum concentration at which the oil inhibited the cell proliferation in all the cell lines. Cells were treated with different concentration of the oils ranging from 2-10 µg/mL. DMSO was used as a solvent for the dilution of oil, which was also used as an experimental control. Mitomycin-C was used as positive controls at a concentration of 1×10^{-5} µg/mL. After 48 h treatment, cell growth was evaluated by MTT assay [17-18]. MTT solution of 50 µL (5 mg/mL of PBS) was added to each well and the plates were incubated for 3 hours at 37^0C in dark. The media was aspirated and 150 µL of MTT solvent (4 mM HCl, 0.1% Nondet P-40, all in isopropanol) was added to each well to solubilize the formazan crystals. The absorbances of plates were measured on ELISA reader (Benchmark, BioRad) at a wavelength of 570 nm. Each sample was performed in triplicate, and the entire experiment was repeated thrice.

The percentage cell inhibition was calculated using the following equation:

$$\text{Cell proliferation inhibition (\%)} = A_{control}\text{-}A_{sample}$$

III.RESULTS AND DISCUSSIONS

Antibacterial Activity

The in-vitro antibacterial activity of *Artemisia amygdalina* essential oil was determined by the presence of inhibition zones through agar well diffusion method and MIC test by Agar dilution assay. Results of antimicrobial activity by agar well diffusion method are presented in the form of table-1. The essential oil of *Artmesia amygdalina* showed significant antibacterial effect against the entire test microorganisms used for screening. Oil was mainly effective against *Bacillus subtilis* with highest inhibition zone of 24 mm at a concentration of 15 µL (Figure-1). Streptomycin sulphate (1000mg/L) was used as a positive control which showed inhibition zones between 18 -33 mm at a concentration of 6 µL against different microorganisms tested. The MIC value of *Proteus vulgaris* was found between 6.4-12.8 µg/mL. While as MIC of other five tested bacteria was found between 3.2-6.4 µg/mL. No visible growth of any bacteria was found at this concentration as shown in Figure-2. This essential oil also showed a broad spectrum of antimicrobial effects against gram-positive and Gram-negative bacteria tested. The antibacterial activity of this oil against all the tested bacteria is reported first time to the best of our knowledge.

Anticancer Activity

In order to understand the effect of *Artemisia amygdalina* essential oil on human cancer cell lines, experiments were carried using cultured Lung and Hek cancer cell lines by MTT assay (table-2). The oil was found highly

active against both the cancer cell line. But more activity was found against Hek cancer cell line. Percent growth inhibition of 52.3% was obtained at a conc. of 10µg/mL and its IC_{50} value was found to be at a conc. of 9.7µg/mL as is evident from the graph, While as IC_{50} value of Hek cancer cell line was found to be only at a conc. of 7.05 µg/mL(Figure-3). The anticancer activity of this oil is reported first time to the best of our knowledge. As the cytotoxic potential of this essential oil is very high, therefore it will be of great interest to both pharmaceutical and food industries because of their possible use as natural additives to replace toxic synthetic food additives.

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Table-1: In-vitro antibacterial activity of essential oil of *Artemisia amygdalina* and refrence antibiotic determined with agar well diffusion method

S.No.	Test bacteria	5µL	10µl	15µL	Antibiotic	MIC

						(µg/mL)
		Zones of inhibition(in mm)				
01	*Proteus vulgaris* MTCC 426	14±0.34	16±1.0	17±0.57	25±0.0	>6.4
02	*Bacillus subtilis* MTCC 441	14±0.0	16±1.53	24±0.57	33±0.0	>3.2
03	*Staphyloccocus aureus* MTCC 96	11±1.0	13±0.0	15±1.0	19±0.58	>3.2
04	*Klebsiella. pneumonia* MTCC 19	11±1.0	12±1.0	14±0.57	20±0.0	>3.2
05	*Escherichai coli* MTCC 443	10±1.0	11±0.57	12±0.57	18±0.58	>3.2
06	*Pseudomonas aeroginosa* MTCC1688	10±0.0	12±1.0	14±0.58	19±0.57	>3.2

Table-2: In-vitro anticancer activity of *Artemisia amygdalina* essential oil

1.

Tissue type		**Lung**	**HEK Human embryonic kidney**
Cell Line Type		**A-549**	
S. No.	**Conc. µg/mL**	**%Growth Inhibition**	**%Growth Inhibition**
1	2	18.4	34.1
2	5	29.7	41.6
3	8	38.7	54.2
4	10	52.3	68

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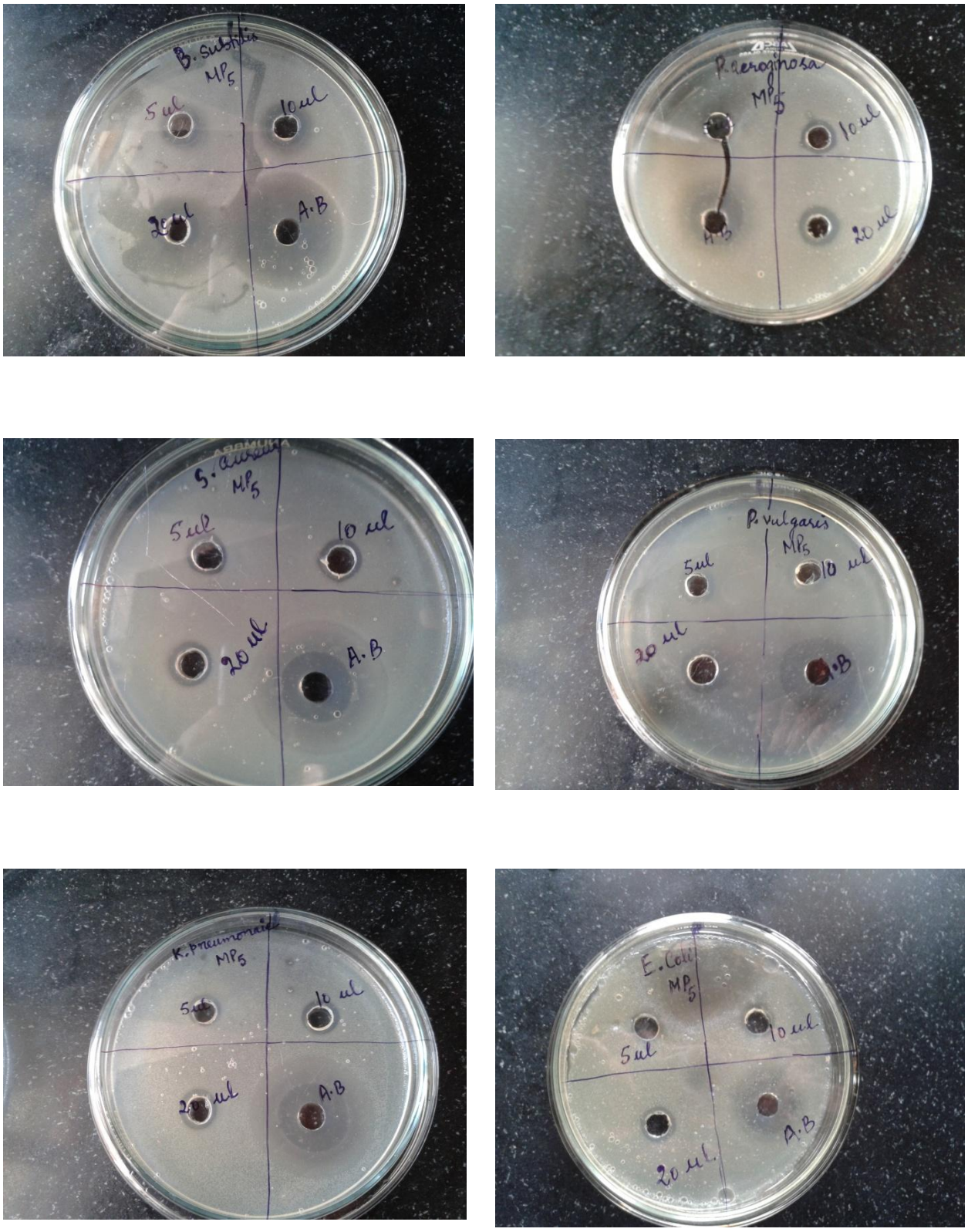

Fig-1: Plates showing zones of inhibition against the six bacterial strains

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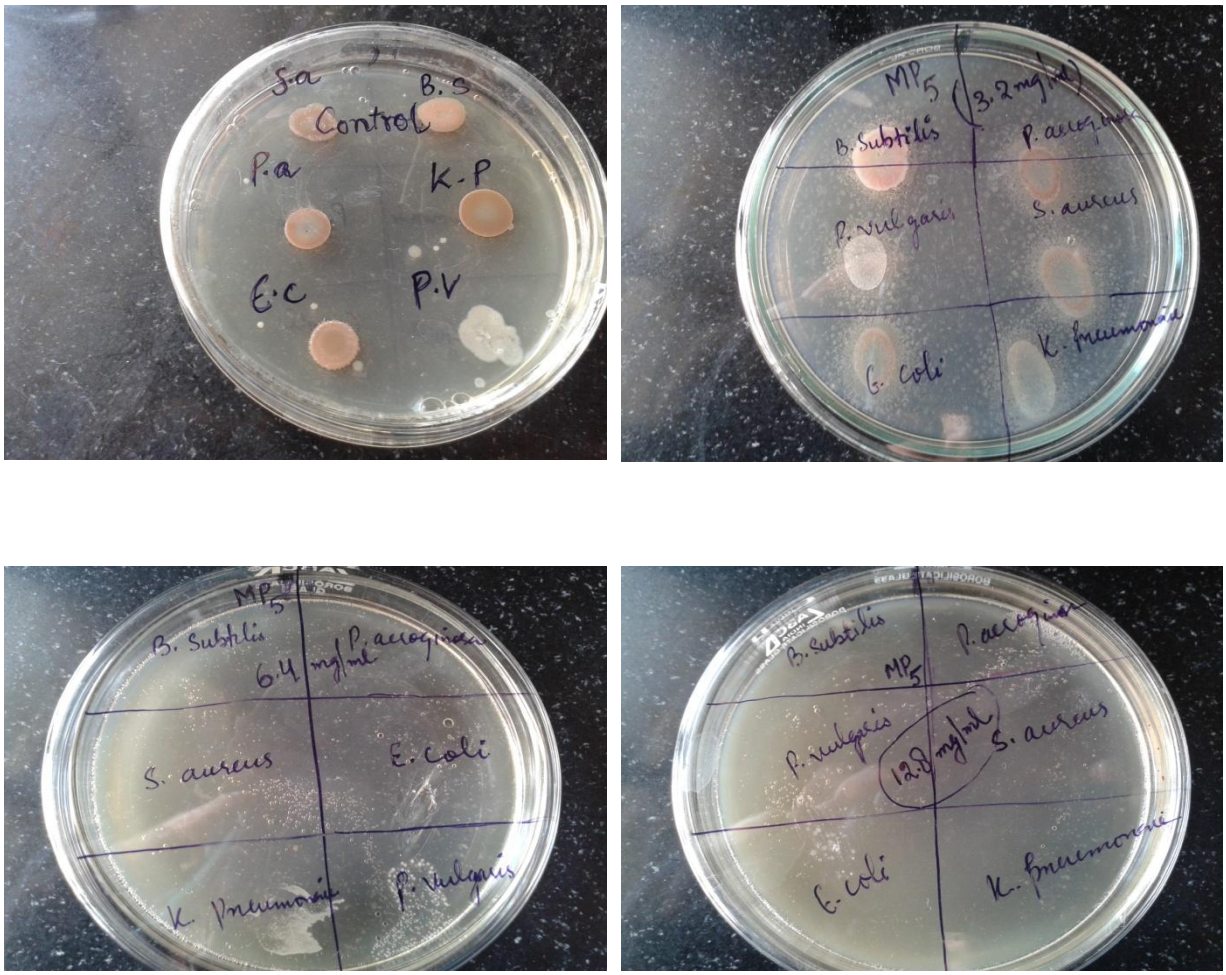

Fig-2: Plates showing minimum inhibitory concentration (MIC)

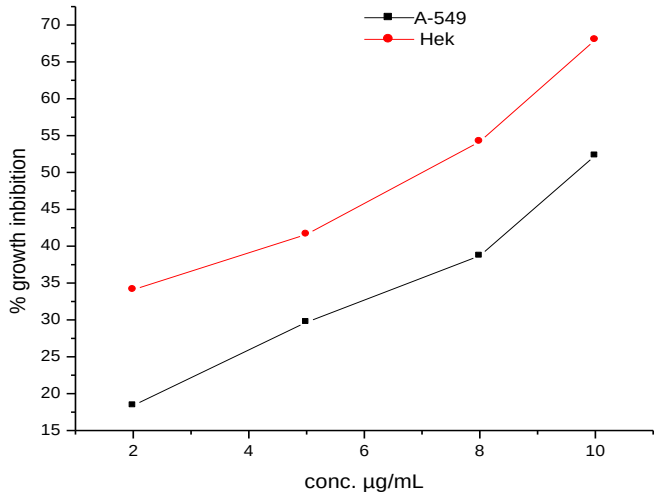

Fig-3: Cytotoxic activity of *Artemisia amygdalina* essential oil against lung and Hek cancer cell line