Supporting Information

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Comparisons of Chemical and Biological studies of Essential Oils of Stem, Leaves and Seeds of *Zanthoxylum alatum* Roxb growing wild in the State of Azad Jammu and Kashmir, Pakistan

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S1. Material and Methods

S. 1.1 Collection of Plant Material

Zanthoxylum alatum has been collected from form District Kotli at High altitude of the State of Azad Jammu and Kashmir in July-September, 2009. The plant was identified and authenticated by the plant taxonomist by Prof. Shafique-ur-Rehman, at the Department of Botany, University of Azad Jammu and Kashmir, and Muzaffrabad. A voucher specimen (AJKBOT-425) has been deposited at the Laboratory of Botany, Department of Botany, University of Azad Jammu and Kashmir, and Muzaffrabad.

S. 1.2. Isolation of Essential Oil

Air-dried plant material of *Zanthoxylum alatum* was ground and subjected to hydrodistllation for 3-5 hours, using a Clevenger-type apparatus as recommended by British Pharmacopeia (1988,). Briefly, the plant was immersed in water and heated to boiling, after which the essential oil was evaporated together with water vapors and finally collected in a condenser. The distillate was isolated and dried over anhydrous sodium sulfate [1, 2].

S. 1.3. GC/MS Analysis

GC/MS analyses of the essential oils were performed using an Agilent Technologies (Little Falls, CA, USA) 6890 N Network gas chromatographic (GC) system, equipped with an Agilent Technologies 5975 inert XL mass selective detector and Agilent Technologies 7683B series auto-injector. Compounds were separated on an HP-5 MS capillary column (30 m× 0.25 mm film thickness 0.25 μm; Agilent Technologies). A sample of 1.0 μL was injected in the split mode with split ratio 1:100. For GC–MS detection, an electron ionization system, with ionization energy of 70 eV, was used. Column oven temperature programme was the same as in GC analysis. Helium was used as the carrier gas at a flow rate of 1.5mL/min. Mass range was 50–550 *m/z*, while injector and MS transfer line temperatures were set at 220 °C and 290 °C, respectively.

S. 1.3.1. Compounds Identification

The identification of components was based on comparison of their mass spectra with those of NIST (National Institute of Standards and Technology) mass spectral library, as well as on comparison of their retention indices either with those of authentic compounds or with literature values [3].

S. 1.4. Antibacterial Assay by Disc Diffusion Method

Nutrient agar (Oxoid, UK) 28 g/L was suspended in distilled water and distributed homogenously and Sterilized. Inoculum (100 μL/100 mL) was added to the medium and poured in sterilized Petri plates. After this, 6mm wicks of paper were laid flat on growth medium containing 100 μL of extract. The Petri plates were then incubated at 37 ºC for 24 hours, for the growth of bacteria. The extracts having antibacterial activity, inhibited the bacterial growth and clear zones were formed. The zones of inhibition were measured in millimeters using zone reader [4].

S. 1.5. Antibacterial Assay by Streak Plate Method

S. 1.5.1. Preparation of Nutrient Agar Plates

About 60-70 mL of nutrient agar (Oxoid) 28 g/L was sterilized and then cooled to 45 ºC and added to pre-sterilized Petri plates (150 mm) and allowed to solidify. Paper wicks were used for the extract to be added (6 mm diameter).

S. 1.6. Determination of Antibacterial Activity

A Petri plate was taken for each essential oil and 20 µl was poured in the well with the sterilized micropipette. Then the four cultures of bacteria including *Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Pasteurella multocida* were streaked one by one from center to the periphery of the Petri plate radially of the well with the help of inoculating loop. The Petri plates were incubated at 37 °C for 24 hours. The inhibition zone diameters (mm) were observed and recorded. Amoxillin drug was used as standard [5].

S. 1.6.1. Growth Medium, Culture and Inoculum Preparation

Pure cultures of the bacteria were maintained on sabouraud dextrose agar (SDA) medium in slant add Petri plates that were pre-sterilized in hot air oven at 180 ºC for 3 h. These cultured slants were incubated at 28ºC for 3-4 days for the multiplication of bacterial strains.

S. 1.7. Antifungal Assay by Disc Diffusion Method

The prepared sterilized growth medium was transferred to the sterilized Petri plates. The Petri plates were then incubated at 28 ºC for 48 hours, for the growth of fungus like *Aspergillus niger, Ganoderma leucedum, Aspergillus flavus and Fusarium solani*. Small filter paper discs were laid flat on growth medium having fungal growth, and 100 µL of extract was applied on each disc. The Petri plates were again incubated. The extracts having antifungal activity exhibited clear zones around the discs. The zones of inhibition were measured in millimeters using zone reader. Fluconazole drug was used as standard [6,7].

S.1.8. Antifungal Assay by Streak Plate Method

S. 1.8.1. Preparation of Potato Dextrose Agar Plates

About 60-70 mL potato dextrose agar (Oxoid, UK) 39 g/L was suspended in distilled water, mixed well and distributed homogenously. Sterilized the medium by autoclaving at 121ºC for 30 min and 15 lb. Potato dextrose agar was then cooled to 45 ºC and added to pre-sterilized Petri plates (150 mm) and allowed to solidify.

S.1.9. Determination of Antifungal Activity

Whatmann No. 1 filter paper was used to cut 6 mm size paper wicks by a punching machine. These discs were sterilized at 121°C for 30 min and 15 lb. At the center of Petri plate a disc was positioned and then 20 µL essential oil sample was soaked on the disc. Then 3-4 days old cultures of fungal strains were streaked one by one over the media plates radially. The Petri plates were incubated at 28°C for 2-3 days. The inhibition zone diameters (mm) were observed and recorded. Fluconazole drug was used as standard [8].

S.1.10. Minimum Inhibitory Concentrations (MIC) of Plant Extracts

The MIC was defined as the lowest concentration of compound that showed no increase in absorbance for all the replicates compared to the negative control. Minimum inhibitory concentration (MIC) of extracts of *Z. alatum* was checked on the selected fungal cultures using micro titer plate-based assay. Resazurin was used as an indicator of cell growth [9].

S. 1.10.1. Micro Dilution Broth Method

For calculation of minimum inhibitory concentration (MIC), which represents the concentration that completely inhibits the growth of microorganisms, a micro dilution broth susceptibility assay was used [14]. A series of dilutions were prepared in the range 0.01– 72.0 mg/mL of the fennel essential oil in a 96-well micro titer plate, including one growth control (NB/SDB + Tween 80) and one sterility control (NB/SDB + Tween 80 + test oil). 160 µL NB and SDB for bacteria and fungi, respectively, were added onto the microplates with 20 µL of the tested solution. Then, 20 µL 5 × 105 CFU/mL (confirmed by viable count) of standard microorganism suspension was inoculated onto the micro plates. The plates were incubated at 37°C for 24 hours for bacteria and at 30°C for 48 hours for fungi. Amoxicillin was used as a reference compound for antibacterial and flumequine for antifungal activities. The growth was indicated by the presence of a white ‘pellet’ on

the well bottom. The MIC was calculated as the highest dilution showing complete inhibition of the tested strains.

S.1.11. Antioxidant Potential of Plant Extracts and Essential Oils

S. 1.11.1. DPPH Radical Scavenging Assay

The 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) assay was carried out spectrophotometrically. Aliquots (50 µL) of various concentrations (10–100 µg/mL) of the essential oil and extract samples was added to 5 mL of a 0.004% methanol solution of DPPH. After a 30 min incubation period at room temperature, the absorbance was read against a blank at 517 nm:

$$I(\%) = 100 \times (A_{blank} - A_{sample}/A_{blank})$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance of the test compound. Extract concentration providing 50% inhibition (IC_{50}) was calculated from a graph plotting percentage inhibition against extract concentration.

S. 1.11.2. Antioxidant Activity Determination in Linoleic Acid System

The antioxidant activities of fennel essential oil, methanol and ethanol extracts were also determined in terms of measurement of percentage inhibition of peroxidation in the linoleic acid system, following the method described by Iqbal and Banger [10] with some modifications. Essential oil and extracts (5 mg) were added to a solution mixture of linoleic acid (0.13 mL), 99.8% ethanol (10 mL) and 10 mL 0.2 M sodium phosphate buffer, pH 7. The total mixture was diluted to 25 mL with distilled water. The solution was incubated at 40 °C for 175 h. The extent of oxidation was measured by the peroxide value, using the colorimetric method[10].

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