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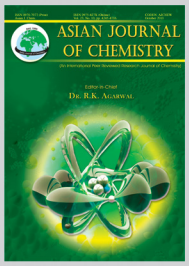

Comparative Study of Antibacterial Activity and Mineral Contents of Various Parts of *Verbena officinalis* Linn.

DILDAR AHMED[1,*], MUHAMMAD ASHRAF CHAUDHARY[1], AKHTAR RAZA[1], ABDUL WAHEED[1], SHAHID REHMAN KHAN[2] and MUHAMMAD IKRAM[1]

[1]Department of Chemistry, Forman Christian College, Lahore, Pakistan
[2]Applied Chemistry Research Centre, PCSIR Laboratories, Lahore, Pakistan

*Corresponding author: E-mail: dildarahmed@gmail.com

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Verbena officinalis Linn., a wild herb found in Pakistan and many other parts of the world, is known for its rich folkloric and traditional medicinal applications. The present study deals with antibacterial activities and mineral content of different parts of the plant. Antibacterial activities of the ethanolic extracts of leaves, stems and roots were studied against five ATCC standard strains which include *Staphylococcus aureus* (ATCC25923), *Escherichia coli* (ATCC25922), *Pseudomonas aeruginosa* (ATCC27853), *Enterococcus faecalis* (ATCC29212) and *Acinetobacter baumannii* (ATCC29213) and eight clinical isolates including MRSA (n = 4) and MDR *Salmonella typhi* (n = 4). All the plant parts showed highest efficacy against *S. aureus*, while stems exhibited, in general, better antimicrobial activity than leaves and roots. Sodium, potassium, magnesium, iron, cadmium, nickel, copper, zinc, manganese, chromium, cobalt and lead were estimated in leaves, roots, stems, seeds and seed husk of *V. officinalis*. All plant parts are good source of potassium, which is approximately 4 times higher than sodium, seed husk being the richest in potassium showed highest potassium to sodium ratio (4492/897). Roots contain higher values of iron (1763.14 ppm) and manganese (117.29 ppm), while seed husk contains higher quantities of magnesium (778.53 ppm) and zinc (54.00 ppm). Chromium, cobalt and lead were not found in any part of the plant studied.

Key Words: *Verbena officinalis*, Antimicrobial activity, Mineral elements.

INTRODUCTION

Growing resistance in pathogenic microorganisms against existing antibiotic drugs is an alarming problem worldwide[1-3]. As a result, the search for new antimicrobial drugs is one of the most important areas of research today. Plants, which are the oldest source of therapeutic agents, have once again become one of the main subjects of investigation worldwide[4,5]. During the last decade, a rapidly growing number of researchers have focused their attention towards the study of antimicrobial activities of herbal samples, crude extracts as well as pure natural products[6-14].

Verbena officinalis Linn. is a well-known plant of the family Verbenaceae, which is a large family of perennial herbaceous plants. There are conflicting reports about the number of genera and species of this family[15,16]. According to one report Verbenaceae consists of about 30 genera and some 1,100 species[17]. The genus *Verbena comprises* about 200-250 species worldwide[18-20]. *Verbena officinalis* is distributed throughout the plains of subcontinent, Pakistan and India and up to 7,000 ft. on Himalaya from Kashmir eastwards[21]. It is also found in North Africa and throughout Europe[22] and has been introduced to many other parts of the world such as North America and South Africa[23]. In Pakistan, the plant is fairly common near water in waste lands and cultivated fields in northern and western regions, between 500 and 2000 m[24]. *Verbena officinalis* is erect perennial, 25-100 cm tall, somewhat woody at base and branched above, with leaves oblong to oblong-lanceolate, 3.5-8.0 cm long, 1.5-3.5 cm broad[24]. The flowers are small to medium-sized and are usually borne on spikes[18]. The plant that has an old history is reputed for its immense medicinal importance[25]. It has been used in folk medicine for the treatment of various diseases such as skin burns, abrasion, gastric diseases[26], rheumatism arthritis, rheumatoid arthritis[27,28], common cold[29], inflammation[30], acute dysentery, enteritis, amenorrhea, depression, Alzheimer's disease[31] and stone kidney formation[32]. Leaves are used as a febrifuge and tonic and roots as a cure for scorpion and snake bite[24]. Additionally, the plant is part of a number of medicinal formulations used for the treatment of cancer[33], liver diseases[34], folliculitis[35], scald head[36], prostatitis[37], nephrosis[38], phagocytosis[39] and cosmetics[40].

Phytochemical studies on the plant have shown that it contains terpenoids, flavonoids, iridoids and glycosides[41-44] which explain the efficacy of the plant as remedy for various ailments. In Pakistan, the local healers use *V. officinalis* (popularly known as *Kori booti* or bitter herb) to treat a number of conditions such as fever, depression, malaria, diphtheria, hepatitis, influenza and liver complaints. In our survey conducted in Lahore, the second largest city of Pakistan, hakeems (practitioners of herbal medicine) confirmed the claimed effectiveness of the herb against various illnesses. The immense ethnomedicinal reputation of the plant tempted us to carry out antimicrobial investigation and to determine mineral elements in various parts of the herb. The study presents a comparison of efficacy of leaves, stems and roots against a number of bacteria and evaluates mineral content in leaves, stems, roots, seeds and seed husk.

EXPERIMENTAL

The whole plant of *V. officinalis* was collected from suburb of Lahore, Pakistan in July 2008. The botanical identification of the plant was confirmed by the taxonomist Professor Jalal Ahmed, Government Tibbia College, Lahore. A sample of the plant was kept at the Department of Chemistry, Forman Christian College, Lahore.

Extract preparation: The leaves, stems, roots, seed and seed husk were separately dried under shade for 2 weeks. For antimicrobial study, the dried and powdered leaves, stems and roots were extracted with 95 % ethanol at room temperature (100 mL × 10 days × 3). In each case, the extracts were filtered and the filtrates were combined followed by the evaporation of the solvent under reduced pressure using rotary evaporator. The dried ethanolic extracts of leaves, stems and roots (3 g each) was then dissolved in 1,2-propanediol to prepare stock solutions (100 mL each).

Antimicrobial study

Microorganisms: In the antimicrobial study, 13 strains of various microorganisms were used to determine mean zones of inhibition, which included 5 ATCC reference strains and 8 clinical isolates. The ATCC reference strains were *Staphylococcus aureus* (ATCC25923), *Enterococcus faecalis* (ATCC29212), *Escherichia coli* (ATCC25922), *Pseudomonas aeruginosa* (ATCC27853) and *Acinetobacter baumannii* (ATCC29212). The clinical isolates included methicillin-resistant *Staphylococcus aureus* (MRSA, four strains) and multidrug-resistant (MDR) *Salmonella typhi* (four strains). Minimum inhibitory concentrations were determined against the five ATCC bacteria, three MDR *S. typhi* and three MRSA clinical isolates. The reference bacteria and clinical isolates were identified by standard morphological, cultural and biochemical profile (API-20E, bioMerieux, France)[45].

Agar well diffusion assay for mean zones of inhibition: To evaluate antimicrobial susceptibility of the ethanolic extracts of the leaves, stems and roots of *V. officinalis*, agar well diffusion assay was used as recommended by Clinical and Laboratory Standards Institute (CLSI)[46] with slight modification. In each experiment, the bacterium under test was spread on the Mueller-Hinton Agar (MHA) medium (Merck, Germany) in a Petri dish (10 µL) and the Petri dish was incubated at 37 ºC for 24 h to obtain uniform well-isolated colonies of the bacterium. To prepare a bacterial suspension, 4-5 morphologically identical colonies of the bacterium were transferred into a test tube containing autoclaved normal saline (NS) solution (5 mL). The turbidity of the suspension was matched with 0.5 McFarland solution used as a standard.

The bacterial suspension was carefully transferred into Petri dishes (90 mm diameter) containing a thoroughly mixed and autoclaved MHA medium (20 mL each). The plates were allowed to dry for a few minutes at room temperature. With the help of a sterile cork borer (9 mm diameter) five wells were made in each MHA plate, three for leaves, stems and roots extracts and two for positive and negative controls. After removing the material from the wells with a sterile syringe needle, the wells were labeled. Then, 140 µL of each extract and controls were transferred into their corresponding wells with the help of micropipettes. The plates were, then, incubated at 37 ºC for 24 h, after which zones of inhibition (ZI) were measured with the help of digital Vernier calipers. Phenol (6 %) and 1,2-propanediol (50 %) were used as positive and negative control, respectively. The tests were replicated three times and the average calculated.

Determination of minimum inhibitory concentrations (MICs): Minimum inhibitory concentration values were determined for ethanolic extracts of leaves, stems and roots of *V. officinalis* against all the abovementioned ATCC standard microorganisms and six clinical isolates used to determine zones of inhibition. From the stock solutions of leaves, stems and roots extracts (3 g/100 mL each), different dilutions were prepared by doubling dilution method as 50, 25, 12.5, 6.25, 3.125, 1.563 and 0.781 (%, w/v). The wells were made and labeled. Then, 140 µL of different concentrations were added in the wells with the help of sterile micropipettes. 50 % propane-1,2-diol was used as negative control while 6 % phenol as positive control. The Petri plates were incubated at 37 ºC for 24 h, after which the plates were observed to determine minimum inhibitory concentrations of leaves, stems and roots against the tested microorganisms. The experiment was repeated three times to ensure the reproducibility.

Determination of mineral elements: The mineral elements were determined in the leaves, stems, roots, seeds and seeds husk of *V. officinalis* by the standard spectrophotometric method as reported previously[14]. The atomic absorption spectrophotometer used for mineral elements analysis was Varian Model AA240 (AAS). In each case, a weighed sample (5.0 g) was heated on flame to remove moisture and volatile matter. Then it was heated in a furnace at 600 ºC for about 4 h which converted the sample into ash. The ash was dissolved in conc. HNO_3 (12 mL) and the total volume was made 100 mL by adding twice distilled water. The content was then filtered to get a clear solution which was used for the analysis. The experiment was done in triplicate and the results were averaged.

RESULTS AND DISCUSSION

Antimicrobial studies: Antimicrobial activities of the ethanolic extracts of leaves, stems and roots of medicinal herb *V. officinalis*, collected from Lahore (Pakistan), were studied

TABLE-1
MEAN ZONES OF INHIBITION OF ETHANOLIC EXTRACTS OF STEM, LEAVES AND ROOTS OF *Verbena officinalis* AGAINST DIFFERENT PATHOGENS

Microorganisms	Mean zones of inhibition (mm)*			
	Stems	Leaves	Roots	Standard
Staphylococcus aureus ATCC25923	33.3	29.3	30.5	30.0
Enterococcus faecalis ATCC29212	24.5	13.5	15.0	21.0
Acinetobacter baumannii ATCC29213	18.5	18.0	16.5	32.0
Pseudomonas aeruginosa ATCC27853	19.5	20.5	16.5	30.5
Escherichia coli ATCC25922	13.5	15.2	13.4	23.0
MDR-*Salmonella typhi* strain 1	25.5	21.5	17.5	32.0
MDR-*Salmonella typhi* strain 2	22.6	24.0	20.5	30.0
MDR-*Salmonella typhi* strain 3	28.0	23.5	27.5	30.0
MRSA strain 1	24.5	22.0	18.5	26.0
MRSA strain 2	26.0	23.3	19.0	26.0
MRSA strain 3	19.0	22.0	18.0	26.0

*Values are the mean of triplicate determination.

using standards methods against various strains of reference and clinically isolated bacteria and the results are presented in Table-1 (mean zones of inhibition) and Table-2 (minimum inhibitory concentrations). The ethanolic extracts of all the parts of *V. officinalis* were considerably effective against the tested microorganisms. Table-1 shows that the ethanolic extract of stems of the herb, in general, exhibited better antimicrobial activity than leaves and roots. *Staphylococcus aureus* proved to be most susceptible to all the extracts. The stems extract exhibited remarkable toxicity against MRSA strain 4 while its activity against MRSA strains 1 and 2 is also good. Table-1 reveals that the stems and roots extracts are considerable effective against MDR *Salmonella typhi* strain 3. As the MIC values indicate (Table-2), *Escherichia coli* ATCC25922 required higher concentrations of all the extracts to inhibit its growth (0.15 mg/mL), while a dose of as low as 0.02 mg/mL is sufficient to kill many of the tested microorganisms. Although the herb possesses a wide range of antimicrobial properties, its efficacy against the gram-positive *S. aureus* and MRSA strains is especially notable. *Staphylococcus aureus* is one of the chief causes of various infections including food-born and skin diseases[46-48]. MRSA is responsible for a number of infections difficult to treat, which include the septic arthritis, endocarditis, staphylococcal scalded skin syndrome and it also acts as an ocular pathogen[49-53]. he stems of the herbs are considerably effective against *Enterococcus faecalis* (ATCC 29212) which causes skin and soft tissue infections[54]. The efficacy of the herbs against various pathogens may be attributed to terpenoids and flavonoids present in it[55].

Determination of mineral elements: Mineral elements (Na, K, Mg, Fe, Cd, Ni, Cu, Zn, Mn, Cr, Co, Pb) in the leaves, roots, stems, seeds and seed husk of *Verbena officinalis* were determined with atomic absorption spectrophotometer and the results are shown in Table-3. The remarkable aspect of the finding is that the quantities of K are approximately four times higher than those of Na with seed husk being five times richer in K than Na (4492 ppm of K against 897 ppm of Na). This should make the herb useful for the patients of hypertension[56]. The herb is a good source of Fe as well, where roots contain notably higher quantity (1763 ppm) than leaves (836.74 ppm), stems (324.39 ppm), seeds (893.25 ppm) and seeds husk (476.21 ppm). Iron is a component of many enzymes, hemoglobin (blood protein) and myoglobin (muscle protein)[57,58]. Roots also contain higher amounts of Mn (117.29 ppm), Cu (27.57 ppm) and Cd (1.54 ppm) with compared to other part studied. Seed husk contains higher quantities of Zn (54.00 ppm) and Mg (778.53 ppm) along with K, than other parts of the herb. All of these mineral elements play important role in human body. Seed husk is particularly rich in Zn, which is a component of about 70 enzymes and plays a variety of roles in human body[59]. It is necessary for proper ocular function[60,61] and has antioxidant

TABLE-2
MINIMUM INHIBITORY CONCENTRATIONS (MICs) OF ETHANOLIC EXTRACTS OF STEM, LEAVES AND ROOTS OF *Verbena officinalis* AGAINST DIFFERENT PATHOGENS

Microorganisms	MICs* (mg/mL)		
	Stem	Leaves	Roots
Staphylococcus aureus ATCC25923	0.08	0.08	0.08
Enterococcus faecalis ATCC29212	0.08	0.08	0.08
Acinetobacter baumannii ATCC29213	0.02	0.02	0.02
Pseudomonas aeruginosa ATCC 27853	0.04	0.02	0.04
Escherichia coli ATCC25922	0.15	0.15	0.15
MDR-*Salmonella typhi* strain 1	0.02	0.02	0.02
MDR-*Salmonella typhi* strain 2	0.15	0.08	0.02
MDR-*Salmonella typhi* strain 3	0.02	0.04	0.08
MRSA strain 1	0.04	0.08	0.04
MRSA strain 2	0.04	0.08	0.04
MRSA strain 3	0.03	0.08	0.04

*MIC values are the mean of triplicate determination.

TABLE-3
MINERAL ELEMENTS IN DIFFERENT PARTS OF *Verbena officinalis* (ppm)

Element	Leaves	Stems	Roots	Seeds	Seeds husk
Na	998.98	1191.34	1041.84	845.32	897.13
K	3974.29	4233.13	3927.58	3688.54	4492.08
Mg	714.37	732.84	679.72	686.95	778.53
Fe	836.74	324.39	1763.14	893.25	476.21
Cd	1.00	1.47	1.54	1.24	1.19
Ni	7.03	6.86	8.15	8.23	8.24
Cu	13.35	13.76	27.57	15.69	18.44
Zn	34.00	42.00	38.00	40.00	54.00
Mn	54.44	28.07	117.29	57.76	30.09
Cr	–	–	–	–	–
Co	–	–	–	–	–
Pb	–	–	–	–	–

properties[62]. Magnesium is a part of a number of enzymes and plays an important role in the regulation of blood sugar levels and blood pressure and is necessary for the transmission of nerve impulses[63]. Copper is a component of various enzymes and plays role in the synthesis of collagen, regulation of normal cardiovascular and immune functions[64,65]. No sample was found to contain chromium, cobalt and lead.

Conclusion

Verbena officinalis is a herb of immense ethnomedicinal importance. Our *in vitro* antimicrobial studies validate its applications in the treatment of various infectious diseases. It should particularly be effective to cure infections caused by *S. aureus* and MRSA. In order to isolate, characterize and analyze the actual natural products responsible for antimicrobial action, activity-guided screening of the herb is required.

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