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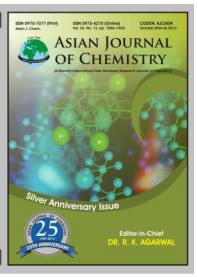

A Comparative Study of *in vitro* Total Antioxidant Capacity, *in vivo* Antidiabetic and Antimicrobial Activity of Essential Oils from Leaves and Seeds of *Zanthoxylum armatum* DC.

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Zanthoxylum armatum DC. is a pharmaceutically important plant that has been claimed to be effective against various diseases in the folk medicinal system of Indian subcontinent. A detailed study regarding total antioxidant capacity (TAC) and radical scavenging, antidiabetic and antimicrobial effects of the essential oils from leaves (ZAL) and seeds (ZAS) of this plant were investigated. Essential oils obtained from steam distillation process were subjected to multiple assays. Total phenolic content values were found to be 28.46 and 29.90 mg/L gallic acid equivalent for ZAL and ZAS, respectively. A linear correlation was observed between the per cent inhibition of ABTS radical cation and the amount of essential oils with R^2 = 0.996 and 0.997 for ZAL and ZAS, respectively. The per cent superoxide anion radical scavenging activity was found to be 44.75 and 45 % and reducing power in terms of FRAP values were 0.094 and 0.684 mM $FeSO_4$ for ZAL and ZAS, respectively. All samples exhibited good metal chelating activities, the per cent inhibition of the complex formation was found to be 70.47 and 72.17 for ZAL and ZAS, respectively. None of the concentration of essential oils from different parts of *Z. armatum* showed any antidiabetic activity. The oils also showed antimicrobial and antifungal activities comparable to chloramphanicol. The data obtained from oils demonstrate the powerful antioxidative, radical scavenging and antimicrobial properties of the plant.

Key Words: Essential oil, Antioxidant Potential, Radical Scavenging, *Zanthoxylum armatum*.

INTRODUCTION

Zanthoxylum armatum, locally known as 'timber' is a wild species, which is native to Laran, Passin, Dir, Murree, Saidpur, Hazara, Kalapani, Jehlum and Swat valley. It is hard thorny dense tree normally available on banks of stream and rivers. *Z. armatum* is extensively used in the Indian system of medicine, as carminative, stomachic and anthelmintic. The bark is pungent and stick from the plant is used in preventing toothache[1]. Essential oil of fruits of *Z. armatum* has been reported to exhibited good antibacterial, antifungal and anthelmintic activities[2]. The fruits and seeds are used as aromatic tonic in fever, dyspepsia, carminative, stomachic, anthelmintic and expelling roundworms[3-5]. The volatile oil is used as antidiaorrehal, antiseptic, deodorant and anticatarrhal[6-8]. The pharmaceutical companies generally use timur fruit for making tooth paste[5]. *Z. armatum* proved pharmacological base for its medicinal use in the gastrointestinal, respiratory and cardiovascular disorders[9]. Chemical composition of essential oils isolated from different species of *Zanthoxylum* was found important to have fungicidal activity. 47.33 and 10.83 % of monoterpenes and sesquiterpenes were found, respectively in the essential oil of *Z. armatum*[10,11]. The diverse anti disease activities claimed for *Zanthoxylum armatum* extracts/oils and the increasing demand for antioxidant compounds from natural sources encouraged us to undertake a comprehensive study of the antioxidant and radical scavenging activities, antidiabetic and antimicrobial effects of the plant.

EXPERIMENTAL

All the chemicals used were of analytical reagent grade. The solvents used were obtained from E-Merck (Germany). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), linoleic acid, sodium acetate, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, 2,2'-azino*bis*-(3-ethylbenzothiazoline)-6-sulphonic acid diammonium salt (ABTS), gallic acid, quercetin, Tween 20 (polyoxyethylene-sorbitan monolaurate), iron(II) sulphate, potassium thiocyanate, 1,1-diphenyl-2-picrylhydrazil (DPPH), 2,4,6-tripyridyl-s-triazine (TPTZ), Folin-Ciocalteau's reagent (FC reagent) were purchased from Fluka (Switzerland).

The plant material (leaves and seeds) of *Z. armatum* was collected from Abbotabad, Pakistan. Water-cleaned and shade-dried plant material was subjected to steam distillation to

extract essential oils. The samples obtained were used either neat or in diluted form in various antioxidant and radical scavenging assays.

Antidiabetic activity: Rabbits were selected for antidiabetic study. Standard conditions were maintained in the animal house regarding its temperature, relative humidity along with dark and light cycle. The rabbits were provided with the feed at regular intervals including green leafy vegetables *viz*. *Daucus carota* (carrot), *Cucurbita maxima* (Cucumber). Tap water was provided in earthen pots.

Acute oral toxicity: Acute oral toxicity of all essential oils was carried out adopting "up and down procedure" as per given in the OECD guidelines.

Experimental design: After a week of acclimatization, the rabbits were made diabetic by injecting intravenously alloxan monohydrate 150-mg/Kg-body weight. This dose permanently destroys the β-cells of pancreas and produces diabetes mellitus. After 8 days injection of the alloxan monohydrate, rabbits with blood glucose level from 200 to 500 mg/100 mL were selected for the evaluation of antidiabetic activity. Different concentrations (50 and 200 µL of EO/mL) were prepared in carboxy methyl cellulose 1 % (CMC) in water. Seven groups *viz*. A, B, C, D, E, F and G of five rabbits each were made. Group A acted as control and was not given any treatment, but 10 mL water. Group B was also treated as control and given 10 mL of 1 % CMC solution in water. Group C and D received an application of a dose of 50 and 200 µL/kg body weight EO from leaves of *Zanthoxylum armatum*, respectively. Group E and F received an application of a dose of 50 and 200 µL/kg body weight EO from seeds of *Z. armatum*, respectively. Group G acts as positive control group. This group was administered with locally available drug, glibenclamide (Glib.). This drug was used in 0.5 mg/mL in concentration diluted also in 10 mL of 1 % CMC.

Total antioxidant capacity: Total soluble phenolic compounds were determined by standard method, using gallic acid as a standard phenolic[13]. FRAP (Ferric reducing antioxidant power) values were determined following the method of Benzie and Strain[12]. Final results were expressed as FRAP values (mM $FeSO_4 \cdot 7H_2O$). ABTS scavenging activity was determined by following the standard method with minor changes[14]. DPPH free radical scavenging activity of the plant oils was measured by using the standard method[15]. Total antioxidant activity of the essential oils was determined according to the standard method[16]. Superoxide anion radical scavenging activity was determined using the standard method[17]. Metal (Ferrous ion) chelation by plant samples was estimated according to the standard method[18].

RESULTS AND DISCUSSION

Per cent yields and total phenolic contents: The per cent yields and total phenolic contents of the sample oils were calculated (Table-1). In comparison with ZAL and ZAS about two times greater per cent yield of oil was found for ZAS sample. Both oils were equal in phenolic contents. It is quite obvious that in spite of low per cent yields, the TPC values were quite good, showing richness of the oil samples with phenolic components. High TPC contents indicated high antioxidant and radical scavenging capacities for both the samples. Polyphenolic compounds constitute an important and diverse group of phytochemicals including phenols, phenol propanoids, lignins, flavonoids, stilbenes. These compounds perform diverse functions such as metal chelation, free radical scavenging and antioxidative actions[19].

TABLE-1
PER CENT YIELDS AND TOTAL PHENOLIC CONTENT (TPC) OF ESSENTIAL OILS FROM LEAVES, AND SEEDS OF *Zanthoxylum armatum*

Plant name	*Zanthoxylum armatum*	
	Leaves	Seeds
Weight of specimen (Kg)	1.56	2.995
Weight of oil (g)	1.35	38.45
Yield (%)	0.86	1.29
Color of oil	Clear brownish pale	Clear yellowish
TPC (mg/L GAE)	28.46	29.90

ABTS radical scavenging capacity and relationship between TPC: ABTS radical cation produced as a result of reaction between ABTS and potassium persulfate in aqueous solution at physiological pH has considerable stability and sensitivity towards crude and specific antioxidants[20]. The reduction potential of ABTS radical cation is similar to that of hydroxyl radical cation. So in test environment it may be taken as equivalent to hydroxyl radical produced *in vivo* during certain disorders and metabolic reactions. ABTS radical scavenging ability of the test samples was evaluated using ABTS radical cation decolorization assay.

Fig. 1 shows the dose dependent per cent inhibition of ABTS radical cation by the two samples. A significant correlation $R^2 = 0.996$ and 0.997 for ZAL and ZAS, respectively was found between per cent inhibition and amount of the sample.

Due to health-promoting effects of antioxidants a general recommendation to the consumer is to increase the intake of foods rich in antioxidant compounds, *viz*. polyphenols, flavonoids[21-30]. Phenolic compounds, especially flavonoids and anthocyanins, are very important antioxidants because of their natural origin and their ability to act as efficient free radical scavengers[31-34]. Small berries have been reported to be rich sources of phenolic compounds such as phenolic acids as well as anthocyanins, proanthocyanidins and other flavonoids, which display potential health promoting effects[35-41].

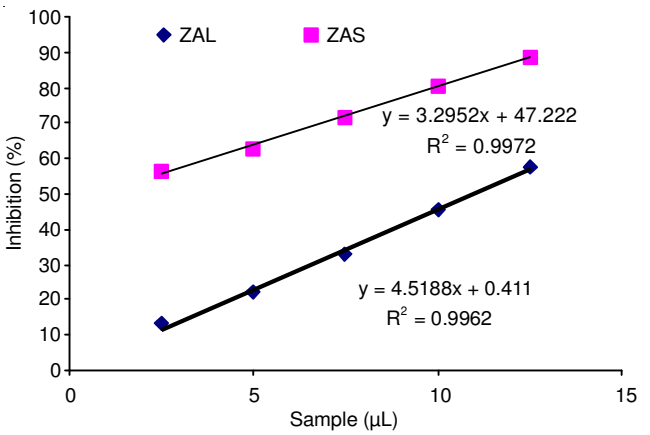

Fig. 1. Comparison of dose dependent per cent inhibition of ABTS radical cation by essential oils from leaves, roots and stem of *Zanthoxylum armatum*

Total phenolic contents in terms of gallic acid equivalents of all the extracts were determined using Folin-Ciocalteu's method. The extracts showed high GAE values. The amount of total phenolics for ZAL and ZAS samples were found to be 28.46 and 29.90 mg/L gallic acid equivalent, respectively. High values of TPC obtained for all the samples demonstrated presence of various phenolic acid components in these samples.

It is also evident from the data that ABTS radical cation decolorization assay is more linearly related to TPC. Attempts have been made to derive a relationship between the phenolic contents and antioxidant activity. Controversial results have been obtained regarding a linear relationship between TPC and antioxidant activity[42-44].

The present study showed a relatively good relationship between TPC and antioxidant activity determined through ABTS radical cation decolorization assay and FRAP Assay. Non-acquisition of absolutely linear relationship between TPC and the two assays may be due to different response of different phenolics in Folin-Ciocalteau reagent. Heinonen *et al.*[45] pointed the difference in the pH of the medium of assays and the reduction potential of the oxidized species. Furthermore the antioxidant activity strongly depends upon the chemical structure of phenolic compounds. Therefore, no definite quantitative relationship could be obtained for general application to all the plant extracts.

DPPH, lipid peroxyl and superoxide anion radicals scavenging activities: DPPH and lipid peroxide free radicals have been used to evaluate reducing properties and to assess free radicals chain breaking abilities of phyto-chemicals. Fig. 2 demonstrates the kinetics of DPPH radicals scavenging by ZAL, ZAS, blank and Trolox (10 µM). Both EOs showed time dependant quenching of DPPH radicals in a similar way. The absorbance continued to decrease with almost a uniform gradient throughout the time span of 0.5 h showing the presence of a good amount of slow reacting antioxidant components in both the mixtures.

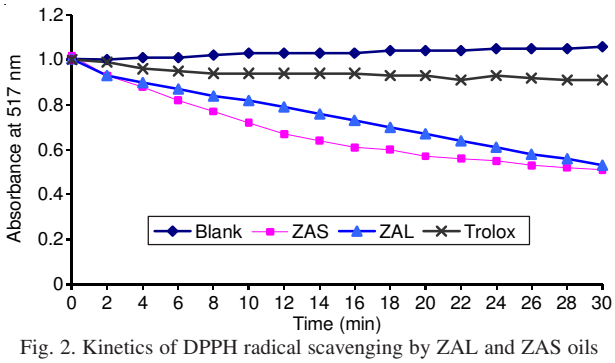

Fig. 2. Kinetics of DPPH radical scavenging by ZAL and ZAS oils

In biosystems, unsaturated fatty acids are always at stake due to free radicals attack on the bio-membranes which results in membrane lipid peroxidation, decrease in membrane fluidity, loss of enzymes and receptor activity and damage to membrane proteins leading to cell inactivation, Dean and Davies[46]. Many disorders like hyper-glycaemia have been ascribed to development of oxidative stress due to increased lipid peroxide production[47]. Lipid peroxidation values of the extracts were found using linoleic acid emulsion system. Linoleic acid after its aerial oxidation to peroxyl radicals converts ferrous to ferric form. The extent of conversion is assessed through iron(III) complex with thiocyanate spectrophotometrically. The antioxidative components in proportionate to their amount halt this conversion by trapping peroxyl radicals. Fig. 3 shows that both samples had considerable resistance to lipid per oxidation which is quite comparable with that of Trolox (10 µM).

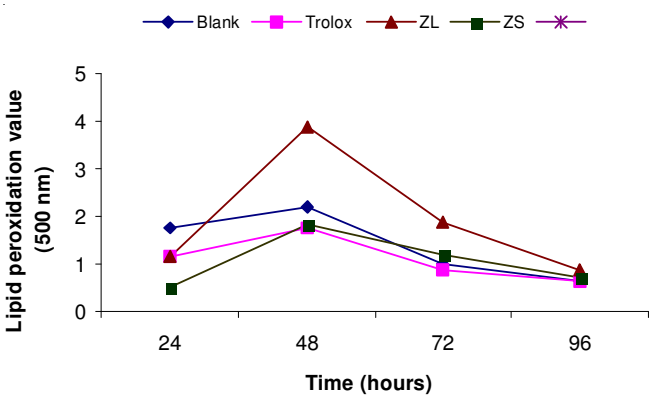

Fig. 3. Lipid per oxidation by ZAL and ZAS oils

Superoxide (SO) anion radical is one of the important ROS which is produced first after oxygen is taken inside the body. The subsequent dismutation of superoxide leads to the formation of other injurious ROS. So the capacity of samples to scavenge ROS can play a very crucial role in determining the overall strength of antioxidant activity. The per cent superoxide anion radical scavenging activity was found to be 44.75 and 45 % for ZAL and ZAS sample, respectively.

Reducing and metal chelating activities: FRAP assay was employed to estimate the ferric reducing activity of the samples using $FeSO_4$ as the standard reducing agent. At low pH, reduction of ferric tri pyridyl triazine (Fe III TPTZ) complex to intense blue coloured ferrous form can be examined by measuring the change in absorption at 593 nm. Being a non specific reaction any half reaction that has lower redox potential, under the test conditions, than that of Fe(III)/Fe(II) reaction, will convert Fe(III) to Fe(II). The change in absorbance is therefore, reflects cumulative reducing power of the electron donating antioxidants present in the reaction mixture. The FRAP values were found to be 0.094 and 0.684 mM $FeSO_4$ for ZAL and ZAS, respectively. The metal chelating activity was found by determining the chelating activity of the sample with ferrous ion in the presence of ferrozine (a ferrous chelating agent). In the presence of chelating components of the sample the formation of Fe(II)-ferrozine complex, which may be monitored at 562 nm spectrophotometrically, is halted. The per cent inhibition of the complex formation was found to be 70.47 and 72.17 for ZAL and ZAS, respectively.

Antibacterial and antifungal activity: Agar well diffusion method was performed to calculate zone of inhibition for both the samples. The results obtained (Table-2) showed that both the samples are quite effective against all the organisms tested.

Antidiabetic activity: As far as antidiabetic activity is concerned none of the concentration of any oil showed hypoglycaemic activity (Table-3).

TABLE-3
EFFECT OF DIFFERENT DOSES OF EOS OF *Zanthoxylum armatum* ON ORAL GLUCOSE TOLERANCE IN ALLOXAN TREATED RABBITS

Treatments	Blood glucose level of Alloxan induced rabbits (mg/dl)	Blood Glucose level of experimental groups after eight days (mg/dl)		
		5 h	10 h	24 h
Water	222	225.6 ± 0.50	233.6 ± 0.50	242.4 ± 0.92
1 % CMC solution	226	235.7 ± 0.50	243.2 ± 0.50	252.4 ± 0.2
50 µL/kg *Zanthoxylum armatum* seeds	220	223.6 ± 1.02	227.0 ± 0.58	233.2 ± 0.91
50 µL/kg *Zanthoxylum armatum* leaves	218	221.8 ± 0.58	226 ± 0.70	233.6 ± 0.74
200 µL/kg *Zanthoxylum armatum* seeds	220	219.21 ± 0.86	227 ± 1	234 ± 0.83
200 µL/kg *Zanthoxylum armatum* leaves	215	228.8 ± 1.15	231.4 ± 1.02	237.6 ± 0.92
Glib 0.5 mg/mL	342	137 ± 0.70	131.6 ± 0.50	111.6 ± 0.50

Values are mean ± SEM for n = 5.

TABLE-2
ANTINACTERIAL AND ANTIFUNGAL ACTIVITY OF ESSENTIAL OILS FROM LEAVES AND SEEDS OF *Zanthoxylum armatum*

Strain/plant part	Zones of inhibition (cm)		
	ZAL*	ZAS*	Chloromphenicol* (1 µg/mL)
Bacillus cereus	1.8	0.9	1.0
Staphylococcus aureus	1.6	1.2	1.2
Salmonella typhimurium	1.8	2.1	2.4
E. coli	2.0	2.2	0.8
Pseudomonas aeruginosa	1.4	1.8	2.0
Enterobacter aerogenes	2.1	2.1	1.6
Streptococcus pyogenes	1.4	1.8	1.8
Micrococcus roseus	2.2	1.9	2.0
Aspergillus niger	2.8	1.4	2.5
Penicillium chrysogenum	2.0	2.3	2.0
Saccharomyces cerevisiae	2.4	2.6	2.1
Rhizopus oligosporus	2.0	2.0	2.0
Rhodutrula minuta	2.4	1.6	2.6

*100 µL of each oil and standard drug were used in the experiment.

Conclusion

The data presented here shows that extracts have great antioxidant and radical scavenging activity and thus may be used as a good source of natural antioxidants. Although the *in vivo* efficacy of *Zanthoxylum armatum* oils against diabetes mellitus is not appreciable but the samples exhibited antimicrobial potential.

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