Assessment of antifungal potential against plant pathogen in Zanthoxylum armatum DC. oil

Zulfa Nooreen[1*], Fariha Sumayya[2], Ram Swaroop Verma[3], Indrajeet[4], Sudeep Tandon[3], Ateeque Ahmad[3]

[1]PSIT-Pranveer Singh Institute of Technology (Pharmacy) Bhauti Kanpur Uttar Pradesh, India-209305

[2]Integral Informatic and Research Center-1 (IIRC-1) Intergral University Lucknow Uttar Pradesh-226026, India

[3]Phytochemistry Division, CSIR-Central Institute of Medicinal and Aromatic Plants, P.O.-CIMAP, Lucknow, India -226015

[4]Crop Production and Protection Division, CSIR-Central Institute of Medicinal and Aromatic Plants, P.O.- CIMAP, Lucknow, India-226015

***Corresponding Author**

Dr Zulfa Nooreen (khan.zulfanooreen7860@gmail.com)

Abstract

Fruits of *Zanthoxylum armatum* contains number of bioactive compounds and are beneficial for health due to antioxidant, anticancer, antifungal and other properties. In this research, the essential oil and absolute constituents antifungal activity of both the samples have been studied. A comparative chemical composition of the essential oil and absolute of mature fruits of *Z. armatum* was analyses by GC-FID and GC-MS. The essential oil contained linalool (65.8%), limonene (8.7%), and (*E*)-methyl cinnamate (7.6%) as major components, whereas absolute was characterised by higher contents of palmitoleic acid (35.5%), linalool (28.0%), (*E*)-methyl cinnamate (9.7%) and palmitic acid (6.0%). The essential oil of *Z. armatum* fruits showed promising activity and showed 100% effective in *Curvularia lunata* at the concentration of 1%. Essential oil of fruits was stable and showed greater potential in biological activities.

Keywords: *Zanthoxylum armatum*, Rutaceae, Chemical composition, Essential oil, Absolute.

Introduction

Zanthoxylum armatum DC. (Rutaceae) is dioecious, pantropical plant bear sharp thorns on either the stem or the foliage found in India, Pakistan, Bhutan etc. In India it available at 5000ft in Himalayas and trans-indu Punjab areas and in Uttarakhand 5000-7000 ft in areas of kumaon (Jain et al. 2001). *Z. alatum* fruits possess anthelminthic, stimulating and fish poisoning properties. The powdered fruits, when mixed with vinegar, provide treatment for dental diseases and scabies. The plant is an insecticide and root decoction is used by tribes in managing remedies in snake bite and various hepatic problem while crushed seed as aromatic tonic in fever, dyspepsia, stomachic and cholera. The volatile oil has the characteristics of eucalyptus oil and is used as an antiseptic, a deodorant, and an anticatarrhal. Traditionally the plant is used for abdominal colic, asthma, cancer, cholera diabetes, cough, treatment of heat diseases, manage the blood flow, swelling, tootache, gum diseases and other mouth problems (Nooreen and Prajapati).It also exhibits good antibacterial, antifungal, and anthelmintic activities. The compounds reported in north Indian *Z. alatum* are *α*-thujene, *α*-pinene, benzaldehyde, sabinene, *β*-pinene, myrcene, *cis*-3-hexenyl acetate, *α*-phellandrene, *α*-terpinene, *β*-phellandrene, limonene, *m*- cymene, *trans*-β-ocimene, *cis*-linalool oxide, *trans*-linalool oxide, *α*-terpinolene, linalool, hotrienol, *cis*-limonene oxide, *trans-p*-menth-2-en-1-ol, camphor, *cis-p*-menth-2-en-1-ol, cryptone, and terpinene-4-ol. The essential oil showed antifungal property against several fungi such as *Aspergillus* sp., *Alternaria* sp., *Cladosporium* sp., *Penicillium* sp. and *Helmintho sporium* sp, and also showed good inhibition of *Bacillus subtilis* (Jain et al. 2001). The oil is also be use as a preservative in food commodities to prevent fungal growth (Prajapati et al. 2015) The essential oil of the plant commonly contains linalool, linalyl acetate, citral, geraniol, methyl cinnamate, limonene, sabinene (Singh et al. 2011). It has been reported that some Zanthoxylum species possess good antifungal activity like *Z. elephantiasis* and *Z. fagara*. They showed effect as standat component nistatin against *M. canis* (Hurtado et al. 2003). Apart from volatile constituents, *Z. armatum* fruits contain tambulin, prudomestin, ombuin, ascorbic acid and ombuin showed broad-spectrum antibacterial effect in Gram positive and Gram negative strains. The hexane and ethyl acetate fractions exhibited moderate antibacterial efficacy against selected pathogenic microbes (Nooreen et al. 2017). *Z. armatum* volatile oil and extracts consisting of

mainly limonene, linalool and flavonoids and other compounds (Nair and Ramidi). In addition to antimicrobial properties, *Z. armatum* seed extract was recognized for larvicidal against as *A. aegypti, C.quinquefasciatus,* and *A. stephensi* and antihelmintic activities (Tiwary and Mehta). Therefore, our work had the objective to characterize the fruits essential oil and its absolute constituents through GC and GC-MS analysis and assess the biological activities such as antifungal and antioxidant activities in order to explore the possibility of sustainable utilization of the essential oil, absolute and oleoresin of an important traditional medicinal plant.

MATERIAL AND METHODS

Plant material

The dried fruits of *Z. armatum* were purchased from the local market in Lucknow, India and identified by the Scientist, Botany and Pharmacognosy Department, CSIR-CIMAP, Lucknow. A voucher specimen (ZA/F/1) has been deposited in the herbarium of our institute.

Isolation of essential oil

The essential oil of crushed shade dried mature fruits and fruit pericarp of *Z. armatum* (500 g) was procured by steam distillation in a Clevenger apparatus (*Borosil Glass* Works *Ltd, Mumbai).*The yield of the essential oil (ZA/V) was 1.5% (v/w) and essential oil of pericarp (ZA/VP) 1.0% (v/w) respectively on a dry weight basis. Obtained oil was procured and stored in glass tight container for further analysis

Preparation of Oleoresins and Absolute

Z. armatum crushed fruits (50 g) was extracted with hexane (three times) for 12 h. After complete extraction, the solvent was distilled to obtain a viscous oleoresin (6.7 g). For absolute, oleoresin was mixed with 50 ml of absolute alcohol and agitated for 1 h. The mixture was freeze out for 6-7 h then filtered by Buchner funnel. The crude mass was again mixed with absolute alcohol. This process was repeated three times. The solute was collected and concentrated. The absolute (ZA/A) were kept at 4°C until analysis and activity. The oleoresin and absolute were preparation as per the method reported by Tandon et al 2006

Physical properties of essential oil

The physical properties such as specific gravity, optical rotation and refractive index were measured using standard procedures using instruments KEM model DA–500 (Kyoto Electronics

manufacturing Co., Ltd, Tokyo, Japan), Anton Paar model MCP-150 (Anton Paar, Graz,Austria), Anton Paar model Abbemat 3200 (Anton Paar, Graz, Austria), respectively.

Gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) analysis Analysis of volatile oil has been done by Gas chromatography-FID and Gas chromatography-Mass Spectroscopy. Aligant 7890 instrument has been used. Constituents of oil was evaluatedby using ELITE-5 column and Flame Ionisation Detector. Column contains stationary phase as5% phenyl)-polymethylsiloxane (internal diameter 30m x 0.25mm and 0.25µm thickness.Temperature of oven was regulated from 60°C -120°C afterword rise in 3°C min^{-1} andterminating hold time of 10 min. 290°C was the temperature of detector and injector as well.Carrier gas hydrogen was used at 1.0mL/min. 0.3µL (diluted with hexane 1:3) of injection wasmade along the split ratio of 1:50 (Morocco et al. 1994)

Antifungal assay: Food Poison Technique

In this study, fruit pericarp essential oil (ZA/VP), fruit absolute (ZA/A) and fruit essential oil (ZA/V) and of *Z. armatum* (Essential oil obtained from division of biochemistry, used as Positive control) were screened for antagonistic activity against the growth of four phytopathogenic fungi (*Fusarium chlamydosporum*, *Macrophomina phaseolina*, *Rhizoctonia solani*, and *Curvularia lunata*) on Potato Dextrose Agar (PDA) using food poison techniques (18-20). The plates were prepared by adding different concentrations (0.2, 0.5 and 1.0%) of the essential oils and absolute of *Z. armatum* into 10 mL of the PDA medium into 9-cm Petri plates at 40–45 °C. To ensure proper mixing of essential oil, 0.05% tween-80 was added. A 6 mm disc of different test fungi was placed on the PDA filled Petri plates. All Petri-plates were incubated at 25±2 °C. This process was repeated three times. The percentage of inhibition of colony diameter was measured every day (5 days average data). All tests test were carried out in triplicate. The PDA plates without essential oil served as a control. Percentage of the fungal mycelia growth inhibition was calculated as follows.

$$\% \ Scavenging = \frac{Absorbance\ of\ Control - Absorbance\ of\ Sample}{Absorbance\ of\ Control} \ X\ 100$$

Where, Ac = absorbance of the control and As = absorbance of the sample.

Nitric oxide scavenging assay

Volatile oil was tested at various concentrations by method used by Marcocci et al 1994 by doingminute modifications. Sodium nitropruside (SNP) generate nitric oxide which can be determined by griess reagent. Generated nitric oxide coordinate with oxygen to give nitrite ions and calculated by using griess reagent. Chromophores absorbance was recorded during diazotization of nitrite with sulphanilamide and successive coupling with naphthyl ethylenediamine dichloride. This was recorded at 546 nm. Sample was mixed with SNP (mM) of 200µL in phosphate buffer (PBS) for thirty minutes at room temperature. Proceeding to process 50 µL of incubated sample was taken and 100 µL griess reagent was dissolved. Ethanol was taken as control sample. Obstruction in nitrite formation by sample (oil and absolute) was observed and calculated by taking ascorbic acid as standard. The absorbance was measured at 546 nm and percent inhibition was recorded.

$$\% \ inhibition = \frac{Absorbance\ of\ Control - Absorbance\ of\ Sample}{Absorbance\ of\ Control} X\ 100$$

Results

Physicochemical composition

The essential oil of the fruits of *Z. armatum* was extracted by conventional hydro-distillation using the Clevenger-type apparatus. The yield of the essential oil (ZA/V) was 1.5% (w/w) and essential oil of pericarp (ZA/VP) 1.2% (v/w) respectively on dry weight basis and absolute, ZA/A was (4.7 g). The fruit essential oil was light yellow viscous oil. A total of 22 and 13 constituents were identified in the fruits essential oil and absolute, respectively by retention index and mass spectral data. The relative concentrations of the volatile components are presented in Table 1, according to their elution order on an HP-5 fused silica capillary column. Components of the fruits essential oil were linalool (65.8%), limonene+ β-phellandrene (8.7%), and *trans*-methyl cinnamate (7.6%), while absolute composition was dominated by palmitoleic acid (35.5%), linalool (28.0%), *trans*-methyl cinnamate (9.7%), and palmitic acid (6.0%). Minor constituents of fruits oil and absolute were as *cis*-linalool oxide (1.1%, 1.0%), *trans*-linalool oxide (1.3%, 1.0%), cryptone (2.6%, 0.2%), and α-terpineol (1.4%, 2.6%). Major components of the fruits

essential oil reported earlier from different places are presented in Table 1. The physical properties like optical rotation [α] D^{20} , refractive index: nD^{20} and specific gravity (25 °C) of *Zanthoxylum armatum* essential oil, essential oil of pericarp and absolute was determined and found that +8.56°, +8.72°, +7.36°, 1.4774, 1.4823, 1.5011 and 0.9523, 0.9522, 0.9870
respectively (Table 2.).

Table 1. Chemical composition of the fruit essential oil and absolute of *Zanthoxylum armatum*

S. No.	Compound[a]	Retention Index		Content (%)	
		Experimental	Literature	Essential oil	Absolute
1.	*α*-Thujene	925	924	0.1	-
2.	*α*-Pinene	930	932	0.1	-
3.	Camphene	943	946	t	-
4.	Sabinene	973	969	0.7	-
5.	*β*-Pinene	978	974	0.1	-
6.	Myrcene	992	988	0.9	-
7.	*α*-Phellandrene	1005	1002	0.1	-
8.	*α*-Terpinene	1018	1014	0.1	0.3
9.	*p*-Cymene	1024	1020	0.2	-
10.	Limonene + *β*-Phellandrene	1030	1024	8.7	0.9
11.	*cis*-Linalool oxide (furanoid)	1073	1067	1.1	1.0
12.	*trans*-Linalool oxide (furanoid)	1090	1084	1.3	1.0
13.	Linalool	1102	1095	65.8	28.0
14.	Terpinen-4-ol	1179	1174	0.4	0.4
15.	Cryptone	1188	1183	2.6	0.2
16.	*α*-Terpineol	1191	1186	1.4	2.6
17.	*trans*-Methyl cinnamate	1381	1376	7.6	9.7
18.	*β*-Caryophyllene	1424	1417	0.2	t
19.	Caryophyllene oxide	1589	1582	0.7	0.7
20.	Palmitoleic acid	1952	1953	0.1	35.5
21.	Palmitic acid	1972	1971	0.1	6.0

[a]Mode of identification: Retention index (RI) and MS (GC/MS).

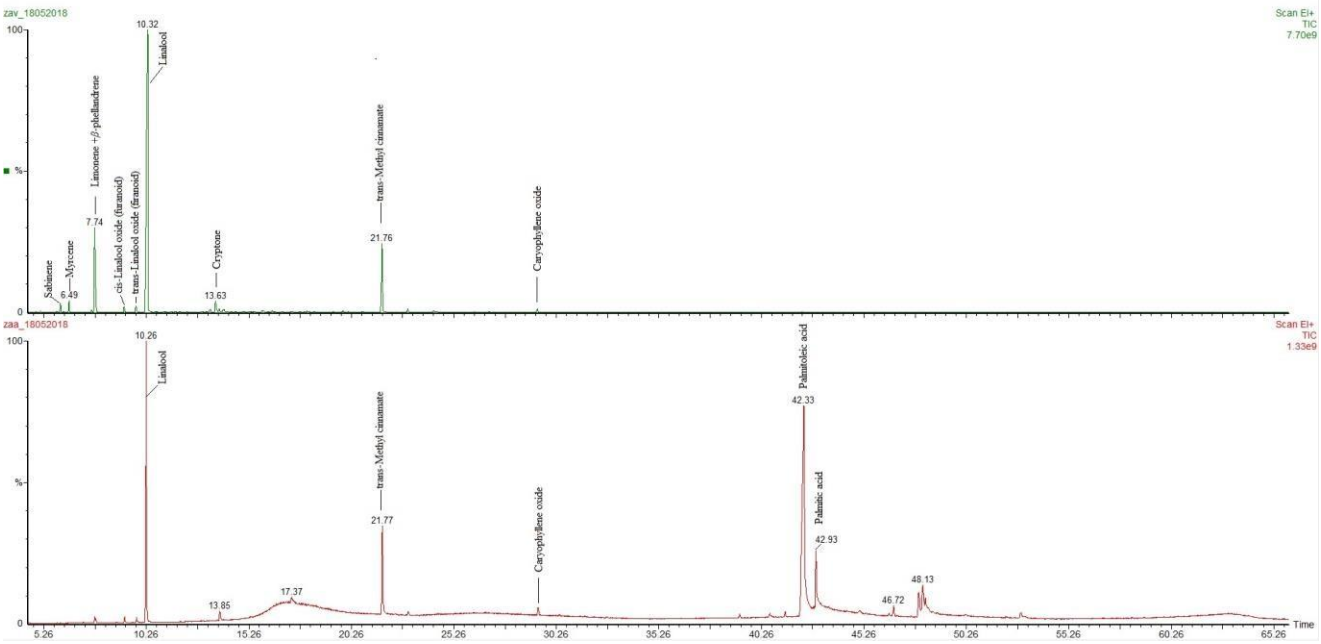

Figure 1. Gas chromatographic profile (TIC) of essential oil (ZAV) and absolute (ZAA) of *Z.armatum* fruits

Table 2. Physical parameters of *Zanthoxylum armatum* essential oil and absolute.

Sample code	**Optical rotation (T = 20°C, Cell length=100mm)**	**Refractive index (T = 20°C)**	Specific gravity (25 °C)
ZA/V	+8.56°	1.4774	0.9523
ZA/Vp	+8.72°	1.4823	0.9522
ZA/A	+7.36°	1.5011	0.9870

Antifungal activity

The essential oil of *Z. armatum* (ZA/VP) inhibited the growth of all four fungi at different concentrations of 0.2, 0.5 and 1.0 %. At 0.2 %, all four test fungi *F. chlamydosporum*, *M. phaseolina*, *R. solani* and *C. lunata* were moderately inhibited, ranging from 5.95 to 31.29 except*Curvularia lunata* which is inhibited 70.60%. At 0.5% concentration 43.74-49.84 % inhibition

except *C. lunata*, It shows the 100% of inhibition against *C. lunata* at 0.5% of *Z. armatum* seed oil. At 1.0% of this essential oil results in 66.72–100 % of inhibition of all the four test fungi. The bar diagram clearly shows the percent of inhibition in applying 0.2 %, 0.5%, and 1.0% of essential oil in continuously 5 days (Fig.1). In the test of ZAV essential oil, 8.1 to 59.10 % inhibitions at 0.2%, all four test fungi, at 0.5%, 33.60-89.23 inhibition all four test fungi, but at 1.0%, 78.7 to 100% inhibition all these fungi (Fig. 2). In the test ZA/VP and ZA/V are effective, while in the case of ZA/A no mycelia growth inhibition was found all these test fungi at 0.2, 0.5 and 1.0% concentration. The essential oil of *Z. armatum* (positive control) also shows antifungal activity at different concentration of essential oil. At 0.2 %, all four test fungi, moderately inhibited, ranging from 4.2 to 22.0 except *Curvularia lunata* which is inhibited 75.0%. At 0.5% concentration 32.0 to 100% and at 1.0% of this EO results 68.0 to 100 % of inhibition all the fourtest fungi. (Table.1)

Table 3. Effect of *Zanthoxylum armatum* essential oil at different concentration against plant pathogenic fungi by the food-poison technique. ZA/VP (Essential oil of fruit pericarp), ZA/A (Essential oil of fruit) ZA/A (Absolute of fruit)

Fungus	Percentage of mycelia zone inhibition at different concentration[a]											
	ZA/VP			ZA/A			ZA/V			Positive control		
Percentage of EO Conc.	0.2	0.5	1.0	0.2	0.5	1.0	0.2	0.5	1.0	0.2	0.5	1.0
Fusarium chlamydosporum	31.2	49.8	79.3	NI	NI	NI	37.1	53.7	78.7	22.0	46.5	82.0
Macrophomina phaseolina	18.7	43.7	86.9	NI	NI	NI	8.1	33.6	89.3	20.0	41.0	88.0
Rhizoctonia solani	5.9	46.4	66.7	NI	NI	NI	40.9	59.1	97.3	4.2	32.0	68.0
Curvularia lunata	70.6	100	100	11.2	13.7	14.2	59.1	89.2	100	75.0	100	100

[a]Average of three replicate, Conc.- concentration, NI- No inhibition

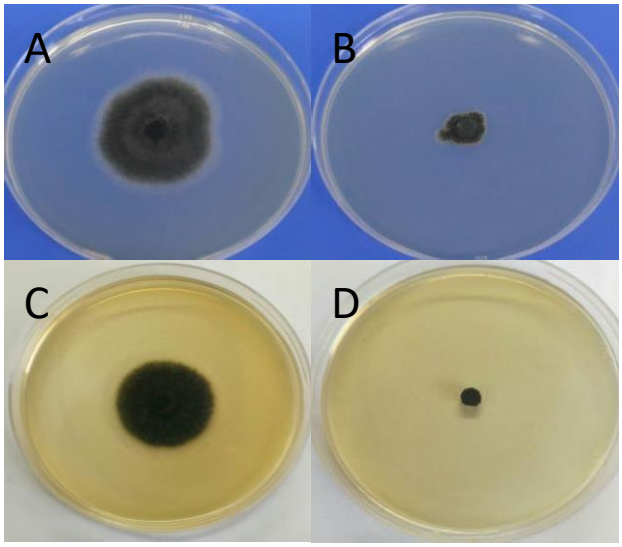

Figure 2. Growth inhibition by diffusion assay of *C. lunata* with 1% of *Z. armatum* EO. (four days old culture) (A). Controle and (B) Growth inhibition by ZA/VP. (C) Control and (D) growth inhibition by ZAV.

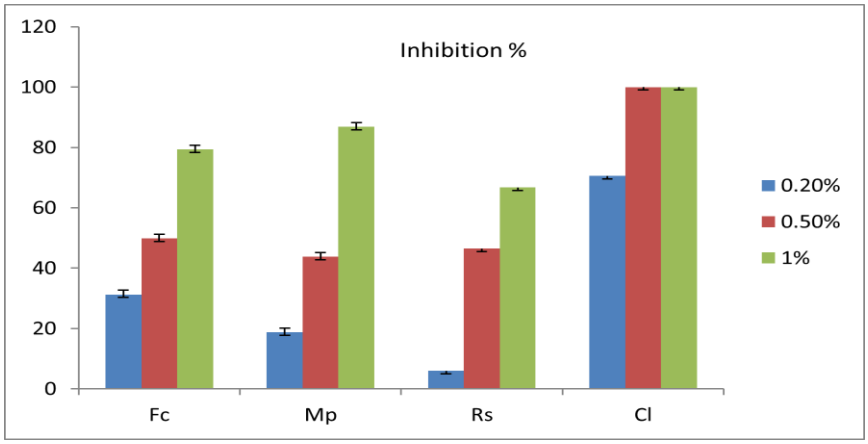

Figure 3. Percentage of radial growth inhibited by crude essential oil of Pericarp of *Z. armatum* (ZA/VP) against four phytopathogenic fungi at 0.2, 0.5, and 1.0 % /mL after 5 days incubation at 25 ± 2°C (average data of five days). *Fc*, *Fusarium chlamydosporum*, *Mp*, *Macrophomina phaseolina*; *Rs.*, *Rhizoctonia solani*; *Cl*, *Curvularia lunata.*

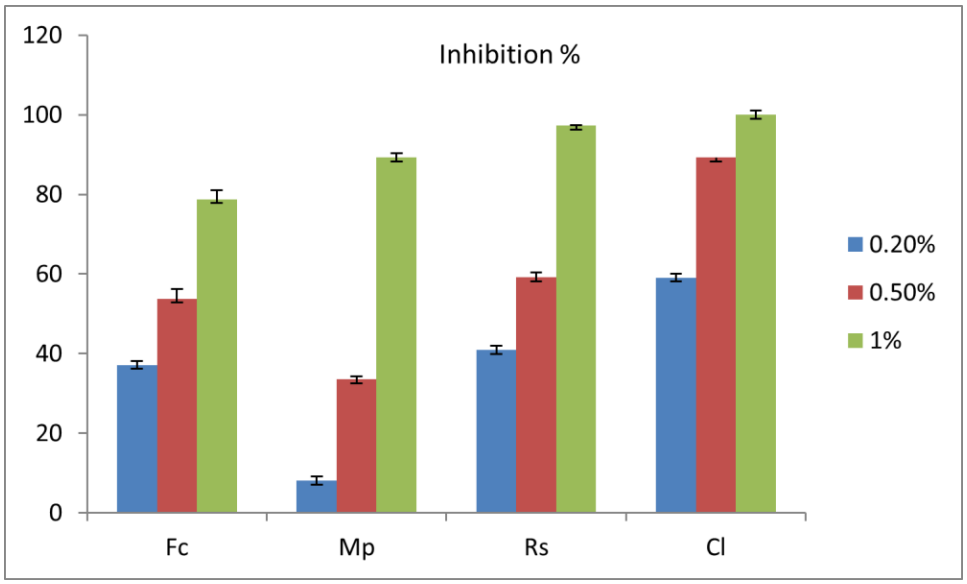

Figure 4. Percentage of radial growth inhibited by crude essential oil (EO) of *Z. armatum* (ZA/V) against four phytopathogenic fungi at 0.2, 0.5, and 1.0 % /mL after 5 days incubation at 25±2°C. (average data of five days). *Fc*, *Fusarium chlamydosporum*, *Mp*., *Macrophomina phaseolina*; *Rs.*, *Rhizoctonia solani*; *Cl.*, *Curvularia lunata.*

Antioxidant activity

All the samples were evaluated for the *in-vitro* antioxidant activity in triplicate manner and the average data is mentioned. All the samples showed a dose-dependent response. In DPPH scavenging assay ZA/A showed maximum effect as compare to others whereas in the case of Nitric oxide assay ZA/Vp showed maximum antioxidant activity compare to others.

Table 4 DPPH inhibition assay of ZA/V_P (Essential oil of fruit pericarp), ZA/V (Essential oil of fruit) ZA/A (Absolute of fruit)

S.No.	Sample Name	% DPPH Radical scavenging (IC_{50})			
		50 µg	100 µg	200 µg	400 µg
1	ZA/V	16.85±5.9	22.60±8.2	30.45±11.0	42.14±3.9
2	ZA/A	22.98±9.6	30.07±5.2	38.50±8.6	44.06±10.1
3	ZA/V_P	17.41±4.0	21.95±8.1	28.91±4.2	42.60±2.6
4	Vit-C	42.50±1.2	82.08±0.5	92.45±2.1	97.80±1.9

Table 5 Nitric oxide scavenging of ZA/V_P (Essential oil of fruit pericarp), ZA/A (Essential oil of fruit), ZA/A (Absolute of fruit)

S.No.	Sample Name	% inhibition of Nitric Oxide (IC_{50})			
		50 µg	100 µg	200 µg	400 µg
1	ZA/V	14.07±7.2	43.68±2.8	48.54±4.0	56.31±5.1
2	ZA/A	32.52±4.5	53.15±5.1	66.26±2.6	70.14±4.6
3	ZA/V_P	25.23±5.4	59.46±7.1	68.68±5.9	72.81±4.6
4	Vit-C	64.47±2.7	76.08±2.1	86.45±1.5	95.32±1.6

Discussion

The fruit essential oil was light yellow viscous oil. A total of 22 and 13 constituents were identified in the fruits essential oil and absolute, respectively by retention index and mass spectral data. *Zanthoxylum armatum* fruits volatile oil contains linalool as major chemical constituent as reported in many research paper while seed and leaves are also gaining interest due to their good pharmacological activities. In order to see the variation in essential oil and antifungal activity of pericarp of fruit (ZA/Vp) and whole fruit (ZA/V)was used, we also prepared the absolute (ZA/A)of the plant. Here in this study antioxidant and plant pathogen especially antifungal activity were performed. The essential oil of Z. armatum shows antifungal property against various fungi like C. lunata, F. chlamydosporum, M. phaseolina, R. solani. Among these test fungi, it was most effective against C. lunata at 1.0% with essential oil of ZA/VP, while in the case with essential oil of ZA/V, C. lunata and R. solani were more effective at 1.0%, comparison to ZA/VP, and comparatively less against F. chlamydosporum, and M. phaseolina. Using synthetic molecules or formulations in the management of fungal disease in plants may hamper the good properties and environment also. Considering these factors now researchers are focusing on the natural remedies. Alternative idea of using essential oil may become success in field level.

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Declaration

The author (s) have no conflict of interest

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