JNCS
Journal of Nepal Chemical Society January 2021, Vol. 42, No. 1, 125-131
ISSN: 2091-0304 (print)
DOI: https://doi.org/10.3126/jncs.v42i1.35363
RESEARCH ARTICLE

Phytoconstituents and Biological Activities of *Zanthoxylum armatum* Fruit Extract

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(Received: September 30, 2020; Revised: January 5, 2021; & Accepted: January 15, 2021)

Abstract

The essential oil and methanol extract of the fruit of *Zanthoxylum armatum* DC were extracted by hydro-distillation in the Clevenger apparatus and cold percolation technique respectively and their chemical and biological studies were performed. The chemical compositions of essential oil were analyzed by GC-MS analysis. The most abundant ingredient was linalool (75.31%) followed by E-methyl cinnamate (11.73%) and limonene (9.45%). The nature of the functional groups present in the extract was analyzed by performing FTIR analysis. The methanol extract showed the presence of alkaloids, flavonoids, glycosides, polyphenols, terpenoids, volatile oils, tannins, and saponins. Biological activities of the oil and extract were evaluated by performing brine shrimp bioassay, antibacterial activity test, and antifungal activity test. Essential oil (LC_{50} = 76.70 µg/mL) and methanol extract (LC_{50} = 62.25 µg/mL) were found highly cytotoxic against brine-shrimp nauplii. The essential oil showed potent antibacterial activity against gram-positive bacteria *Bacillus subtilis, Micrococcus leutus,* and *Staphylococcus aureus* with the zone of inhibitions 11 mm, 16 mm, and 17 mm respectively and moderate antibacterial activity against the gram-negative bacteria *Klebsiella pneumonia, Enterobacter cloacae,* and *Pseudomonas aeruginosa* with the zone of inhibitions 9 mm, 5 mm and 6 mm respectively. The methanol extract was found to be effective against gram-positive bacteria only. Both oil and extract showed moderate antifungal activity against bacterial strains.

Keywords: *Zanthoxylum armatum*, essential oil, extract, GC-MS analysis, antibacterial

Introduction

The genus *Zanthoxylum armatum* DC belongs to the family Rutaceae is a sub deciduous, xerophytic, aromatic, and branched tree or shrub[1]. It is known as "Timoor" in Nepali, Tejphal in Hindi, Tejovati, or Tumbru in Ayurved, and Prickly ash in English. It has a maximum length of up to about 5 m with compound, alternate imparipinnate leaves, and flowers are minute, polygamous, and pale yellow [2]. Fruits are drupe and small with red color, splitting into two when ripe [3]. Seeds are shining black, rounded, and non-endospermic [4]. It is mostly distributed in the Himalayas from Jammu to Bhutan, Nepal, Pakistan, India, and some other countries at an altitude up to 2500 m. In Nepal, it is distributed from east to west at an elevation range of 1100-2500m [5].

The different parts of this plant such as leaves, fruits, seeds, stem, bark, and roots have been used for the treatment of various ailments and their significant medicinal properties were further reported through various scientific investigations [6]. It shows antioxidant, anti-inflammatory, analgesics, antimicrobial and insecticidal activity. Various scientific investigations revealed that *Z. armatum* is more effective for the treatment of liver (hepatoprotective), controlling diabetes and blood pressure [6]. It is also used for the treatment of pneumonia, teeth infection, curing gum diseases, stomachache, gas problems, indigestion, and as a carminative [7]. Its fruits and seeds are edible and are used as potherb spices and flavoring agents [8]. Seeds of this aromatic plant contain mainly volatile oils and different phytoconstituents like alkaloids, tannins, terpenoids, glycosides, amino acids are present in leaf and seed extracts [9]. In this study, the extract was prepared and essential oil was extracted from *Zanthoxylum armatum* fruits.

Materials and Methods

Collection of plant materials

The fresh fruits of *Zanthoxylum armatum* DC were collected from Lalitpur district, Nepal (altitude 1400m) in September 2018. The taxonomic identification of

the plant was confirmed at the Department of Botany, Amrit Campus, Kathmandu, Nepal.

Extraction process

The essential oil from the fruits (500 g) of *Zanthoxylum armatum* was extracted by hydrodistillation for 6 hours using a closed type Clevenger apparatus for extraction of oils lighter than water. The collected oil was dried over anhydrous Na_2SO_4 and stored at 5 ºC until analysis. The marc was subjected to a cold percolation technique using methanol as solvent (500mL × 24 hrs × 5 times). The methanol extract was concentrated using a rotary evaporator. The concentrated extract was dried and stored in a sealed glass vial in a refrigerator until further experiment was performed.

Phytochemical screening

The methanol extract of the fruit was subjected to various qualitative tests by following a standard protocol to identify the presence of different classes of secondary metabolites present in the extract [10].

Gas chromatography-mass spectrometry(GC-MS) analysis

The qualitative and quantitative analysis of extracted essential oil of *Z. armatum* was performed on Shimadzu made GC-MS equipped with RTX-5 MS column (30 m × 0.25 mm internal diameter, 0.25 µm film thickness). Helium was used as a carrier gas at a flow rate of 1 mL min^{-1} . An injection volume of the sample was 1µL with a split ratio of 1:90. The initial column temperature was maintained at 40 ºC. The instrument was operated in the electron impact (EI) mode of ionization at 70 eV and ion source temperature 170 °C. Volatile constituents of the oil were identified by comparing the mass spectra available in a database (NIST 2017). The identified constituents were also confirmed by comparing it with the literature [11,19,20].

FTIR spectroscopic analysis

FTIR spectrum of methanol extract of *Z. armatum* fruits was recorded in a sophisticated computer-controlled FTIR spectrometer (Shimadzu made, Tracer 100). 10 mg of extract was loaded and scanned at the range of 4000-400 cm^{-1}.

Biological assays

Cytotoxicity

The brine shrimp lethality assay of the essential oil and methanol extract of *Z. armatum* was performed to evaluate cytotoxicity by following the standard protocol [12]. About 50 mg of eggs of brine shrimp (*Artemia salina*) were sprinkled on the beaker filled with artificial seawater and illuminated with a table lamp (60 watts) for 48 hours by adjusting the temperature at 30 °C. Stock solutions were prepared by dissolving 20 mg of each extract and oil separately in DMSO in two separate test tubes. From each stock solution, solutions of 250 µg/mL, 125 µg/mL, 62.5 µg/mL, 31.25 µg/mL, 15.625 µg/mL and 7.81 µg/mL were prepared by serial dilution method. 2.5 mL of each concentration was transferred into test tubes, three for each concentration. Similarly, 2.5 mL of DMSO was taken in three test tubes as a blank. Labeling of test tubes was done and then they were kept for 24 hours to evaporate the solvent (DMSO). Then, the solvent was evaporated by standing overnight. After complete evaporation of the solvent, ten matured shrimps in 5 mL artificial seawater were transferred to all test tubes containing samples. Similarly, three controlled vials were taken and ten matured nauplii were introduced in each vial. After 24 hours of illumination under a table lamp (60 Watt), the numbers of survivors were counted with the help of disposable pipettes. The LC_{50} value (lethal concentration for 50% mortality) was determined using probit regression. The compound of LC_{50} values less than 1000 ppm are considered as potentially pharmacologically active [12].

Antibacterial activity

Inhibition of the bacterial growth by the samples was tested by using the agar well diffusion method [13,15]. The bacterial strains used in the assay were *Klebsiella pneumonia* (KCTC 2242), *Bacillus subtilis* (KACC 170477), *Micrococcus leutus* (KACC 1377), *Pseudomonas aeruginosa* (KACC 10232), *Enterobacter cloacae* (KAC 13002), and *Staphylococcus aureus* (KCTC 1916). About 100 µl suspension of tested microorganisms were spread on the Muller-Hilton Agar medium. Wells having a diameter of 6 mm were made on the agar plates using the sterile cork borer. Essential oil and the methanol extract (0.3 mg/well) were loaded separately in the wells along with solvent dimethyl sulfoxide (DMSO). Ciprofloxacin (0.3 mg/well) was used as a positive control and DMSO as a negative control. The plates were then incubated at 37ºC for 32 hrs. After incubation, the plates were observed for the diameter of the zone of inhibition (ZOI) in mm around the well. All tests were performed in triplicate.

Antifungal activity

Pure colonies of the fungal specimens (*Aspergillus flavus, Fusarium solani*) were inoculated into sterile

Potato Dextrose Broth (PDB) and were kept in an incubator at 28ºC for 72 hrs for proper growth. After culture, 100 µL of the broth was inoculated into the sterile Muller-Hilton Agar plate and spread uniformly. Following this, the disc previously soaked with 15µL of the sample (essential oil and methanol extract) were kept on the surface of the plate and kept at room temperature for 30 minutes to allow proper diffusion of the extract onto the medium. Miconazole was used as positive control and methanol was used as a negative control. The plates were left at 28ºC for 48 hrs and the results were observed. The experiment was done separately for essential oil and methanolic extract of the plant [14].

DPPH radical scavenging activity

DPPH radical scavenging activity of the extract and essential oil was determined by following the method of Sharma *et al.,* (2015) [15]. Exactly weighed 7.886 g of the DPPH was dissolved in 100 mL methanol to prepare the 0.2mM solution. Briefly, for methanol extract, essential oil, and ascorbic acid (positive control), different concentrations (12.5, 25, 50, 100, and 200 µg/mL) were tested. From each concentration, 2 mL volume was taken and 2 mL of 0.2mm DPPH solution was added. The absorbance of each final solution was taken on a UV-Visible spectrophotometer (UV professional double beam, Shimadzu made) at 517 nm after 30 minutes. The experiment for both methanol extract and essential oil was performed in triplicate and the percentage radical scavenging activity was calculated by using the following equation.

$$\% \text{ radical scavenging activity} = (A^0 - A^1/A^0) \times 100\%$$

where, A^0 = Absorbance of the control (1mL MeOH + 0.5 mL DPPH).

A^1 = Absorbance of the sample

The antioxidant activity result of each methanol extract, essential oil, and the positive control, ascorbic acid was expressed as an IC_{50} value. i.e. inhibitory concentration of the sample required to scavenge 50% of the free radicals and was calculated from the linear regression curve of concentration versus the percentage of free radical scavenging activity.

Results and Discussion

Extraction

Hydro distillation of fruits of *Z. armatum* yielded 7.8±0.5% (v/w) essential of light brown color having strong fragrance. The percentage yield of the extract was found to be 8.5%.

Phytochemical analysis

Phytochemical screening revealed that the fruit of *Z. armatum* is rich in bioactive secondary metabolites. The qualitative result of the screening is presented in table 1. The presence of these metabolites in the plant helps to show antimicrobial activities through different mechanisms [15].

Table 1*: Phytochemical screening of fruit extract of Zanthoxylum armatum*

Flavonoids	+	Tannins	+
Alkaloids	+	Volatile oils	+
Glycosides	+	Coumarins	-
Polyphenols	+	Saponin	+
Terpenoids	+	Protein and amino acids	-
Quinones	-	Reducing sugar	-

Key: + = Present - = Absent

GC-MS analysis

The GC-MS analysis revealed the presence of at least six different compounds representing 99.27% of the oil (Table 2). Among the identified compounds, 3 were monoterpenes, 1 monoterpene alcohol, 1 ester, and 1 bicyclic sesquiterpene. Linalool (75.31%, peak no. 4) was the major constituent followed by E–methyl cinnamate (11.73%, peak no. 5) and limonene (9.45%, peak no. 3). To the best of our knowledge, *Z. armatum* fruits from Nepal contain the highest percentage (75.31%) of linalool [8,16,18-22].

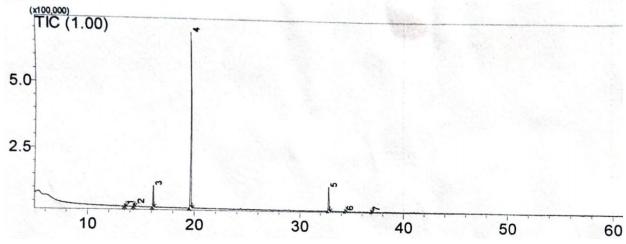

Figure 1: *GC-MS chromatogram of essential oil of Z. armatum*

Table 2: *Chemical constituents of essential oil from Z. armatum fruits*

Peak No.	Compound	Retention time	Area %	Class of the compound
1	β-Pinene	13.544	0.74	Monoterpene
2	Myrcene	14.388	1.16	Monoterpene
3	Limonene	16.173	9.45	Monoterpene
4	Linalool	19.676	75.31	Monoterpene alcohol
5	E-methyl cinnamate	32.771	11.73	Ester
6	Cis-α-Bergamotene	34.351	0.88	Bicyclic sesquiterpene

FTIR spectroscopic analysis

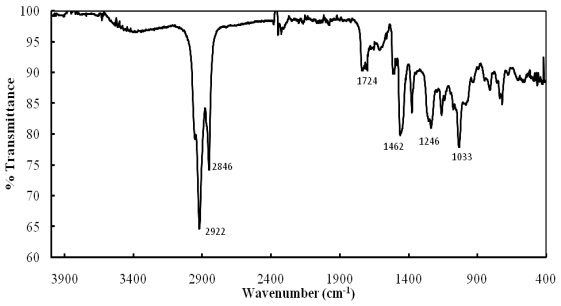

Figure 2: *FTIR analysis of methanol extract of Z. armatum fruits*

FTIR analysis was performed to identify the nature of functional groups of active components present in the methanol extract of *Z. armatum* fruits. FTIR spectroscopic analysis results (figure 2) revealed the presence of various characteristic functional groups at different peak values. The strong absorption peak at 2922 cm^{-1} and 2846 cm^{-1} showed the presence of C-H stretching. The peak at 1724 cm^{-1} represented the presence of the C-O stretch. The peak value at 1462 cm^{-1} represents the presence of C=C stretch. The band at 1246 cm^{-1} denotes the O-H absorption of alcohol and phenol. Similarly, the peak at 1033 cm^{-1} may arise due to the presence of a C-N bond.

Biological assays

In the toxicity test, a brine-shrimp bioassay of the samples was performed and LC_{50} values were calculated. LC_{50} values for the essential oil and methanol extract were found to be 76.70µg/mL and 62.25 µg/mL respectively. LC_{50} value is the lethal concentration dose required to kill 50% of the shrimps. Values less than 1000 µg/mL are supposed to be pharmacologically active [32]. So, *Z. armatum* is found to be cytotoxic against brine-shrimps as shown by the LC_{50} values much below the accepted maximum potential value of 1000.

The results of the antibacterial screening and antifungal screening of the oil and extract of the plant are presented in tables 3 and 4 respectively. The antibacterial and antifungal activities against the pathogens examined were assessed by the presence or absence of inhibition zones.

The result of the antibacterial sensitivity test reveals that essential oil has high antibacterial activity against both gram-positive and gram-negative bacteria as compared to the standard ciprofloxacin. Gram-positive bacteria *Bacillus subtilis*(11mm), *Micrococcus leutus* (16mm), *Staphylococcus aureus* (17mm) are found to be more sensitive than gram-negative bacteria *Klebsiella pneumonia* (9mm), *Enterobacter cloacae* (5mm), and *Pseudomonas aeruginosa* (6mm)towards the essential oil. The presence of the highest amount of linalool (75.31%), E-methyl cinnamate (11.73%), and limonene (9.45%) in the oil could be responsible for the high antibacterial activity of the essential oil from Nepal. Pattanaik e*t al.,* (1997) and Alviano *et. al*. (2005) reported the high antibacterial activity of linalool [17,23]. Moderate antibacterial activity had been previously reported for the essential oil of *Z. armatum* fruit [17]. The methanol extract of *Z. armatum* fruit showed moderate antibacterial activity compared to the essential oil. The extract could inhibit the growth of gram-positive bacteria only (*Bacillus subtilis, Micrococcus leutus, and Staphylococcus*

Table 3: *Antibacterial activity of the volatile oil and methanol extract of Z. armatum fruits*

Test organism (100µL)	Zone of inhibition in mm			
	Essential oil (0.3 mg/well)	MeOH extract (0.3 mg/well)	Ciprofloxacin (0.3 mg/ well)	DMSO
Klebesiella pneumonalae	9	NA	24	NA
Bacillus subtilis	11	7	26	NA
Micrococcus leutus	16	9	35	NA
Enterobacter cloaceae	5	NA	32	NA
Pseudomonas aeruginosa	6	NA	33	NA
Staphylococus aureus	17	11	38	NA

Note: NA = Not active (No ZOI was observed), Diameter of the well is 6 mm

Table 4: *Antifungal activity of the volatile oil and methanol extract of Z. armatum fruits*

Test organism (100µL)	Zone of inhibition in mm			
	Essential oil (15µL)	MeOH extract (15µL)	Miconazole	Methanol
Aspergillus flavus	9	7	30	NA
Fusarium solani	5	4	29	NA

aureus) with ZOI inhibition ranging 7-11 mm. Gram-negative bacteria *Klebsiella pneumonia, Enterobacter cloacae,* and *Pseudomonas aeruginosa* are found to be ineffective against the extract. Essential oil and the extract both showed moderate antifungal activity against the fungal strains *Aspergillus flavus* and *Fusarium solani* with ZOI ranging from 4 to 9 mm (Table 4). Previous studies also reported the moderate antifungal activity of *Z. armatum* [18,19].

DPPH radical scavenging activity

The free radical scavenging activity of *Zanthoxylum armatum* fruit extract, essential oil, and the standard ascorbic acid was determined by using the DPPH assay. IC_{50} values (Table 5) were calculated by using the equations of the graphs obtained from the plot between % radical scavenging activity and concentration. IC_{50} values of both extract and the essential oil are higher than that of the standard ascorbic acid (IC_{50} value = 39.55µg/mL). If the IC_{50} value of samples is closer to that of ascorbic acid, then the samples are considered more potent in terms of free radical scavenging capacity [26]. Free radical scavenging capacity of extract (IC_{50} value = 97.28 µg/mL) is found to be more than that of essential oil (IC_{50} value = 174.47 µg/mL). This variation is probably due to the difference in constituents present in the extract and essential oil. Essential oil chiefly contains the monoterpenes (Table 2) and the extract mainly contains the phytoconstituents having an antioxidant activity (Table 1).

***Table 5**: % radical scavenging at different concentrations and IC_{50} values*

Concentration (µg/mL)	**% Free radical scavenging**		
	Methanol extract	Essential oil	Ascorbic acid
0	0	0	0
12.5	9.88	4.76	48.58
25	19.64	16.46	54.93
50	35.75	24.29	67.76
100	63.11	42.11	88.08
200	86.94	49.71	96.02
IC_{50} value (µg/mL)	97.28	174.47	39.55

The presence of flavonoids and polyphenols in the extract may be responsible for the antioxidant activity [31]. Nooren *et al.,* (2017) showed that flavonoids like tambulin are chiefly present in *Z. armatum* fruits [24]. Brijwal *et al.,* (2013) and Phuyal *et al.,* (2020) reported that the fruits of *Z. armatum* possess high antioxidant activity and this is due to the high amount of total phenolic and total flavonoids present in them [25,26]. Our findings are in agreement with Negi *et al.,* (2012), Upadhaya *et al.,* (2010), and Dhami *et al.,* (2018) where the essential oil of *Z. armatum* fruits is found to more potent in terms of radical scavenging activity [27-29]. Singh *et al.,* (2013) also reported that the essential oil of *Z. armatum* fruits from India exhibited high scavenging ability of DPPH radicals [30].

Conclusion

Chemical and biological studies of the essential oil and methanol extract of the fruit of *Z. armatum* have been carried out. GC-MS analysis of the oil showed a high concentration of Linalool (75.31%) followed by E-methyl cinnamate (11.73%) and limonene (9.45%). The FTIR spectroscopic analysis of the methanol extract showed the presence of C-H, C=C, O-H, and C-N bonds. Phytochemical screening of the methanol extract revealed the presence of alkaloids, flavonoids, glycosides, polyphenols, volatile oils, terpenoids, tannins, and saponins. From the study of brine shrimp bioassay, essential oil and extract were found to be cytotoxic against brine-shrimp nauplii having LC_{50} values (µg/mL) 76.70 and 62.25 respectively. Essential oil displayed high antibacterial activity against gram-positive bacteria and moderate antibacterial activity against gram-negative bacteria. However, the methanol extract is found to be effective against gram-positive bacteria only. Both oil and extract showed moderate antifungal activity against bacterial strains.

Acknowledgments

The authors are thankful to the Department of Chemistry, Amrit Campus, Tribhuvan University for providing the laboratory facility. Special thanks go to the Department of Plant Resources, Thapathali, Kathmandu, and the Central Department of Chemistry, Tribhuvan University, Kirtipur for providing the GC-MS and FTIR facility respectively.

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