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GC-MS analysis, antibacterial, antioxidant and brine shrimp lethality assay of *Zanthoxylum armatum* DC.

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Abstract
Fruit part of *Zanthoxylum armatum* DC. was subjected to extraction of essential oil by hydro distillation in Clevenger apparatus. Dried and powdered plant was subjected to extraction with methanol. The composition of essential oil so collected were determined by GC-MS system and showed the presence of 20 different compounds. The most abundant were *β*-Linalool (60.00%), Methyl cinnamate (16.28%) and 3-Carene (9.69%). Antibacterial and antioxidant activity of the essential oil of *Z. armatum* were studied. Oil exhibited moderate antibacterial activity. *Z. armatum* showed efficient DPPH antioxidant activity with IC_{50} 58.5± 4.2µg/mL. The LD_{50} of the sample was found to be 35.1µg/mL in Brine shrimp lethality assay.

Keywords: *Zanthoxylum armatum* DC., Essential oil, GC-MS, Activity

Introduction
Zanthoxylum armatum DC. belongs to the family Rutaceae, an important family from economic point of view. It is a wild species and locally known as Timur [1]. It is widely distributed in the various countries like Nepal, India, China, Pakistan, Bhutan, Bangladesh, Japan, Korea, Taiwan, Laos, Myanmar, Thailand, Vietnam and Indonesia [2].
It is a sub deciduous aromatic shrub or small tree upto 5 m high, with pale brown corky bark and with numerous long straight spines on branchlets and leaf stalks, with pinnate leaves and with bisexual small yellow flowers in short branch lateral cluster and seed shining black and prefers semi shady or no shade for growth [2, 3].
Traditionally, leaves and fruits are used for mouth fresh and tooth care while bark is used for intoxicating the fishes and leaves, fruits and barks are also used as spice. Plants essential oils, commonly used as fragrances and flavouring agents for foods and beverages [4]. The volatile oil is employed as an antidiarreheal, antiseptic, deodorant and anticatarerhal. The oil has a good tenacity and is appreciated for its fixative qualities. The essential oil composition can provide much more knowledge regarding the medicinal properties and active constituents of this plant [1].
In Nepal 8 species of this genus is reported which are *Z. armatum, Z. acanthopodium, Z. bungeanum, Z. nepalenses, Z. nitidum, Z. avalifolium, Z. simulans* and *Z. oxyphyllum* [3].
The reported compounds of *Z. armatum* are asarinin, fargesin, α and β- amyrins, β-sistosterol-β-D-glucoside, berberine, dictamnine, xanothplanine, armatumamide, α-pinene, β-pinene, α-terpinene, α-phellendrene, linalool, caryophyllene, etc. [1, 3, 5].
This present study of essential oil as well as methanolic extract of fruit of *Z. armatum* has been carried out to determine major constituents through GC-MS and study its antioxidant and antibacterial activities.

Experimental
Collection of Plant Materials
The plant material (fruits) was collected from Baglung District. The plants were identified by Department of Botany, Amrit Campus, Lainchour, Kathmandu.

Preparation of Plant Extracts
The clean and dried fruits were grinded to powder and further proceeded via cold percolation process for 7 days for three times with 2.5 liters methanol. The methanol extract was concentrated by evaporation on rotavapour. Plants extracts were stored at 4°C.

Extraction of Essential Oil

The mature fruits of *Zanthoxylum armatum* were crushed for hydro distillation and subjected to a Clevenger apparatus for three hours. By this process about 3ml of pale yellow coloured essential oils were collected and stored in a sealed glass vials at low temperature(0-4°C) prior to analysis.

GC-MS Analysis

The essential oils sample of *Zanthoxylum armatum* was subjected to GC-MS analysis. GC/MS analysis was performed on a gas chromatography mass spectrometer GCMS-QP2010 under the following condition: injection volume 1µL with split ratio 1:50; Helium as a carrier gas with a Rtx-5MS column of dimension 30m×0.25mm×0.25µm, temperature programmed at 40, 200 and 280ºC with a hold time of 2.0, 3.0 and 4.0 min identification was accompanied by comparison of MS with those reported in NIST 05 and FFNSCI.3 libraries. It was performed in Department of Food Technology and Quality Control, Nepal Government, Babarmahal, Kathmandu, Nepal.

Antioxidant Assay (DPPH method)

First of all, 1mg of sample to be tested was dissolved in 1ml methanol to get stock solution of concentration 1mg/ml. 100µl of these solutions were added to 100µl of 0.1mM DPPH (prepared in methanol) and was left for 30 minutes in dark room. After 30 minutes, their absorbance was taken at 517 nm against DPPH and DMSO as a blank. Quercetin was prepared as standard. The extracts or essential oil, which do not show antioxidant property was discarded and for the sample with the yellow colour (more than 50% inhibition then control) was taken for further testing as they were expected to be the potential antioxidants.

Different concentration of the extracts were prepared by two-fold dilution method to find the IC_{50} value.

Antibacterial activity assays

Antimicrobial assay of extracts of plants was performed by agar well diffusion method in Muller Hilton Agar (MHA) and the minimum bactericidal concentration of those extract was determined by micro dilution method. All the strains of bacteria was cultured in Nutrient broth (NB) and incubated at 37 ^{0}C for 18 hours. After incubation each stain were diluted with sterile distilled water. The turbidity of dilution was compared with 0.5 McFarland standards (approximately 10^8 CFU/ml). The suspensions were then diluted (1:100) in Muller Hilton Broth (MHB) to obtain 10^6 CFU/ml. Prepared inoculums were incubated for 30 minutes at 37 ^{0}C prior to use.

Plant extracts (30 µl) were loaded into the respective wells with the help of micropipette. The solvent (50% DMSO) was tested for its activity as a control at the same time in the separate well. The Neomycin 20 µg/ml was used as a positive control. The plates were then left for half an hour with the lid closed so that extracts diffused to the media. The plates were incubated overnight at 37 °C. After proper incubation (18-24 hours) the plates were observed for the zone of inhibition around well which is suggested by clean zone without growth. The ZOI were measured with the help of the ruler and mean was recorded for the estimation of potency of antibacterial substance.

Determination of Minimum Bactericidal Concentration

The Minimum Bactericidal Concentration (MBC) was determined by micro dilution method. The methanol extracts were diluted by two fold to get series of concentrations from 0.048 to 25 mg/ml in freshly prepared sterile nutrient broth. 20µl of the microorganism suspension (correspond to 10^6 CFU/ml) was added to each of the sample dilutions. These were incubated for 18 hours at 37°C and each tube content was subculture in fresh nutrient agar separately and minimum bactericidal concentration was determined that showed no growth at all.

Determination of the Minimum Inhibitory Concentration

The smallest amount of compounds required to kill or inhibit the growth of micro-organism *in vitro* can be determined by the dilution method. This amount is referred as minimum inhibitory concentration (MIC). It is a measure of potency which is expressed in terms of either µg or mg/ml. A stock solution of 25 mg/ml was prepared. This was serially diluted to obtain various ranges of concentrations between 25 mg/ml to 0.048 mg/ml.

Brine Shrimp Lethality Assay

Ten nauplii were exposed to each of different concentrations of the plant extract and number of survivors were calculated the percentage of death after 24 hours.

Result and Discussion

GC-MS Analysis

GC-MS analysis of essential oils of fruits of *Zanthoxylum armatum* shows the presence of 20 different compounds. The chemical compound identified in essential oils of the fruits of the *Z. armatum* plant are presented below:

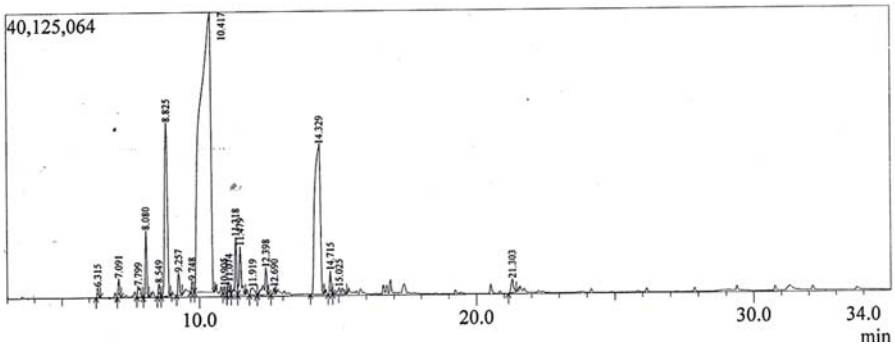

Fig 1: Chromatogram of essential oils of fruits of *Z. armatum*

The major constituents present in the essential oils sample were *β*-linalool (60.00%), cinnamic acid, methyl ester (16.28%) and 3-carene (9.69%). Constituents of essential oils of *Z. armatum* are tabulated as follows.

Table 1: List of compounds in essential oils *Z. armatum*

S.N.	Name of the compounds	Molecular Formula	Molecular Weight	Retention Time	Area %	Height %
1.	Annulene	C_8H_8	104	6.315	0.31	1.09
2.	α-Pinene	$C_{10}H_{16}$	136	7.091	0.55	198
3.	β-Phellandrene	$C_{10}H_{16}$	136	7.799	0.58	1.12
4.	β-Myrcene	$C_{10}H_{16}$	136	8.080	2.02	6.94
5.	2-Carene	$C_{10}H_{16}$	136	8.549	0.41	1.38
6.	3-Carene	$C_{10}H_{16}$	136	8.825	9.69	18.08
7.	γ-Terpinene	$C_{10}H_{16}$	136	9.257	0.69	2.34
8.	2-Carene	$C_{10}H_{16}$	136	9.748	0.34	1.19
9.	β-Linalool	$C_{10}H_{18}O$	154	10.417	60.00	28.98
10.	Citronellol acetate	$C_{12}H_{22}O_2$	198	10.905	0.24	0.74
11.	n-Nonadecanol	$C_{19}H_{40}O$	284	11.074	0.45	1.29
12.	Terpinen-4-ol	$C_{10}H_{18}O$	154	11.318	2.06	5.80
13.	α-Terpineol	$C_{10}H_{18}O$	154	11.479	2.18	4.95
14.	β-Citronellol	$C_{10}H_{20}O$	156	11.919	0.59	0.74
15.	Piperitone	$C_{10}H_{16}O$	152	12.398	1.42	2.76
16.	Phellandral	$C_{10}H_{16}O$	152	12.690	0.24	0.66
17.	Methyl cinnamate	$C_{10}H_{10}O_2$	162	14.329	16.28	15.51
18.	Caryophyllene	$C_{15}H_{24}$	204	14.715	0.69	2.35
19.	5-methyl-3-(1-methylethenyl)-Cyclohexene	$C_{10}H_{16}$	136	15.025	0.58	0.68
20.	Tridecanoic acid	$C_{13}H_{24}O_2$	212	21.303	0.70	1.44
					100.00	100.00

Antibacterial activity

Table 2: Antibacterial activity of the essential oils of the *Z. armatum*

Sample	MIC Values					MBC Values				
	SA	EC	EC**	MRSA	KP	SA	EC	EC**	MRSA	KP
Neomycin*(µg/ml)	0.078	0.156	1.25	0.156	0.0195	1.25	0.625	1.25	5	0.156
Oil(mg/ml)	12.5	1.56	0.78	1.56	0.39	12.5	6.25	12.5	6.25	25
Extract(mg/ml)	-	-	-	-	0.78	-	-	-	-	12.5

*Control Antibiotics
SA = *Staphylococcus aureus* (ATCC) 25923
EC = *Escheriachia coli* (ATCC) 25922
EC** = *Escheriachia coli* MDR
MRSA = Methicillin resistance *Staphalococcus aureus* (MRSA)
KP = *Klebsiella pneumoniae* (MDR)

Antioxidant Activity

The antioxidant potential is in an inverse relation with IC_{50} value, which can be calculated from linear regression of the % inhibition verses antioxidant activity. Lower the IC_{50} value indicates high antioxidant activity.

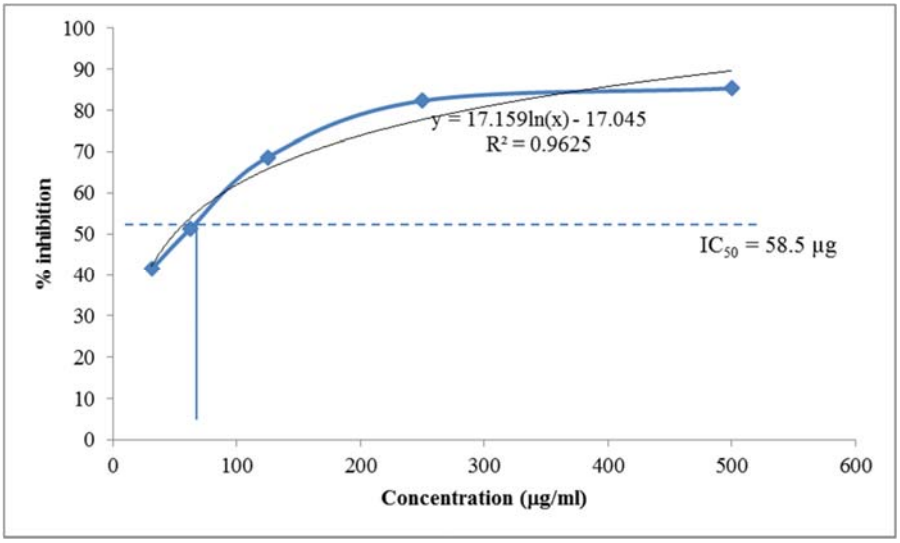

Fig 2: Graphical representation of DPPH assay of the methanolic extract of *Z. armatum*

The IC_{50}±SEM of the sample was found to be 58.5± 4.2µg/mL and the standard, quercetin was 2.28±0.1µg/mL.

Brine Shrimp Lethality Assay

The LD_{50} of the sample was found to be 35.1µg/mL in Brine shrimp lethality assay.

Conclusion

The major constituents present in the essential oils sample were *β*-linalool (60.00%), methyl cinnamate (16.28%) and 3-carene (9.69%). *Zanthoxylum armatum* showed moderate antibacterial property against *Staphylococcus aureus* (ATCC) 25923, *Escheriachia coli* (ATCC) 25922, *Escheriachia coli* MDR, Methicillin resistance *Staphalococcus aureus* (MRSA) and *Klebsiellap neumoniae* (MDR). The IC_{50}±SEM of the sample was found to be 58.5± 4.2µg/mL, from DPPH antioxidant activity. In Brine shrimp lethality assay, the LD_{50} of the sample was found to be 35.1µg/mL.

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