Phytochemical and Biological Studies on *Zanthoxylum Armatum* of Nepal

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Abstract

Phytochemical and biological screening of stem extracts of *Zanthoxylum armatum* of Nepal were carried out. Presence of sterols, triterpenes, volatile oils, coumarins, alkaloids, flavonoids, flavonic glycosides, saponins and tannins were detected in phytochemical screening. β-sitosterol and lupeol were isolated from the hexane fraction and β-sitosterol glucoside was isolated from the ethyl acetate fraction. Lupeol was identified by comparison of melting point, Co-TLC and ^{1}H-NMR spectrum with authentic sample. β-sitosterol and β-sitosterol glucoside were characterized by their melting points and Co-TLC with authentic samples. In brine-shrimp bioassay, hexane and methanol fractions exhibited bioactivity, whereas ethyl acetate and methanol fractions showed proper bioactivity in antimicrobial bioassay.

Keywords: *Zanthoxylum armatum*, cytotoxic activity, anti-bacterial property, β-sitosterol, Lupeol, β-sitosterol glycoside

Introduction

Zanthoxylum armatum known in Nepali language as timur belongs to Rutaceae family. It grows throughout Nepal in between 1500-2400m altitude. *Z. armatum* is an armed, erect shrub or small tree up to 6m high with dense foliage. Plants based remedies have been part of traditional health care in most parts of the World for thousands of years and there are increasing scientific interest in medicinal plants as source of novel agents to fight infectious diseases [1]. Since *Z. armatum* plant is medicinally important there are large numbers of bioactive compounds are isolated and identified. Different types of chemical constituents such as alkaloids, flavonoids, terpenoides, Glycosides, sterols, tannins, ligands, coumarins, amides and essential oils are reported in this plant [2].

The bark, fruits and seeds of *Z. armatum* are extensively used in indigenous system of medicine as a carminative, stomachic and anthelminthic. The seed and bark are also used as an aromatic tonic in fever, dyspepsia. Because of their deodorant, disinfectant and antiseptic properties, the fruits are used in dental troubles, their lotion for scabies and also used to ward-off houseflies [3]. Besides this it is also used as a flavouring agent in the confectionery industry and in the manufacture of soft drinks [4]. Anti tumour activities, antistress activities, antinflammatory and antioxtidant activities were evaluated in different parts of this plant [11, 12, 13]. Essential oil of *Z. armatum* possesses leech repellent activity [10].

Experimental

Phytochemical screening

The plant material was collected from Rolpa district and identified from Central Department of Botany, Tribhuvan University, Kirtipur. Stems of the plants were cut into small pieces and air dried and

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grinded to make powder. 100 gm. of air dried plant material was extracted successively by hexane, ethylacetate and methanol by soxhlet extraction method. Each fraction was concentrated to reduce its volume up to 50 ml. The method employed for phytochemical screening was based on the procedure given by Prof. I. Ciulei [9]. The different classes of natural compounds present in the extracts are identified by their colour rections with the different specific reagents.

Isolation and chacterization of Compounds

Air dried powder material (2kg) was extracted successively with solvent of increasing polarity by soxhlet extraction method. Firstly, it was extracted with hexane and was concentrated under reduced pressure to obtain the hexane extract (10 gm). The marc was again extracted with ethylacetate and concentrated under reduced pressure to obtain ethylacetate extract (12 gm). Finally, it was extracted with methanol and concentrated under reduced pressure to obtain a methanolic extract (30 gm.).

The Hexene extract (7 gm) was adsorbed on equal amount of silica gel and this mixture was loaded on silica gel (100 gm, E-merck, 60-120 mesh) packed in column having internal diameter 4 cm with the adsorbent height 45 cm. The column was eluted with gradients of hexane, ethylacetate and methanol to obtain number of fractions and concentrated in a rotatory evaporator.

Fraction 4 obtained by eluting with 2% ethylacetate in hexane gave compound ZA_1 which was concentrated and the purity was checked by TLC. For purification, the residue was washed with hexane and dissolved in pure ethylacetate and added a few drops of hexane to the drop-wise till turbid. White Crystalline substance was obtained after 24 hrs. It was filtered and washed with hexane. The compound was UV inactive and it was observed by charring with conc. H_2SO_4.. The compound gave positive Libermann- Burchard test of greenish red colour indicating itself as sterol and found a single spot on TLC with R_f value 0.40 in 20% ethylacetate in hexane. It was soluble in Chloroform, ethylacetate and methanol. The melting point was found to be 134^0C.

Fraction 6 obtain from 10% ethylacetate in hexane was concentrated in a rotatory evaporator. The residue was washed with hexane and recrystallized with methanol for purification. The compound ZA_2 was found to be single spot on TLC (in 30% ethylacetate in hexane) with R_f value 0.68 and melting point 216^0C. The compound was soluble in ethylacetate and chloroform and gave positive Libermann-Buchard test (pink colour for triterpene) indicating to be a triterpenoid compound.

Ethylacetate extract (10 gm) was adsorbed on equal amount of silica gel and loaded on silica gel column as same as hexane extract. Fraction 10 obtained on elution with 50% of ethylacetate in hexane was reduced the volume by rotatory evaporator. Several times of washing with cold methanol to this residue, a white amorphous compound ZA_3 was obtained. The compound gave a positive Molish's test. The melting point was above 200 and R_f value 0.64 (in 1:3, MeOH: $CHCl_3$).

Brine-Shrimp Bioassay

The procedure followed for the brine-shrime bioassay was carried out according to McLaughlin *et al* procedure [7]. This procedure involves introducing the newly hatched Brine shrime nauplii to the crude plant extract. The method determines the LC_{50} values (μg/ml) for crude extract which is less than 1000 ppm are considered as potentially pharmacologically active.

Antibacterial activity

The antibacterial susceptibility tests measure the ability of an antibacterial agent to inhabit the bacterial growth. This process evaluated the effectiveness of antimicrobial substance by determination of zone of inhibition (ZOI), minimum inhibitory concentration (MIC) of the antibacterial agent and minimum bacterial concentration (MBC) for bacteria and minimum fungicidal concentration (MFC) for fungi [8].

Results and Discussions

Phytochemical screening

The results obtained from phytochemical screening are given bellow.

Phytochemical Screening for Non- polar Solvent (Hexane) Extract

S.N.	Group of Compounds	Results
1	Volatile oils	+
2	Basic Alkaloids	-
3	Carotenoids	-
4	Flavone Aglycones	-
5	Emodins	-
6	Quinones	-
7	Coumarins	-
8	Sterols and Triterpenes	+
9	Fatty acids	+

Phytochemical Screening for Semi-polar Solvent (Ethyl acetate) Extract

S.N.	Group of Compounds	Results
1.	Volatile oils	+
2.	Basic Alkaloids	-
3.	Carotenoids	-
4.	Flavone Aglycones	-
5.	Glycosides	+
6.	Quinones	-
7.	Coumarins	+
8.	Sterols and Triterpenes	+
9.	Fatty acids	+

Phytochemical Screening for Polar Solvent (methanol) Extract

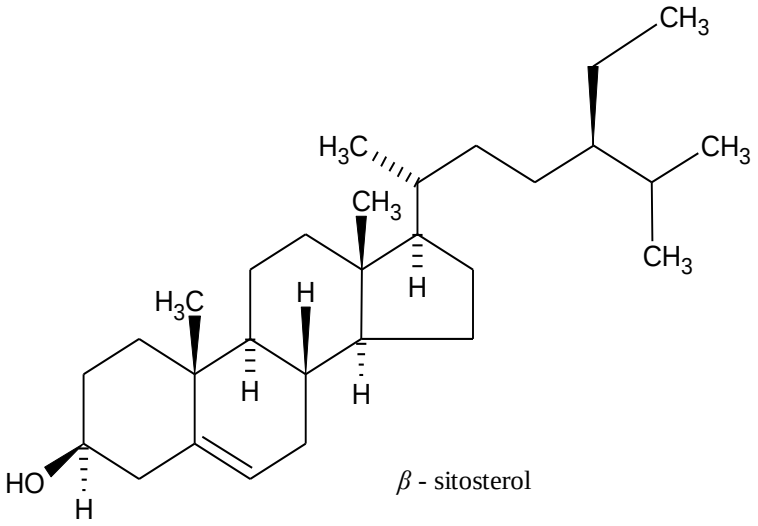
S.N.	Group of Compounds	Results	S.N.	Group of Compounds	Results
1.	Polyphenols	+	7.	Coumarins	-
2.	Reducing compounds	-	8.	Sterols and Triterpenes	-
3.	Alkaloids	+	9.	Resins	+
4.	Coumarins	-	10.	Tannins	+
5.	Flavonic glycosides	+	11.	Saponins	+
6.	Flavonoids	+			

+ = Present - = Absent

Identification of isolated compound

Compound ZA$_1$

It is white crystalline compound, soluble in chloroform, ethyl acetate and methanol, melting point 134^{o}C and R_f Value 0.40 (in 20% EtOAc in hexane). The compound was identified as β-sitosterol from its melting point, Co-TLC with authentic sample [6].



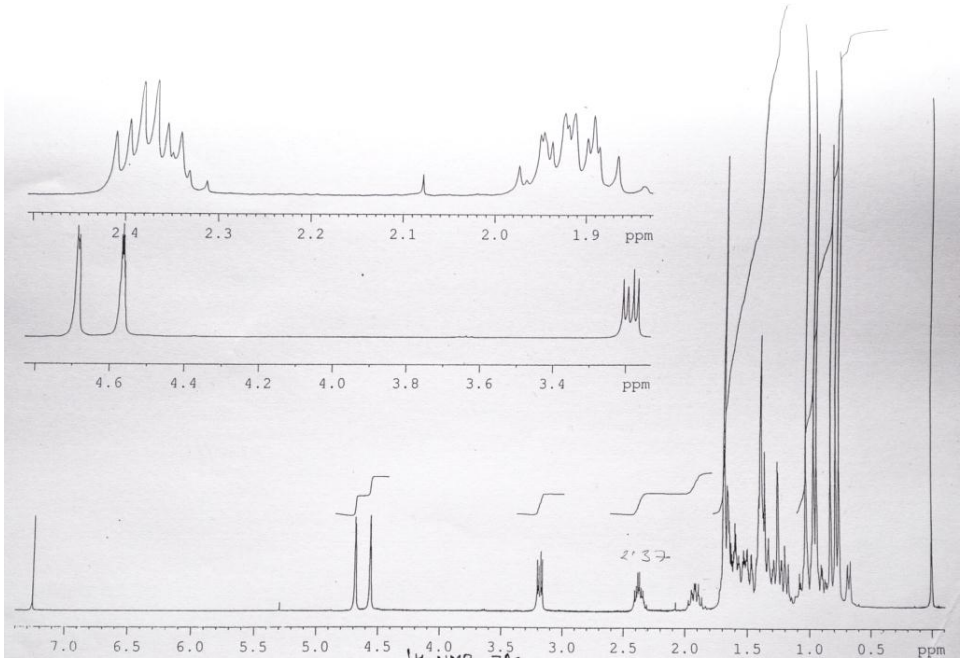
β - sitosterol

Compound ZA$_2$

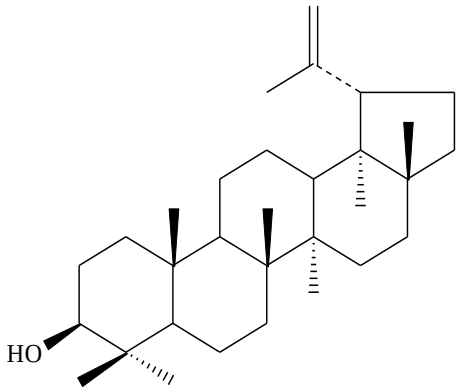
It is white crystalline compound, soluble in chloroform and ethyl acetate, R_f Value 0.68 in 30% ethyl acetate in hexane, R_f 0.46 in 1:1 ethyl acetate: toluene, melting point 216^{o}C and gave a positive Libermann-Buchard test indicating it to be a triterpenoid compound. The compound was identified as Lupeol by comparison with its melting points, Co -TLC and ^{1}H-NMR Spectrum with authentic sample. Lupeol exhibited anticancer properties [5].

^{1}H-NMR Spectrum (δ values in ppm):

0.76 (S, 3H), 0.79 (S, 3H), 0.83 (S, 3H), 0.945 (S, 3H), 0.965 (S, 3H), 1.31 (S, 3H), 1.31 (S, 3H), 1.67 (3H, dd, J = 1.0Hz), 0.68 (d, J = 9Hz, H),3.18 (dd, J = 10.0Hz, 10.0, Hz, H), 4.58 (td, J = 1.0, 1.0, 1.0Hz, H), 4.68 (d, J = 2.2Hz, H), 2.37 (td, J = 6.0, 11.0, 11.0Hz, H), 1.385 (d, J = 8Hz, H) 7.25 (H)



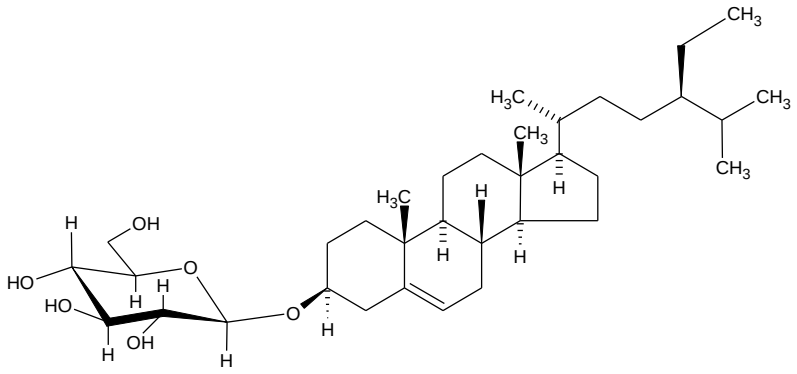
H-NMR-ZA_2 (Lupeol)



Lupeol

Compound ZA_3

It is white compound, soluble in mixture of methanol and chloroform, R_f Value 0.64 in 25% MeOH in $CHCl_3$, melting point above 200^oC and gave the positive Molisch's test indicating it to be a glycoside. The compound was identified as β-sitosterol glycoside by comparing its melting point and Co-TLC with authentic smple.



β – sitosterol – β – D – glycoside

Brine Shrimp Bioassay

From the brine shrimp bioassay of all extracts; it was concluded that hexane, and Methanol fractions were toxic against brine shrimp nauplii (LC_{50} value less than 1 x 10^3 is Toxic and more than $1x10^3$ is less toxic).

S. N.	Extracts	LC_{50} (µg/ml)
1	Hexane	215.44
2	Ethyl Acetate	1293.20
3	Methanol	298.80

Antimicrobial Screening

The diameter of Zone of inhibition (ZOI) produced by plant fraction on particular bacteria was measured for the estimation of potency. Methanol fraction exhibited potent antibacterial activity than ethyl acetate fraction whereas hexane fraction was found to be inactive towards bacteria.

Conclusion

Presence of volatile oils, sterols, triterpenes, fatty acids, glycosides, coumarins, polyphenols, alkaloids, flavonoids, resins, tannins and saponins is determined from the phytochemical screening of the plant extract of *Z. armatum.*

β-sitosterol and Lupeol were isolated and identified from the hexane extract, whereas β- sitosterol glycoside from ethyl acetate extract.

Brine Shrimp Bioassay indicated that hexane and methanol extract exhibited cytotoxic activity except ethyl acetate extract.

Bioassay of ethyl acetate and methanol fractions against the different gram positive and gram negative bacteria showed proper anti-bacterial property in different concentration whereas hexane fraction was ineffective to both types of bacteria.

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