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Two New Bioactive Furoquinoline Alkaloids from *Zanthoxylum armatum*

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The *n*-hexane, dichloromethane, ethyl acetate and *n*-butanol soluble fractions of *Zanthoxylum armatum* were screened for antifungal activity against *Aspergillus flavus* and *Aspergillus niger*. The dichloromethane soluble fraction of *Zanthoxylum armatum* inhibited the growth of *Aspergillus flavus* by 22.5 % and *Aspergillus niger* by 41.2 %, while the ethyl acetate soluble fraction compound decreased the growth of *Aspergillus flavus* and *Aspergillus niger* by 55.5 and 61 % respectively. The *n*-hexane and *n*-butanol fractions showed low inhibitions against these fungi. Further the chromatographic separation of the ethyl acetate fraction led to the isolation of two new furoquinoline alkaloids; 4-methoxy-12,12-dimethylcyclopenta furoquinoline-10-carbaldehyde and methyl 4-methoxy-12,12-dimethylcyclopenta furoquinoline-10-carboxylate. The isolated compounds were tested for antioxidant activities. The compound **1** was found more potent in both DPPH and ABTS radical scavenging assay.

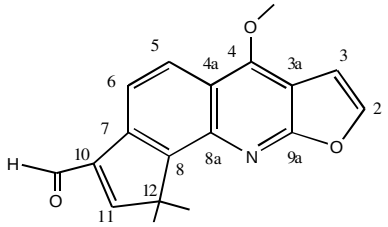
Keywords: *Zanthoxylum armatum*, Furoquinoline alkaloids, Antifungal, Antioxidant.

INTRODUCTION

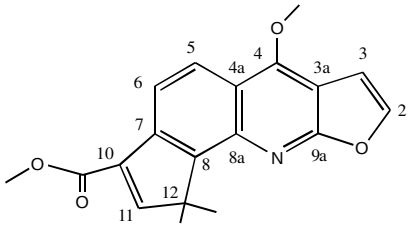
Zanthoxylum is a genus of about 250 species of deciduous and evergreen trees and shrubs belonging to family Rutaceae, native to warm temperate and subtropical areas worldwide[1]. Various plants of the genus *Zanthoxylum* have widely been used by tradition healers against several ailments like colic, flatulent, gastro-intestinal disorder and intestinal paralysis as well as in snake bite remedy. Anciently, the Zulus used the powdered bark of these plants to relive tooth ache and against T.B[2]. *Z. gillnet* is used in Ghana and Nigeria as an antiseptic and analgesic[3]. *Z. allantoids* have been used in myocardium disorder attenuation, bone-injury alleviation and cold resistance[4]. Some of the species of this genus are reported to contain alkaloids, especially benzophenanthriidines and exoprofinic alkaloid liriodenine, which show remarkable anti-tumour activity and potent inhibiter of topoisomerase II, respectively[5-7]. *Z. schinifolium* possesses apoptogenic activity against human acute leukaemia[8]. *Z. armatum* is widely distributed in siren valley, Mansehra, Pakistan. Its Hindi name is Tejbal while local name is Timber. In Pakistan, the only one species of zanthoxylem genus is found[9]. The essential oil of fruits of *Z. armatum* shows antibacterial, antifungal, anthelmintic activities and also used as leech repellent and a cure in skin diseases[10,11] while the seeds and roots are stomachic and vermifuge[12-14]. The plant has also find uses in the treatment of chest and cardiac pain, for piles and tamers. A product naming "*Thumburu*" from this plant is used in treating numbness and atrophy. In Unani (Greek) medicine, the fruit seeds and bark are used as a carminative, stomachic and anthelmintic[15]. The bark is also used as a remedy for toothache[16]. Phytochemically, the plant is reported to contain *cis*-9-hexadecenoic, monoterpenes, enoic acids and palmatic acids, tambuletin[17,18] along with the sesamin, asarinin and limonene and zanthonitrile[19]. Four alkenoic acids were obtained from the hexane extract of the seed of this plant previously[20]. In the present study two new compounds **1** and **2** were isolated from the ethyl acetate soluble fraction of *Zanthoxylum armatum* (Fig. 1). Further the different solvent soluble fraction of *Zanthoxylum armatum* and the isolated compounds were screened for antifungal and antioxidant activities.

EXPERIMENTAL

Aluminium TLC plates (20 × 20, 0.5 mm thick) pre-coated with silica gel 60 F_{254} (20 × 20 cm, 0.2 mm thick; E. Merck, Darmstadt, Germany) were used for TLC to check the purity of the compounds. Column chromatography (CC) was carried out using silica gel of 230-400 mesh (E. Merck, Darmstadt, Germany). Preparative TLC glass plates (20 × 20, 2 mm thick)



4-methoxy-12, 12-dimethylcyclopenta furoquinoline-10-carbaldehyde (**1**)



Methyl 4-methoxy-12,12-dimethylcyclopenta furoquinoline-10-carboxylate (**2**)

Fig. 1. Structures of compounds **1** and **2** isolated from *Zanthoxylum armatum*

pre-coated with silica gel 60 F_{254} (0.5 mm layer thickness; E. Merck, Germany) were used for the purification of semi pure compounds. Ceric sulphate and potassium permanganate solutions were used as visualization reagents. The UV spectra were recorded on Shimadzu UV-2700 spectrophotometer (Shimadzu, Japan) in MeOH. Mass Spectra was recorded on Bruker TOF Mass spectrometers (Billerica, USA) using electrospray ionisation (ESI). The ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker DPX-400 NMR spectrometer (Billerica, USA). Further assignments were made by DEPT, COSY, HMQC and HMBC experiments.

The whole plant of *Zanthoxylum armatum* was collected from Siran valley, Hazara division, District Mansehra, in 2008 and was identified by Professor Dr. Manzoor Ahmad, Plant Taxonomist, Department of Botany, Government Degree College Abbotabad, Pakistan, (Accession No. C-0028).

Extraction and isolation: The shade dried whole plant of *Zanthoxylum armatum* was ground and extracted with methanol at room temperature. The combined methanolic extract was evaporated under reduced pressure to obtain a thick greenish gummy material (crude). It was suspended in water and successively partitioned with suitable solvents to yield *n*-hexane, dichloromethane, ethyl acetate and *n*-butanol soluble fractions, respectively.

The ethyl acetate soluble fraction was subjected to column chromatography over silica gel (70-230 mesh) eluting with *n*-hexane: EtOAc (1:19-19:1), EtOAc (100 %), EtOAc: MeOH (1:19-5:1), in increasing order of polarity to obtain compound **8** fractions E01-E08.

The subfraction Et07 gave two spots on TLC with good R_f value and hence was rechromatographed over silica gel column chromatography eluted with *n*-hexne: EtOAc (5:1-3:2) to get the two spots separately which were placed in fume hood to get fine crystals of compound **1**.

Subfraction E08 gave several spots on TLC and was loaded over silica gel column chromatography eluted randomly with *n*-hexane: EtOAc (10:1-3:2) to afford compound **2** as white needles.

4-Methoxy-12,12-dimethylcyclopenta furoquinoline-10-carbaldehyde (1): White crystal; m.p. 210-212 °C; U.V (MeOH) λ_{max} (log ε): 221 (3.11), 249 (3.22), 236 (3.33), 255 (3.27), 266 (3.46), 278 (3.36), 310 (3.92), 318 (3.95), 385 (4.15); IR (KBr, ν_{max}, cm^{-1}): 2960, 2910, 2850, 2820, 1640, 1625, 1610, 1585, 1510, 1380, 1325, 1245, 1091, 1034; ^{1}H NMR and ^{13}C NMR ($CDCl_3$, 400 and 100 MHz) Table-1; HR-EI-MS *m/z* 293.54 $[M + H]^+$ Calcd. for $C_{18}H_{15}NO_3$, 293.32).

Methyl 4-methoxy-12,12-dimethylcyclopenta furoquinoline-10-carboxylate (2): White needles; m.p: 223-224 °C; U.V (MeOH) λ_{max} (log ε): 240 (3.27), 249 (3.24), 272 (3.83), 312 (4.20), 324 (3.90) and 332 (4.15) nm; IR (KBr, ν_{max}, cm^{-1}): 2934, 2862, 1715, 1620, 1615, 1600, 1595, 1510, 1365, 1336, 1253, 1092, 1025; ^{1}H NMR and ^{13}C NMR ($CDCl_3$, 400 and 100 MHz) Table-1; HR-EI-MS *m/z* 323.46 $[M + H]^+$ Calcd. for $C_{19}H_{17}NO_4$, 323.34).

TABLE-1
^{1}H AND ^{13}C NMR DATA OF COMPOUNDS **1** AND **2** (δ IN ppm, OBTAINED IN $CDCl_3$)

No.	Compound 1		Compound 2	
	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
2	143.13	7.61 d (2.9)	146.45	7.68 d (3.2)
3	105.78	7.02 d (2.9)	107.47	6.89 d (3.2)
3a	109.21	–	108.28	–
4	157.69	–		157.35
4a	123.48	–	121.30	–
5	119.89	7.91 d (8.4)	117.55	8.02 d (8.5)
6	129.26	7.69 d (8.4)	125.66	7.91 d (8.5)
7	143.96	–	138.34	–
8	148.14	–	152.18	–
8a	145.17	–	140.82	–
9	–	–	–	–
9a	165.39	–	161.91	–
10	146.56	–	131.74	–
11	161.51	7.42 s	155.73	7.42 s
12	53.08	–	49.87	–
CH_3	27.81	1.38 s	25.65	1.40 s
OCH_3	62.32	4.46 s	62.39	4.41 s
CH_3OCO	–	–	55.41	3.72 s
H-C=O	196.53	10.09 s	–	–
Ac	–	–	159.57	–

Antifungal assay: The antifungal activity of different solvent soluble fractions of *Zanthoxylum armatum* was determined by using the the protocol[21]. 1 mg/mL stock solution of crude hexane, dichlormethane, ethyl acetate fraction and *n*-butanol fractions were prepared in DMSO. Furthermore, 1 mL solution of 200 µg/mL was prepared from the stock solution using DMSO as diluting solvent. In the same way, terbinofine stock solution of 1 mg/mL (positive control/antifungal agent) was prepared in DMSO. Moreover, 1 mL solution of the required concentration (200 µg/mL) was prepared from the stock solution in DMSO. Correspondingly, 1 mL DMSO was taken, used as a negative control from the bottle. In order to grow fungus for inoculums preparation, Sabouraud dextrose agar (MERCK) was used, composed of peptone complex 10 g/L, glucose 40 g/L and agar 15 g/L. 6.5 g SDA media was dissolved in 100 mL distilled/autoclaved water in flask for fungus growth and autoclaved at 121 °C for 15 min. 4 mL of this media was poured in the all the autoclaved test tubes and marked up to

10 cm in the Laminar flow cabinet for two fungal strains. From the required concentration (200 µg/mL) of the solution, 67 µL of extract solution was put in all the 4 test tubes, which were specified and duplicate for the two fungal strains. In the same way, the terbinofine solution of 67 µL (for positive control) of the required concentration (200 µg/mL) was put in all the two test tubes (one for each) of the two fungal strains. Similarly, 67 µL DMSO (negative control) was poured in another set of two test tubes (one for each) of the two fungal strains. After the completion of this whole process, all the test tubes were place in the laminar flow in slanting position for solidifying the media in the test tubes at room temperature. After the solidification process, 15 spores from 7 days old culture were placed of each fungus strain in all the test tubes (extract + control) and were specified very carefully for each strain. All the test tubes were packed air tightly and were placed in incubator at 36 °C for 7 days. Their growth was measured after 7 days and calculated their % inhibition.

DPPH radical scavenging assay: The antioxidant DPPH radical scavenging assay was done according to the standard procedure with some modifications[22]. In the assay 900 µL methanolic solution of DPPH (1 mM), 100 µL from each of the isolated compound previously solublized in methanol was added at various concentrations, from 50 to 500 µg/mL in a final volume of 1 mL. An equal amount of methanol was added to the control. After 20 min, absorbance was recorded at 517 nm. Experiment was done in duplicate and performed in triplicate.

ABTS radical scavenging assay: The 2,2'-azino-*bis*-(3-ethylbenzthiazoline-6-sulphonic) (ABTS) have high ability to act as an electron donor for the reduction of oxo species. ABTS assay was made according to the standard method with some modification[23]. Assay procedure equal volumes of 7 mM ABTS and 35 mM $K_2S_2O_8$ were mixed and incubate for 24 h. 1200 µL of the sample, from each concentration *i.e*., (50, 100, 150, 200, 250, 500 µg/mL each of these concentration was taken in duplicate) was mixed with 1800 µL of ABTS solution was mixed with it for each concentration. Similar process was repeated for ascorbic acid concentration. All test tubes (compounds **1-2** and ascorbic acid) labeled separately were shaken well and incubated in the dark at 25 °C for 0.5 h. The kinetic absorbance was recorded at 734 nm by spectrophotometer after 1 and 6 min for each concentration and mean was taken for each reading. The potential to scavenge the ABTS radical was calculated using the following equation:

$$\text{ABTS radical scavenging effect (\%)} = \frac{(A_1 - A_2)}{A_1} \times 100$$

where A_1 is the absorbance of the control (ABTS solution without test sample) and A_2 is the absorbance in the presence of the test sample.

RESULTS AND DISCUSSION

The *n*-hexane, dichloromethane, ethyl acetate and *n*-butanol fractions were screened for antifungal activities against *Aspergillus flavus* and *Aspergillus niger*. The ethyl acetate soluble fraction showed maximum inhibition and decreased the fungal growth by 55.5 and 61 % against *Aspergillus flavus* and *Aspergillus niger,* respectively, dichloromethane fraction showed moderate inhibition while *n*-hexane and *n*-butanol fractions showed minimum inhibitions against the corresponding fungi (Table-2).

TABLE-2
ANTIFUNGAL ACTIVITY *n*-HEXANE, DICHLOROMETHANE, ETHYL ACETATE, AND *n*-BUTANOL FRACTIONS OF *Xanthoxylum armatum* AGAINST *Aspergillus flavus* AND *Aspergillus niger*

Fractions, solvent and control	*Aspergillus flavus*		*Aspergillus niger*	
	Fungal growth (cm)	Inhibition (%)	Fungal growth (cm)	Inhibition (%)
n-Hexane	12.3	24.6	10.8	35.5
Dichloromethane	7.3	48.9	8.5	43.4
Ethyl acetate	**6.9**	**52.5**	**5.2**	**61.2**
n-butanol	14.8	21.7	15.6	18.4
DMSO	9.0	–	9.0	–
TER	–	–	–	–

Furthermore, the ethyl acetate soluble fraction was subjected to column chromatography over silica gel (70-230 mesh) and preparative TLC to afford compounds **1** and **2** (Fig. 1).

Compound **1** was isolated as white crystals. The EI-MS afforded the molecular ion at *m/z* 293 implying the molecular formula $C_{18}H_{15}NO_3$ which was confirmed by HR-EI-MS ($[M]^+$; found: 293.54 Calcd: 293.32). The presence of 2-quinoline system in the molecule was indicated by the UV absorption at 221, 236, 266 sh, 318 and 385 nm. The IR spectrum also showed band at 1672 cm^{-1} suggesting the presence of α, β-unsaturated aldehyde which was confirmed by the presence of formyl signal at δ 10.09 in ^1H NMR. Other ^1H NMR signals were observed at δ 4.46 (OCH_3), 1.38 (CH_3). A couple of doublet found at δ 7.91 (1H, *J* = 8.4 Hz), 7.69 (1H, *J* = 8.4 Hz) were assigned to H-5 and H-6 proton, respectively. Another couple of doublet displayed at δ 7.61 (1H, *J* = 2.9 Hz) and 7.02 (1H, d, *J* = 2.9 Hz) were characteristics of H-2 and H-3 of the furoquinoline moiety. The proton at β-position of formyl group resonated at 7.42 (1H, s). ^{13}C NMR displayed 17 signals. Signals for aromatic carbons were found at δ 165.39 (9a), 157.69 (C-4), 148.14 (C-8), 145.17 (C-8a), 143.96 (C-7), 143.13 (C-2), 129.26 (C-6), 123.48 (C-4a), 119.89 (C-5), 109.21 (C-3a) and 105.78 (C-3). The α and β carbon of D-ring resonated at δ 146.56 (C-10) and 161.51 (C-11), respectively. The methoxy and methyl carbons were found at δ 62.32 (OCH_3), 27.81 ($2CH_3$) while the peak at δ 53.08 was assigned to C-12. The ^1H and ^{13}C NMR data was confirmed by HMBC experiments which showed ^3J correlations between ^3J correlations was found between H-2 and C-3a and C-9a, H-3 and C-9a, H-5 and C-7 and C-8a, H-6 and C-4a and C-8, H-11 and C-7, C-8 and C=O. Formyl proton showed ^2J correlation with C-10 while methoxy proton showed ^3J correlation with C-4. Similarly ^3J correlation was found between the two methyl protons and C-11 and C-8. According to the above data and HMBC experiments (Fig. 2) the structure of compound **1** was elucidated as 4-methoxy-12,12-dimethylcyclopenta furoquinoline-10-carbaldehyde.

Compound **2** was isolated as white needles. Its molecular formula was suggested as $C_{19}H_{17}NO_4$ from the molecular ion peak at *m/z* 323 in EI-MS spectrum which was also confirmed by HR-EI-MS analysis. The UV absorption at 249, 312, 324

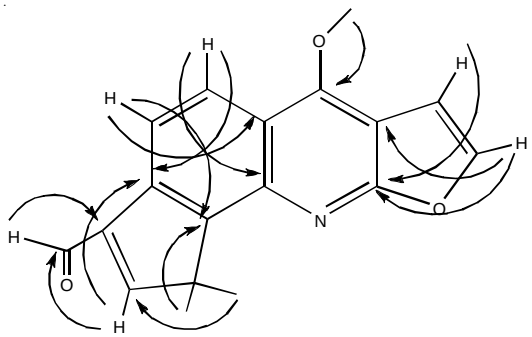


Fig. 2. Important HMBC correlations of compound **1**

and 332 were similar to that of compound **3** and were characteristics of furoquinoline nucleus. The presence of α,β-unsaturated ester in a molecule was revealed by IR bands at 1715 cm^{-1} and was confirmed by ^1H NMR peak at 3.72 ppm. The ^1H NMR spectrum of compound **2** was similar to that of compound **1** except that the aldehydic peak of formyl group was replaced by methoxy peak of ester group. The presence of furoquinoline nucleus was clearly demonstrated from a two pairs of doublet of doublet resonated at δ 8.02 (1H, d, J = 8.5 Hz, H-5), 7.91 (1H, d, J = 8.5 Hz, H-6), 7.68 (1H, d, J = 3.2 Hz, H-2) and 6.89 (1H, d, J = 3.2 Hz, H-3). In addition the presence of methoxy group at C-4 and the methyl grops at C-12 were confirmed by signals at δ 4.35 (3H, s) and 1.40 (6H, s), respectively. ^{13}C NMR data was similar to compound **1** except and additional peak at δ 55.41 for OCH_3 group of ester moiety. According to the above data the structure of compound **2** was assignes as methyl 4-methoxy-12,12-dimethyl-cyclopenta furoquinoline-10-carboxylate which was further confirmed by HMBC experiment (Fig. 3).

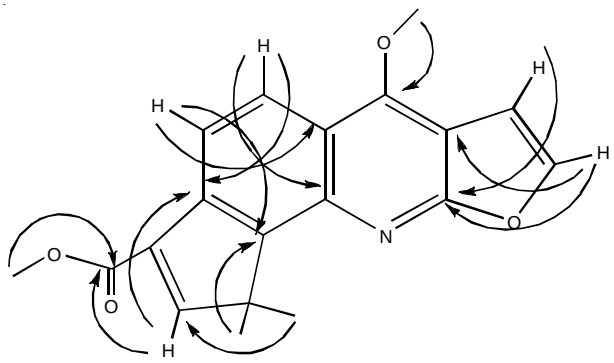


Fig. 3. Important HMBC correlations of compound **2**

The isolated compounds were studied as antioxidant. The various range concentration of compounds **1** and **2** (50, 100, 150 200, 250 500 µg/mL) showed significant DPPH radical scavenging activity compared with ascorbic acid using as standard of same concentration. Furthermore, the compound **2** has less anti-oxidant activity than compound **1** (Fig. 4).

Similar experiments for ABTS (2,2'-azino-*bis*(3-ethylbenzthiazoline-6-sulphonic) radical scavenging assay were designed to verify the antioxidant activity of the isolated compounds **1** and **2** from *Zanthoxylum armatum*. The ABTS (2,2'-azino-*bis*(3-ethylbenzthiazoline-6-sulphonic) radical scavenging assay also support the data that indicated that compound **1** has higher scavenging than compound **2** (Fig. 5).

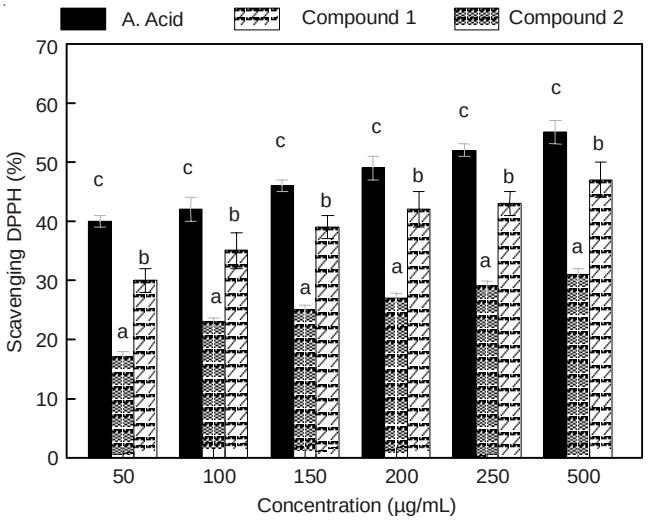


Fig. 4. DPPH scavenging activities of various concentrations of ascorbic acid and pure compounds **1** and **2**. The data represent the mean of three different experiments done in duplicate. The different letters represent statistically different from each other

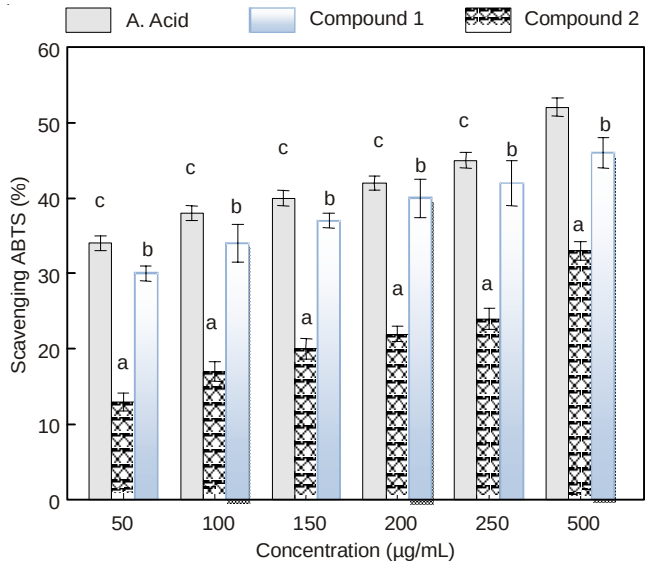


Fig. 5. ABTS scavenging activities of various concentrations of ascorbic acid and isolated compounds **1-6**. The data represent the mean of three different experiments done in duplicate. Each value represents mean ± SEM

Conclusion

In conclusion, it is interesting to note that *Zanthoxylum armatum* that has been used for long time for various purposes; in fact the new scientific report may suggest new applications.

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