CHEMICAL CONSTITUENTS FROM THE BIOACTIVE ETHYL ACETATE FRACTION OF *XANTHIUM STRUMARIUM* LINN.

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خلاصہ

زینتھیم اسٹرویم کی ایتھائیل ایسی ٹیسٹ موجودگی مخالف مائیکروحیاتیاتی اور آکسی ڈینٹ خصوصیات کی حامل پائی گئی۔ جو Ec50 = 937 ug/ml اہمیت کی حامل مخالف Oxident عمل کو ظاہر کرتی ہے۔ اسکاربک تیزاب یا معیار کی محلول کے برخلاف۔ یہ درمیانی درجہ سے اعلیٰ درجہ کا بیکٹریا مخالف اور فنجائی مخالف رویہ اختیار کرتی ہے جو 12-34 m.n کا مخالف علاقہ بناتی ہے۔
Pentacyclic terpenes جس میں B-armyrin اور Oleanolic تیزاب اور Aromatic تیزاب جسمیں Ferulic تیزاب شامل ہے۔ اس حیاتیاتی اہمیت کے اتھائیل ایسیٹیٹ جو کہ Strumarium Xanthinm میں سے حاصل کیئے جاتے ہیں۔ اس مرکب کی شناخت کو مختلف اسپیکٹرواسکوپک طریقہ کار سے جو کہ EIRS – IR اور H-NMR طریقہ کار سے حاصل کیا گیا ہے۔

Abstract

The ethyl acetate fraction of *Xanthium strumarium* Linn. was found possessing antioxidant and antimicrobial potential. It showed significant *in vitro* antioxidant activity (EC_{50}= 937 µg/mL) in contrast of ascorbic acid as standard. It also exhibited moderate to high antibacterial and antifungal activities having zone of inhibition in range of 12-34 mm against pathogenic microorganism. Two pentacyclic triterpenes *β*- amyrin (**1**), oleanolic acid (**2**) and an aromatic acid, ferulic acid (**3**) have been isolated from this bioactive ethyl acetate (EtOAc) fraction of *Xanthium strumarium* Linn. The structures of these compounds were elucidated through various spectroscopic techniques such as IR, EIMS and ^{1}H-NMR techniques.

Introduction

Xanthium strumarium L. belongs to family compositeae (Winter *et al*., 1969). The genus *Xanthium* consist of 25 species and distributed near temperate and tropical region of world (Han *et al*., 2007). The members of genus *Xanthium* L. used to cure herpes, fever, nasal sinusitis, urticaria, headache, arthritis, cancer, and scrofula (Ravindra, 2003). *X. strumarium* L. has been used as a most important plant in herbal medicine in Malaysia, India and China (Prajapati and Purohit, 2003). Previously sesquiterpenoids, alkaloids, and sesquiterpene lactones (Gupta and Gupta, 1975) and Carbohydrates (Craige *et al*., 1976) have been reported from this species. Chemical constituents of this plant possess antimicrobial activity (Jawad *et al*., 1988), antifungal activity (Sastri, 1976) and cytotoxic activity (Kim *et al*. 2003). Fruits are used to treat small-pox; and contain anti-inflammatory agent (Sastri, 1976) and anti-hyperglycemic agent (Hsu *et al*. 1999). Seeds have toxic effects due to choline and hydroquinone (Sastri, 1976). Biological activities of *X. strumarium* extracts were also reported such as anticancer, antitrypanosomal (Anooj and Ajay, 2010), antimalarial (Joshi *et al*., 1997), hepatoprotective (Panday and Rather, 2012) and analgesic properties (Han *et al*., 2007).

The ethnopharmacological and chemotaxonomic importance of the genus *Xanthium* stimulated us to further investigate on *X. strumarium.* Previously we have reported antimicrobial, antioxidant potential and chemical constituents of n-hexane, dichloromethane and n-butanol fraction (Aneela *et al*., 2012). Herein, we have investigated ethylacetate fraction of *X. strumarium* to determine its anti-bacterial, anti-fungal and anti-oxidant activities and possibly active substances.

Experimental
Materials and Methods

General: Gallenkamp melting point apparatus was used to determine melting points. Silica gel (70-230 mesh, Merck) was used for column chromatography (CC), whereas, Pre-coated silica gel (GF-254) was used for TLC. IR spectra were scanned on Jasco-302-A spectrophotometer. EIMS spectra were recorded on Mass spectrometer (Jeol-JMS HX-110). Bruker spectrometer (500 and 300 MHz) was used to record ^{1}H-NMR spectra. The chemical shift values were reported in δ (ppm) relative to tetramethylsilane (TMS) as an internal standard. The coupling constant (*J*) values were given in Hz.

Plant Material: The aerial part of plant material *X. strumarium* were collected from Lakimarwat (NWFP) Pakistan and identified by Dr. Sahar, Department of Botany, University of Karachi (voucher specimen G.H. No.86398, No. 01).

Extraction and Isolation: The freshly collected shade dried aerial parts of plant material *X. strumarium* (10 kg) were ground and extracted with methanol (15 L). The resulting extract was concentrated under vacuum to obtain a crude extract (250 g) which was then successively extracted with *n*-hexane (13 g), dichloromethane (14 g), ethyl acetate (97 g) and *n*-butanol (21 g). The ethyl acetate fraction (97 g) was subjected to CC over silica gel and eluted successively with *n*-Hexane and *n*-Hexane-ethylacetate in an increasing order of polarity to obtained 16 sub-fractions (A-1 to A-16). The sub-fraction A-3, eluted with solvent *n*-hexane-ethylacetate (9:1) was a semi-pure compound which was further purified by preparative TLC (Hexane: ethylacetate, 9:1) to give compound **1** (*β*-amyrin). The sub-fraction A-6 was subjected to CC (with an increasing order of polarity, *n*-hexane, *n*-hexane-EtOAc) which gave ten sub- fractions (A-6-1 to A-6-10). The sub-fraction A-6-3 obtained from solvent (*n*-hexane-ethylacetate, 8:2) was kept overnight in a mixture of solvent (MeOH:$CHCl_3$, 1:1) to obtain compound **2** (oleanolic acid). The sub-fraction A-9 was subjected to CC (with an increasing order of polarity of solvent $CHCL_3$, $CHCl_3$-MeOH) gave six sub-fractions (A-9-1 to A-9-6). Fraction A-9-3 was obtained from solvent system ($CHCl_3$-MeOH, 9:1) to afford compound **3** (ferulic acid).

***Characterization of β –Amyrin* (1):** Colourless solid (6.0 mg). M.P., 195- 196º C; IR (KBr) ν_{max} cm^{-1} : 3352 (OH), 1648 (C=C); EI-MS *m/z* (rel. int.,%): 426 $[M]^+$ (49), 411 (15), 408 (10), 393 (10), 218 (100), 207 (42), 203 (53), 189 (60), 175 (24), 147 (25), 135 (45), 119 (27), 109 (41) and 69 (55); HR-EIMS *m/z* : 426.0100 (calcd. for $C_{30}H_{50}O$, 426.3862); 411.3412 $[C_{29}H_{47}O]^+$, 408.1502 $[C_{30}H_{48}]^+$, 393.223 $[C_{29}H_{45}]^+$, 218.0420 $[C_{16}H_{26}]^+$, 207.3301 $[C_{14}H_{23}O]^+$, 203.1354 $[C_{15}H_{23}]^+$, 189.3010 $[C_{14}H_{21}]^+$, 175.0132 $[C_{13}H_{19}]^+$, 147.1945 $[C_{11}H_{15}]^+$, 135.1093 $[C_{10}H_{15}]^+$, 119.2203 $[C_9H_{11}]^+$, 109.0110 $[C_8H_{13}]^+$ and 69.2015 $[C_5H_9]^+$; ^{1}H- NMR ($CDCl_3$, 500 MHz): *δ* 5.15 (1H, t, *J* = 4.5 Hz, H-12), 3.09 (1H, dd, *J* = 11.0, 4.0 Hz, H-3*α*), 1.08 (3H, s, CH_3), 1.00 (3H, s, CH_3), 0.99 (3H, s, CH_3), 0.94 (3H, s, CH_3), 0.87 (3H, s, CH_3), 0.86 (3H, s, CH_3), 0.85 (3H, s, CH_3), and 0.78 (3H, s, CH_3). All the spectral data were in agreement with values reported in literature (Malik *et al*., 2005).

***Characterization of Oleanolic acid* (2):** Colourless needles (4.0 mg); MP: 195-197°C ; IR (KBr) ν_{max} cm^{-1}: 3410 (OH), 2789 (COOH), 2768 (C-H), 1710 (C=O), 1625 (C=C), and 1066 (C-O); EI-MS *m/z* (rel. int.): 456 $[M]^+$ (4), 248 (100), 207 (23), 203 (31), 189 (10) and 133 (21) ; HR-EIMS *m/z*: 456.3410 (calcd for $C_{30}H_{48}O_3$, 456.3530), 248.1730 $[C_{16}H_{24}O_2]^+$, 207.1643 $[C_{14}H_{23}O]^+$, 203.2735 $[C_{15}H_{23}]^+$, 189.1549 $[C_{14}H_{21}]^+$ and 133.1081 $[C_{10}H_{13}]^+$; ^{1}H-NMR ($CDCl_3$, 300 MHz): *δ* 5.30 (t, *J* = 3.6 Hz, H-12), 3.42 (1H, dd, *J* = 14.0, 4.0 Hz, H-3*α*), 2.80 (1H, dd, *J* =14.1, 4.6 Hz, H-18), 1.20 (3H, s, CH_3), 1.15 (3H, s, CH_3), 0.97 (3H, s, CH_3), 0.94 (3H, s, CH_3), 0.86 (3H, s, CH_3), 0.85 (3H, s, CH_3), and 0.75 (3H, s, CH_3). The studied spectral data coincided with those in literature (Misra *et al.*, 1981).

***Characterization of Ferulic acid* (3):** Colourless solid (3.0 mg); M.P., 168-172 ºC ; IR (KBr) ν_{max} cm^{-1} : 3345 – 2578 (OH, COOH), 1675 (C=O), 1619 and (C=C); EI-MS *m/z* (rel. int. %): 194 $[M]^+$ (10), 149 (24), 163 (32), 123 (19) and 92 (5); HR-EIMS *m/z* : 194.0136 (calcd. for $C_{10}H_{10}O_4$, 194.2110), 149 $[C_9H_9O_2]^+$, 163 $[C_9H_7O_3]^+$, 123 $[C_7H_7O_2]^+$ and 92 $[C_6H_4O]^+$; ^{1}H-NMR ($CDCl_3$, 500 MHz): *δ* 7.48 (1H, d, *J* =14.5 Hz, H-7), 7.21 (1H, d, *J* = 7.8 Hz, H-6), 7.15 (1H, br. s, H-2), 6.59 (1H, d, *J* = 7.9 Hz, H-5). 6.01 (1H, d, *J* = 14.5 Hz, H-8) and 3.89 (3H, s, OCH_3). The given spectral data agreed the literature values (Li *et al*., 1999).

Biological Assay

Preparation of media: The media used for culturing of bacterial strain was Muller Hinton agar and Muller Hinton broth s and fungal strains were culturing on Sabourd dextrose agar (SDA) (Smyth *et al*., 2011).

***Screening of Antibacterial activity*:** Antibacterial activity of ethylacetate fraction was carried out by agar well diffusion method. Muller Hinton broth was autoclaved for 2 h and then culture (10 μl) was transferred into the wells (Perez *et al*., 2009). Test fraction (10 mg/mL) was incubated at 28 °C for 48 hr and then measured zone of inhibition. The standard drug gentamicin was used.

Screening of Antifungal activity: Ethyl acetate fraction was screened for antifungal activity by agar-well diffusion method. The media was shifted aseptically into each SDA plates (Wuthi *et al*., 2011). Test fraction (10 mg/mL) were incubated for 48 hr. at 28 ± 2°C and then measured zone of inhibition. The standard drug gresiofulvin was used.

Minimum Inhibitory Concentration (MIC): MIC was calculated by broth dilution method. Stock solution (100 mg/mL) of extract was made in dist. H_2O. Further two fold serial dilution of extract was made in 100 µL broth afterward 10 μl culture matched with 0.5 Mac Farland index was added to each well. One well as culture control while other as antibiotic control. All plates were incubated at 37 ºC for 24 hours. The MIC was establish when no visible growth was observed (Sherwani *et al*., 2013).

***Screening of anti-oxidant activity*:** Ethylacetate fraction of *X. strumarium* was screened for antioxidant potential by the method of Lee *et al.* (1998). DPPH (1,1-diphenyl-2-picrylhydrazile) solution was made in ethanol. 10 μL EtOAc fraction and 90 μL DPPH were added in 96-well microtiter plates and incubated for 30 min. at 37 ºC. Absorbance was measured through spectrophotometer at wavelength of 515 nm. % inhibition of radicals was determined by following formula.

$$\% \text{ Inhibition} = \frac{(\text{absorbance of the control} - \text{absorbance of the test sample})}{\text{Absorbance of the control}} \times 100$$

Results and Discussion

Compound **1** (β-amyrin) was isolated as colorless solid. The molecular ion peak at *m/z* = 426 was observed by EI-MS and molecular formula $C_{30}H_{50}O$ was obtained by HR-EIMS. Two peaks at *m/z* 218 and 207 was obtained in EIMS due to Diels Alder fragmentations (Misra *et al.*, 1981). The absorption band in IR spectrum at 3352 cm^{-1} is due to –OH group and absorption band at 1648 cm^{-1} supported olefinic carbons (C=C).^{1}H-NMR displayed eight singlet of methyl proton at δ 1.08 -0.78 and a triplet at δ 5.15 (J = 4.5 Hz) is of olefinic protons. A double doublet (dd) at δ 3.09 (J = 11.0 and 4.0 Hz) is the characteristic feature of H-3α (Amzad and Zhari, 2013). All these data showed that compound **1** belongs to pentacyclic triterpene of Δ^{12} β-amyrin series (Malik *et al*., 2005).

Compound **2** (oleanolic acid) was isolated as colorless neddles. The $[M]^+$ peak at *m/z* = 456 was observed by EI-MS and molecular formula $C_{30}H_{48}O_3$ was observed by HR-EIMS. Mass fragments at *m/z* 248 and 207 due to retro Diels Alder fragmention further supported that compound belongs to the pentacyclic triterpenes (Misra *et al.*, 1981). The IR spectrum showed the absorption band at 2789 cm^{-1} due to presence of –COOH and absorption bands at 3410 due to presence of –OH group. while the presence of –COOH group at C-17 was indicated by the mass fragments at *m/z* 203, 189 and 133 whereas –OH group at C-3 with β –orientation is on biogenetic ground. ^{1}H-NMR spectrum displayed seven methyls singlet at δ 1.20-0.75 and a triplet at δ 5.30 (J = 3.6 Hz) of olefinic proton and a dd at δ 3.42 (J = 14.0 Hz, 4.0 Hz) of H-3α oxymethine (Amzad and Zhari, 2013). It was analyzed that compound **2** also belongs to series of Δ^{12} β-amyrin (Misra *et al*., 1981).

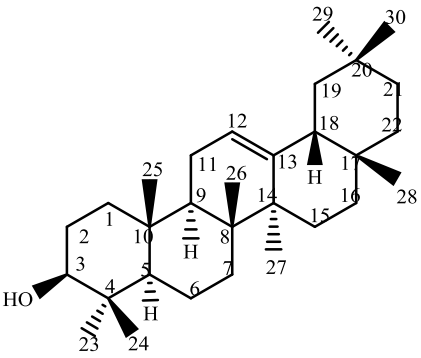
Compound **3** (ferulic acid) was isolated as colorless solid. EIMS displaying $[M]^+$ peak at *m/z* = 194. Mass fragment at *m/z* 149 was observed due to loss of –COOH group, at *m/z* 163 due to loss of $–OCH_3$ and at *m/z* 123 due to loss of olefinic linkage. Absorption band of IR at 1619, 3345 and 2578 cm^{-1} exhibited of -C=C, -OH and -COOH group. ^{1}H-NMR displayed a singlet of methoxy group at δ 3.89 and two doublet of *trans* olefinic protons at δ 7.48 and 6.01 (J = 14.5 Hz). A downfield singlet at δ 7.15 (s, H-2) and two doublet at 7.21 and 6.59 (J = 7.9 Hz) showed *meta* substituted $–OCH_3$ and *para* substituted –OH in aromatic ring (Muhammad *et al*., 2011 and Li *et al*., 1999).

The ethylacetate fraction was evaluated for antimicrobial and antioxidant potential. Antibacterial activity was screened against *B. cereus, B. subtilis, B. thruingiensis, C. coli, E. aerogenes, K. pneumonia, S. typhi, S. paratyphi A, S. paratyphi B,* and *Vibrio cholera* and antifungal activity was evaluated against *A. flavus, A. niger, Helminthosporum, M. canis, Penicillium sp,* and *Penicillium sp* (Table **1**). The ethylacetate fraction showed excellent antibacterial activity within the range of 17 to 34 mm and MIC value ranges 52 to 200 mg/mL. Ethylacetate fraction also showed significant antifungal activity (12-19 mm) and MIC value was observed within the range of 100-320 mg/mL.

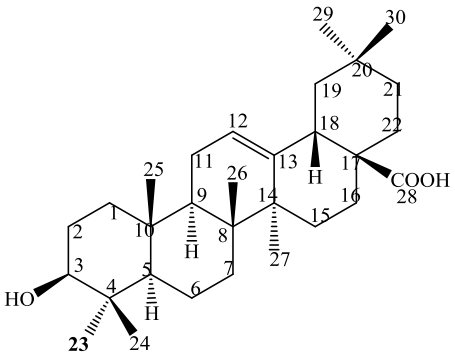
The ethylacetate fraction also showed significant antioxidant potential (Table 2). DPPH free radical showed 71 % inhibition and the result of EC_{50} was 938 ± 0.5 μg/ml.

Conclusion

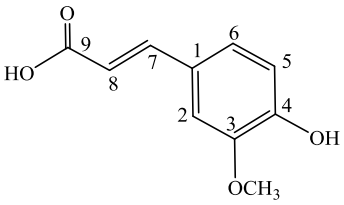
The present study concludes that ethylacetate fraction of *Xanthium strumarium* is biologically active and has potential to inhibit the growth of various pathogenic Gram positive, Gram negative bacteria and is also minimizing fungal infections. It is also exhibited good antioxidant potential.



β-Amyrin (**1**)



Oleanolic acid (**2**)



Ferulic acid (**3**)

Table.1 *In vitro* anti-bacterial and anti-fungal activity of ethyl acetate fraction of *X. strumarium* Linn.

Organism	Zone of inhibition (mm)	Standard drugs	MIC (mg/mL)
Gram positive bacteria		**Gentamicin**	
Bacillus cereus	26 ± 2	>15	88
Bacillus subtilis	34 ± 0	>15	120
Bacillus thruingiensis	30 ± 0	>15	102
Gram negative bacteria			
Enterobacter aerogenes	17 ± 1	>15	200
Klebsiella pneumoniae	22 ± 1	>15	56
Salmonella typhi	18 ± 1	>15	89
Salmonella paratyphi A	19 ± 1	>15	90
Salmonella paratyphi B	20 ± 0	>15	52
Campylobacter coli	19 ± 0	>15	200
Vibrio cholerae	19 ± 1	>15	160
Fungi		**Gresiofulvin**	
Microsporum canis	12 ± 2	>12	312
Aspergillus flavus	19 ± 1	>12	320
Aspergillus niger	15 ± 0	>12	100
Penicillium sp	12 ± 1	>12	149
Rhizopus	16 ± 1	>12	300
Helminthosporum	14 ± 0	>12	212

Table-2: *In vitro* anti-oxidant activity of Ethyl acetate fraction of *X. strumarium* Linn.

Sample	% inhibition	EC_{50} (*u*g/ml)	Standard drug
Ethyl acetate fraction	71 %	938 ± 0.5	Ascorbic acid

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