REPORT

Preliminary phytochemical screening, antimicrobial and antioxidant activities of *Euphorbia milli*

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Abstract: *Euphorbia milii* is a Pakistani herb used for various infectious diseases. In this study we have carried out phytochemical, antibacterial and antioxidant investigation of different extracts/fractions. Phytochemical studies showed the presence of cardiac glycosides, steroids/phytosterols, anthocyanin, proteins, terpenoids, flavonoids and tannins. Susceptibility testing by well diffusion assay of its chloroform and methanol fractions revealed good antimicrobial activity against Klebsiella pneumonia and Staph epidermis. Ethyl acetate fraction of roots also exhibited considerable antimicrobial activity against most of tested pathogens. Various fractions (Hexane, chloroform, methanol and water) of *E. milii* were screen for their antioxidant potential using DPPH radical scavenging assay at different concentrations among these, chloroform fraction exhibited good scavenging activity. The IR spectroscopy of the various extracts/fractions indicated the presence of OH, saturated CH stretching, C=C, C=O, NO_2, C-N, Ar-O, C-O- and R-O- Stretching respectively. The findings provide helpful evidence for the use of *E. milii* in traditional medicines.

Keywords: *Euphorbia milli*, antimicrobial activity, DPPH radical scavenging assay, IR spectroscopy.

INTRODUCTION

The Family Euphorbiaceae consists of 2000 species (Davis *et al*., 1988). The genus *Euphorbia* is the largest genus of medicinal plants and widely cultivated in several parts of the China and Pakistan. The plants are characterized by the presence of milky latex which is more or less toxic. *E. milli* is used for ornamental purpose and have not been reported in folk therapy in Pakistan, however in Nepal the latex is used to treat sprains (Mananhdir *et al*., 2011); while in China it is used to cure hepatitis and abdominal edema. In Brazil *E. milii* is commonly known as "Crown of Thorns". Naturally, *E. milli* crude latex showed to be a potent plant molluscicide (Vasconcellos *et al*., 1986) due to its toxic effect to mammals. Its undiluted latex also found to cause infections to mammalian eyes and skin (Freitas *et al*., 1991). Phytochemical studies of *E. milli* revealed the presence of β-sitosterol, cycloartenol, β-amyrin acetate, lupeol, euphol, triterpenes and flavonoids (Delgado *et al.*,2003; Pan*corbo et al.,* 1972). Diterpene esters of ingenol are potent skin irritants but, in contrast with other closely-related ingenol and phorbol derivatives, no tumor promoting activity is reported (Marston *et al*., 1984). Milliamines isolated from *E. milii* latex exhibited effective molluscicidal activity (Zani *et al*., 1993).

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EXPERIMENTAL

General Experimental

2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical, Streptomycin, Dragendrof's reagent, Fehling's solution A and B and acetic anhydride were obtained from Sigma-Aldrich. All other chemicals and reagents used were of analytical grade.

Plant material

Aerial parts of *E. milli* were collected from Toormang, Razagram area of district Dir, Khyber Pukhtun Khawa province, Pakistan in the month of February, 2010. The plant was identified by a plant taxonomist Dr. Abdur Rashid and a voucher specimen (UOP-545) has been deposited in the herbarium of the Department of Botany, University of Peshawar.

Materials and Methods

The plant materials of *E. milii* were shade dried at room temperature for 15 days. The shade dried plant material (aerial parts) was crushed to make fine powder. The powdered materials were soaked in methanol for 5 days and then subjected to repeated extraction until exhaustion

of plant materials. The extracts obtained were then concentrated under vacuum at temperature below 50°C. MeOH crude extract was poured in water and successively partitioned with *n*-hexane, $CHCl_3$, EtOAc and MeOH to obtained fractions of theses solvents.

Antimicrobial activity

In the present investigation five bacterial strains were used as reported earlier (Uddin *et al*., 2011). Three strains of Gram-positive (*Staphylococcus aureus*, *Strap Epidermis* and *Bacillus subtilis*) and two of Gram-negative (*Escherichia coli* and *Klebsiella pneumonia)* bacteria were used, which were obtained from PNRL laboratories, Institute of Chemical Sciences, University of Peshawar, Peshawar, Pakistan. These organisms were kept in muller-hinton agar at 4°C, prior to subculture. Tests for susceptibility were determined using modified agar well diffusion method to test the antibacterial activity of the different solvent fractions. The muller-hinton agar was used as medium. All the cultures were taken in triplicates at incubation temperature of 37°C for 24 to 72 hours. In a sterile Petri-dish, test organism broth culture (0.6mL) was placed with sterile molten MHA (20mL). Holes were bored in to the medium and each fraction was added (0.2 mL). Streptomycin was the standard antimicrobial agent at a concentration of 2 mg /mL. Inoculation was done for 1 h to make possible the diffusion of the antimicrobial agent into the medium and it was incubated at 37°C for 24 h. The diameter of the zone of inhibition of microbial growth in the plate was measured in millimeter (mm).

Antioxidant assay

The modified literature protocol of Blois (Blois, 1958; Uddin *et al*., 2012) was used for antioxidant assay. Briefly 2, 2-diphenyl-1-picrylhydrazyl (DPPH) solution (1mL; 1 mM) was prepared in methanol and mixed with sample solution (3mL, containing 20-100*u*g) in ethanol. The control was also run which contains only ethanol. The hydrogen atom or electron donation abilities of the each fractions and standards were measured from the bleaching of the purple-colored methanol solution of 2, 2-diphenyl-1-picrylhydrazyl (DPPH). The absorbance was measured at 517 nm after 30 min incubation. Decreasing of the DPPH solution absorbance indicates an increase of the DPPH radical-scavenging activity. Scavenging of free radicals by DPPH as percent radical scavenging activities (%RSA) was calculated as follows.

DPPH% = (Control abs – Extract abs / Control) × 100

Phytochemical profiling

The chemical tests were performed on the water, *n*-hexane, $CHCl_3$ and MeOH crude extracts of *E. milii* followed by the standard procedures (Uddin *et al*., 2011a; Uddin *et al*., 2012b; Rauf *et al*., 2012) for identification of the chemical constituents.

Test for alkaloids

Each fraction (0.2g) was warmed with 2% H_2SO_4 (2.0ml) for two minutes. The reaction mixture was filtered and few drops of Dragendrof's reagent were added to filtrate. Orange red precipitation showed the presence of alkaloids moiety.

Test for tannins

Each extract in small quantity was mixed with water and heated on water bath and filtered. To the filtrate, few drops of $FeCl_3$ and a dark green solution was obtained which indicate the presence of tannins.

Test for anthraquinone

Each extract (0.5g) was boiled with 10% HCl for few minutes. The reaction mixtures was then filtered and allowed to cool. Equal volume of $CHCl_3$ was added to each filtrate along with few drops of 10% NH_3 and heated. Rose-pink color formation was obtained which indicate the presence of anthraquinones.

Test for glycosides

Each extract (0.6g) was hydrolyzed with HCl and neutralized with NaOH solution and few drops of Fehling's solution A and B were added. Red precipitate were formed which indicate the presence of glycosides.

Test for reducing sugars

Each extract (0.5g) was shaken with distilled water and filtered. Few drops of Fehling's solution A and B were added and boiled for few minutes. An orange red precipitate was formed, showed the presence of reducing sugar.

Test for saponins

Each extract (0.2g) was shaken with distilled water (5.0 mL) and boiled. Frothing (appearance of creamy miss of small bubbles) was observed showed the presence of saponins.

Test for flavonoids

Each extract (0.2g) was dissolved in diluted NaOH and few drops of HCl were added. A yellow solution turned into colorless which indicate the presence of flavonoids.

Test for phlobatanins

Each extract (0.5g) was dissolved in distilled water and filtered. The filtrate was then boiled with 2% HCl solution. Red precipitates were obtained which showed the presence of phlobatanins.

Test for steroids

Acetic anhydride (2.0mL) was added to mixture of extract (0.5g) and H_2SO_4 (2.0mL) of each extract. The change of color from violet to blue or green in some samples showed the presence of steroids.

Test for terpenoids

Each extract (0.2g) was mixed with $CHCl_3$ (2.0mL) and carefully conc: H_2SO_4 (3.0mL) was added to form a layer.

Table 1: Antimicrobial activity of the different fractions of *E. milli*

Microorganism	Gram	*n*-Hexane Fraction	Chloroform Fraction	Ethyl fraction	MeOH fraction
Escherichia coli (E. c)	-	NA	NA	NA	NA
Staphylococcus aureus (S. a)	+	NA	NA	NA	NA
Klebsiella pneumonia (K. p)	-	10	12	16	14
Staph epidermis (S. e)	+	10	12	10	12
Bacillus subtilis (B. s)	+	NA	NA	NA	NA

Key: NA – Not active, Well size: 4mm

Table 2: Phytochemical screening of *n*-hexane, chloroform, ethyl acetate and methanolic fractions of *E. milli.*

Class of Constituent	*n*-Hexane Fraction	Chloroform Fraction	Ethyl acetate Fraction	Methanolic Fraction
Alkaloids	-	-	-	-
Anthocyanin and Betacyanin	+	+	+	+
Proteins and Amino acids	+	+	+	+
Cardiac glycosides	+	+	+	+
Steroids	+	+	+	+
Terpenoids	+	+	+	+
Flavonoids	+	+	+	+
Anthraquinone	-	-	+	-
Tannins	+	+	+	+
Phlobatanins	-	-	-	
Saponins	-	-	-	-
Glycoside	-	-	-	-
Reducing sugars	-	-	-	-

Key: – = absent, + = present

Table 3: IR Spectroscopic data of the various fraction of *E. milli*

Components	*n*-Hexane Fraction	Chloroform Fraction	Ethyl acetate Fraction	Methanolic Fraction
	Region (cm^{-1})			
OH	3360.00(B)	-	3358.07	3367.11
CH	2916.37	2918.30	2916.37	2916.37
C=O	1710.86	1732.08	1716.37	1716.65
C=O α, β unsaturated	1687.7	1687.71	1687.71	1616.35
C=O amide	-	1653.00	-	-
C=C	-	-	1608.62	-
NH def	-	1550.05	1416.61	1458.26
NO_2	-	1558.48	1373.32	1375.25
Ar-O	1162.04	1217.91	1238.30	1212.16
C-O-C	1099.13	1015.12	1031.92	1037.72

Reddish brown coloration at the interface was formed which indicate positive results for the presence of terpenoids.

Test for anthocyanin and betacyanin

To each plant fraction (2.0mL), NaOH (1mL, 2 N) was added and heated for 5min at 100°C. Formation of bluish green colour showed the presence of anthocyanin.

Test for proteins and amino acids

To each fraction (2.0mL) of *E. milli* few drops of 0.2% ninhydrin was added and heated for 5 minutes, formation of blue colour indicate the presence of proteins.

Test for cardiac glycosides

Each fraction (2.0 mL) of *E. Milli*, glacial CH_3COOH (1.0 mL) and 5% $FeCl_3$ were mixed together then few drops of conc: H_2SO_4 were added. Greenish blue colour was observed showed the presence of cardiac glycosides.

RESULTS

The preliminary phytochemical screening of *E. milli n*-hexane, $CHCl_3$, EtOAc and MeOH fractions has discovered the presence of secondary metabolites of therapeutic importance. The extract and fractions were found active against one Gram positive and one Gram

negative bacteria (table 1). The major phytochemicals found were cardiac glycosides, steroids/phytosterols, anthocyanin, proteins, terpenoids, flavonoids and tannins. Ethyl acetate extract yielded maximum class of phytochemicals (table 2).

DISCUSSION

Different extracts of E. *milli* showed antibacterial and antioxidant activities which may be due to the presence of secondary metabolite for example flavonoids, terpenoids, tannins, phenolic and saponins (Uddin *et al*., 2011; Rauf *et al*., 2012b). The presence of phlobatanins suggests the diuretic property of the plant (Awoyinka *et al*., 2007). Flavonoids were found in *E. milli* which are water soluble antioxidants which prevent oxidative cell damage telling antiseptic, anticancer, anti-inflammatory effects and mild hypersensitive properties (Okwu., 2004). Phenolic compounds are also present in *E. milli* which are responsible for antioxidant activity (Pietta, 2000). In the present study, all extracts and fractions of *E. milli* inhibited the growth of most of the tested bacterial strains at different potential. The $CHCl_3$ and EtOH extracts have shown relatively greater activity than that of any other extracts at 22mg/mL concentration.

The observed antibacterial effects on the isolates are believed to be, due to the presence of tannins and flavonoids which have been shown to possess antibacterial properties (Awoyinka *et al*., 2007; Cowan, 199). The results obtained are encouraging as the chloroform and ethanol fractions have shown considerable antibacterial activity against the tested organisms. The screening and scientific evaluation of plant extracts against microbes may provide new antimicrobial substances. Plant based antimicrobials possess tremendous therapeutic potentials and lesser side effects as compared with synthetic antimicrobials (Iwu *et al*., 199). Therefore, the present investigation undoubtedly reveals the antibacterial nature of *E. milli* and suggests that it could exploit in the management of diseases caused by these bacteria in human body. Various Fractions obtained were also screened for the radical scavenging activity using DPPH (fig. 1). MeOH and $CHCl_3$ fraction sowed highest scavenging activity while water found the least one.

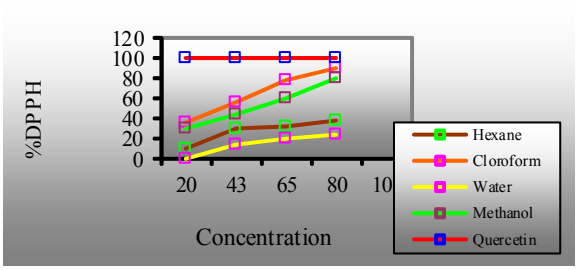

Fig. 1: DPPH radical scavenging activities of fractions/extract of *E. milii.*

The IR spectroscopy also showed the presence of phytochemicals (table 3). The IR gives broad signal at 3358.07, 3360.00 and 3367.11 which indicate the presence of OH stretching. The IR gives strong signals at 2916.37, 2916.37 and 2916.37which indicated the presence CH stretching saturated. The signal observed at the 1732.08, 1716.65, 1710.68 and 1716.65 indicate the presence of C=O (figs. 2-5).

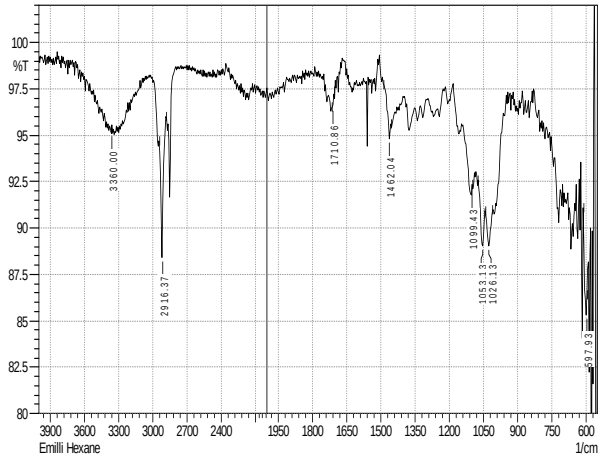

Fig. 2: IR spectra of *n*-hexane extract of *E. milii.*

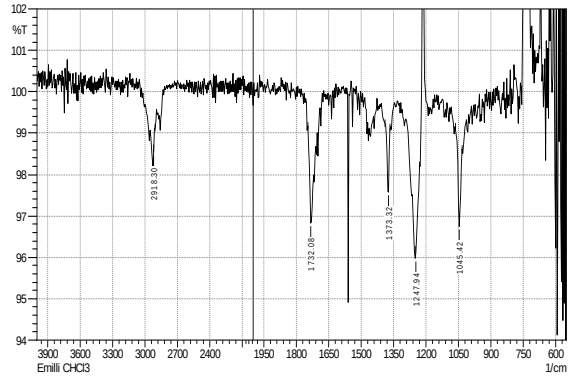

Fig. 3: IR spectra of $CHCl_3$ extract of *E. milii.*

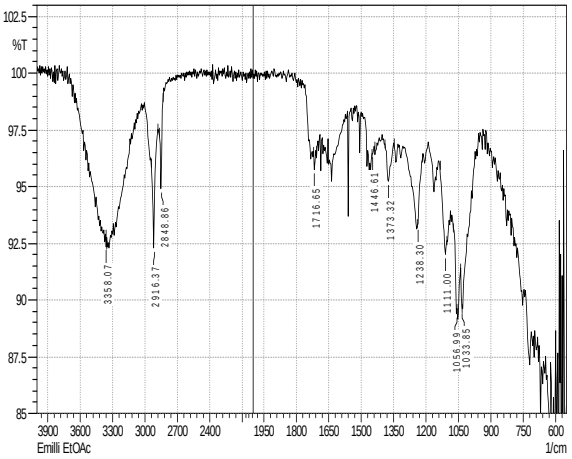

Fig. 4: IR spectra of EtOAc extract of *E. milii.*

The peak observed at 1616.35, 1458.26 and 1416.61 indicate the presence of C=C aromatic system in all the title plants (figs. 2-5).

The peak observed at 1373.32, 1373.32 and 1375.25 indicated the presence of NO_2. The presence of IR peak at 1217.91, 1212.16, 1162. 04 and 1198.72 obtained for *E. milii* showed Ar-O stretching (figs. 2-5).

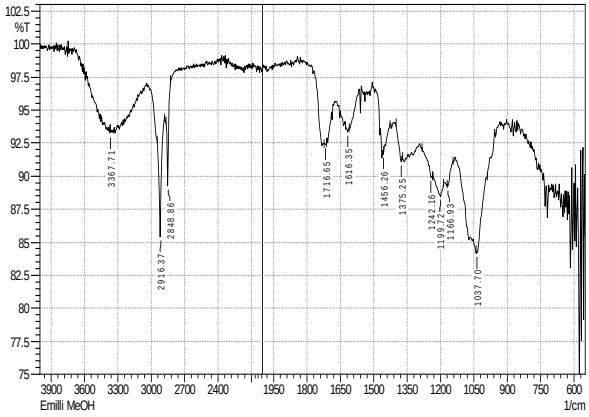

Fig. 5: IR spectra of MeOH extract of *E. milii.*

CONCLUSIONS

Results of preliminary studies showed that *E. mill* has variety of compounds and needs to explore. Interesting results have been obtained during biological screening such as fine antimicrobial, antioxidant activities etc., of different extract / fractions. In addition, this plant requires extensive phytochemical and pharmacological studies.

REFERENCES

Alkindele AJ and Adeyemi OO (1996). Anti-inflammatory activity of the aqueous leaf extrat of *Byrocdarpus coccineus*. *Fitotherapia.*, **78**: 25.

Awoyinka OA, Balogun IO and Ogunnowo AA (2007). Phytochemical screening and *in vitro* bioactivity of *Cnidoscolus aconitifolius*. *J. Med. Plants Res.*, **1**: 63.

Blois MS (1958). Antioxidant determinations by the use of a stable free radical. *Natur*e, **26**: 1199.

Cowan MM (1999). Plant products as antimicrobial agents. *Clin. Microbiol. Rev.*, **12**: 564.

Davis PH, Mill RR and Tan K (1988). Flora of Turkey and the East Aegean Islands. University Press, Edinburgh. **10**: 513.

Delgado I F, De-Carvalho RR, De-Oliveria AC, Kuriyama SN, Oliveira-Filho EC, Souza C. A and Paumgarteen FJ (2003). Absence of tumor promoting activity of *Euphorbia milli* latex on the mouse back skin. *Toxicol. Lett.*, **145**: 175.

Freitas J. CBR, Presgrave OAF, Fingola FF, Menezes MAC, Vasconcellos MC and Schall FJ R (1991). Toxicological study of the molluscicidal latex of *Euphorbia splendens*: Irritant action on skin and eye. Paumgartten. *Mem. Inst. Oswaldo Cruz.,* **87**: 88.

Mananhdir NP and Sanjay M (2011). Plants and people of Nepal. *Timber press Inc. Portland,* pp.88.

Marston A and Hecker E (1984). Active Principles of the Euphorbiaceae. VII. Milliamines H and I, Peptide Esters of 20-Deoxy-5ζ-Hydroxyphorbol from *Euphorbia milii*. *Planta Medica,* **50**: 319.

Manach C, Regerat F and Texier O (1996). (title) *Nutrition Research*, **16**: 517.

Okwu DE (2004). Phytochemicals, Vitamins and Mineral contents of two Nigeria Medicinal plants. *Int. J. Mol. Med. Adv. Sci*.**1**: 378-381.

Panc*orbo* S and Hammer RH (1972). Preliminary phytochemical investigation of *Euphorbia milli*. *J. Pharm. Sci.,* **61**: 954.

Pietta PG (2000). Flavonoids as antioxidants. *J. Nat. Prod.,* **63**: 1035.

Rauf A, Qaisar M, Uddin G, Akhtar S, Muhammad N and Qaisar M (2012). Preliminary Antinociceptive Studies of Methanol Extract of *Euphorbia milli*. *Middle-East J.Med. Plants Res.,* **1**: 09-13.

Rauf A, Muhammad N, Khan A, Uddin N, Atif M and Barkatullah (2012). Antibacterial and Phytotoxic Profile of Selected Pakistani Medicinal Plants. *World Appl. Sci. J.*, **20**: 540-544.

Uddin G, Rauf A, Qaisar M, Latif A and Ali M (2011). Preliminary phytochemical screening and antimicrobial activity of *Hedera halix* L. *Middle-East J. Sci. Res.* **8**: 198.

Uddin G, Rauf A, Arfan M, Ali M, Qaisar M, Saadiq M and Atif M (2012). Preliminary phytochemical screening and antoxidant activity of *Bergenia caliata*. *Middle-East J. Sci. Res.* **11**: 1140.

Uddin G, Rauf A, Rehman TU and Qaisar M (2011). Phytochemical Screening of *Pistacia chinensis* var. integerrima. *Middle-East J. Sci. Res.* **7**: 707.

Vasconcellos MC and Schall VT (1986). Latex of "Coroa de Cristo" (*Euphorbia splendens*): An effective molluscicide. *Mem. Inst. Oswaldo Cruz.* **81**: 475.

Zani CL, Marston A, Hamburger M and Hostettmann K (1993). Molluscicidal milliamines from *Euphorbia milii var. hislopii*. *Phytochemistry*, **34**: 89.

Iwu M, Angela RD and Chin OO (1999). A reprint from Journal of Janicked perspective on New Crops and New Uses. ASHS Press. Alexander.