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Antimicrobial and Phytochemical Screening of an Ethanolic Extract of the Stem Bark of *Calotropis procera* (Ait.) R. Br. by GC-MS

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Shahnaz Sultana[1,*], Andleeb Khan[1], Farah Islam[1], Fakhrul Islam[1], Hassan A. Alhazmi[1], Ibrahim A Khardali[2], Mohd M Safhi[1]

[1]*College of Pharmacy, Jazan University, Jazan, Saudi Arabia*

[2]*Poison Control and Medical Forensic Chemistry Centre, Jazan, Saudi Arabia*

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ABSTRACT

Calotropis procera (Ait.) R. Br. (Asclepiadaceae) is a small, hardy, pubescent, evergreen, erect and compact shrub which grows wild in drier and warm regions, sandy and alkaline soils in southeastern Asia Egypt, tropical Africa, Indochina, Morocco and some regions of Saudi Arabia (Jazan). It is prescribed to treat anasarca, asthma, ascites, bronchitis, cough, cutaneous diseases, intestinal worms, leprosy and eczema. Its bark has an excellent potential for dermal wound healing. GC-MS analysis of an ethanolic extract of the stem bark indicated that the predominant constituent was 9-octadecenamide (12.84%) followed by 12-oleanen-3α-yl acetate (6.56%), methyl commate A (6.38%), 2,3-dihydroxypropyl octadecanoate (5.82%), dimethyl (docosyloxy) butoxy-silane (3.61%), lup-20(29)-en-3β-ol acetate (2.33%), *n*-hexadecanamide (2.17%), stigmasta-5-en-3β-ol (2.08%) and urs-12-ene (2.05%). Forty one compounds were present in trace amounts. The bark ethanolic extract showed mild antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis* and *Streptococcus pyogenes* in high concentrations.

INTRODUCTION

Calotropis procera (Ait.) R. Br., known as Madar, Ashir or Milkweed (Asclepiadaceae), grows mainly in drier and warm regions in sandy and alkaline soils in southeastern Asia including India, Pakistan, Afghanistan, Waziristan, Egypt, tropical Africa, Indochina, Morocco, Senegal and some regions of Saudi Arabia (Jazan). Its growth is luxuriant on rubbish heaps, waste or fallow lands, along roadsides, sea shores and river banks [1]. It is a flowering plant exits as a spreading shrub having a simple stem with few branches. The large, dark-green leaves are sessile or sub-sessile, opposite, ovate, cordate at the base in opposite pairs along smooth stem. Beautiful waxy white flowers have deep purple spots or blotches at the base of each five petals with umbellate lateral cymes and are fragrant. The root is cylindrical, branched, curved, light, woody and grayish white. It produces a fleshy fruit with inflated pod containing several brown seeds with white long silky hair. It exudes a milky white latex when cut or broken [2].

The different parts of the plant are used to treat leprosy, eczema, inflammation, cutaneous infections, syphilis, malarial and low hectic fevers and as an abortifacient. The leaves of *C. procera* are useful as an antidote for snake bite and to alleviate sinus fistula, rheumatism, mumps, burns, injuries, body pain and jaundice. The bark is used to cure wounds, dermatological and bronchial affections and cough [3, 12].

Its root bark contained a norditerpenyl ester calotropterpenyl ester and pentacyclic triterpenoids calotropursenyl acetate, calotropfriedelenyl acetate, quercetin, kaempferol and isorhamnetin[4]. The plant grown in Saudi Arabia showed significant antimicrobial activity [5]. The ethanolic, aqueous and chloroform extracts of leaves and latex of *C. procera* exhibited antimicrobial effects [6, 13]. The bark showed antioxidant, antiplasmodial, anti-inflammatory and gastro mucosal protective properties [7, 8, 9]. An aqueous extract of the stem bark exhibited pronounced potent antimicrobial activity. Calo-protein isolated from the aqueous extracts of *C. procera* exerted broad-spectrum antimicrobial activity [2] and possessed significant antitumor activity against breast cancer [10]. An ethanolic extract of the flowers elicited strong cytotoxic effect t[11].

The sterols and triterpenes isolated from the root bark showed good inhibitory activity against human lung, glioblastoma and prostate cancer cell lines [14]. Seven oxy pregnane oligoglycosides calotroposides H-N were isolated from the root bark. The in vitro growth inhibitory effects of an

n-butanol fraction and calotroposides H-N were evaluated against the lung cancer, glioblastoma and prostate cancer cell lines [15]. The ethyl acetate and methanol extracts of the leaves showed antibacterial activity against *Ps. aeruginosa*, *Vibrio harveyi* and *Aeromons hydrophila* [16]. The present paper describes analysis of chemical constituents of the stem grown in the southern region of Saudi Arabia in Jazan and to investigate antibacterial activity of the same.

MATERIAL AND METHODS

Plant material

The stem bark of *C. procera* was collected from Jazan, Saudi Arabia and identified by Dr. Yahiya Masruhi, Department of Botany, Faculty of Science, and Jazan University. A voucher specimen No. JU/COP/16-2 of the sample is preserved in the herbarium of the Department.

Preparation of the leaves extract

The air-dried coarsely powder of the stem bark (500 g) was exhaustively extracted with ethanol in a Soxhlet apparatus for 30 h. The extract was concentrated on a steam-bath and dried under reduced pressure to get 53 g of dark brown mass. The residue was stored at 4 °C in the dark for subsequent experiments.

GC-MS analysis of the extract

GC-MS analysis was carried out on a Shimadzu Gas Chromatograph instrument fitted with a capillary column TR-5MS (30m x 0.25mm), film thickness 0.25 µm. The carrier gas He, flow rate 1.2 ml/min. The initial temperature was 70°C and then heated at a rate of 15°C per minute to 290°C and held for 16 minutes. The chromatograph was coupled to Shimadzu QP2010 Ultra MS detector 70eV.

Identification of constituents: The most constituents were identified by GC-MS by comparing their retention indices with those of authentic standard available in the library which were in close agreement with the reference samples. Further identification was achieved by the fragmentation patterns of the mass spectra compared with those stored in the spectrometer database using the NIST08 and Wiley 9 built libraries.

Antimicrobial activity

Microbial strains

Pure cultures of pathogenic bacteria species *Escherichia coli, Staphylococcus aureus, Bacillus subtilis* and *Streptococcus pyogenes* were obtained from the Microbiology Department, College of Pharmacy, Jazan University, KSA. All the bacterial cultures were maintained on nutrient agar medium at 4 °C.

Standard antimicrobial substance

Antibiotic tetracycline (50µg/ml) was prepared in dimethyl sulphoxide.

Preparation and sterilization of media

Distilled deionized water was used to prepare nutrient agar media. The media was composed of peptone (5.1 g), sodium chloride (5.0 g), beef extract (1.5 g), yeast extract (1.5 g) and agar (1.5 g). Dehydrated nutrient agar medium (28 g) was accurately weighed, suspended in 1000 ml of distilled water in a conical flask and heated to dissolve completely. Finally, the nutrient agar medium was sterilized by autoclaving at 15-lbs/in^2 pressure for 20 minutes [17].

Methods of preparation of test organisms

The test organisms were maintained on slants of nutrient agar medium and transferred to a fresh slant once in a month. The slants were incubated at 37 °C for 24 hours. Using 10 ml of sterilized normal saline solution, the cells/ mycelium were washed from the slants. A dilution factor was determined which gave optical density of 1.5 at 600 nm. The amount of suspension to be added to each 100 ml agar or nutrient broth was determined by use of test plates or test broth. The test organisms were stored under refrigeration at 4 °C.

Antimicrobial assay

In vitro antimicrobial assay was carried out by Agar well diffusion [18]. A previously liquefied and sterilized nutrient agar was poured into Petri-plates of 100 mm size (to make uniform thickness) and kept for solidifying. Microbial suspensions were spread over the solidified media. Holes were made in each plate with a stainless steel borer having 6 mm ID. Different dilutions

(75 µl) of the ethanolic extract of the stem bark were made having a concentration of 3 µLml-1, 5 µLml-1, 7 µLml-1 and 9 µLml-1 of solution. Tetracycline solution was used as standard. The plates were labeled as Co (control), S (standard), A (*Escherichia coli),* B *(Staphylococcus aureus),* C *(Bacillus subtilis)* and D *(Streptococcus pyogenes)* with four different holes, labeled as 3, 5, 7 and 9 for different concentrations. All dilutions were made in DMSO solvent. The plates were then left for standing for 3 hours at 4 °C for proper diffusion of the drug/test solutions. After diffusion process, all the Petri plates were incubated for 24 h at 37 °C. After 24 h the plates were examined and the diameters of zones of inhibition were accurately measured [19].

RESULTS AND DISCUSSION

The chemical composition of the ethanolic extract of the stem bark of *C. procera* is tabulated in Table 1. The extract was consisted mainly fourteen fatty esters (14.29%), eight aliphatic alkanes (5.1%), six pentacyclic triterpenes (18.92%), sterols (4.96%), four aliphatic amide (14.65%), three heterocyclic compounds (3.25%), two each of aromatic compounds (2.01%) and tetracyclic triterpene (1.69%), one each of aromatic phenol (1.68%), sulphur alcohol (0.31%), amino acid (0.33%), halogenated ester (0.48%), diazao alkane (0.77%), monoterpene (0.71%), sugar derivative (0.46%), sugar ester (0.4%), aromatic acid (1.98%), aliphatic aldehyde oxime (0.34%), glycol derivative (0.34%), sesquiterpene alcohol (0.36%), acid amide (2.17%), cyclic ketone (1.4%), aromatic ester (0.39%), acyclic diterpene (0.63%), acyclic triterpene (0.35%) and vitamin (0.89%).

The predominant constituent was 9-octadecenamide (12.84%) followed by 2,3-dihydroxypropyl *n*-octadecanoate (5.82%), 12-oleanen-3α-yl acetate (6.56%), methyl commate A (6.38%), lup-20(29)-en-3β-ol acetate (2.33%), dimethyl (docosyloxy)butoxy-silane (3.61%), urs-12-ene (2.05%), stigmasta-5-en-3β-ol (2.08%), *n*-hexadecanamide (2.17%), 2,4-bis-(1,1-dimethylethyl)-phenol (1.68%), *n*-docosane (1.14%), 7,9-tert-butyl-1-oxaspiro(4,5)-deca-6,9-diene-2,8-dione (1.4%), *n*-tetracosane (1.46%), methyl *n*-octadecanoate (1.71%), *n*-octadecane (1.0%), *n*-octadecenamide (1.49%), 2-hydroxy-1-(hydroxymethyl)ethyl hexadecanoate, (1.29%), phthalic acid (1.98%), 1H-indole-3-ethanamine (1.59%), 1H-indene,1-hexadecyl-2,3-dihydro- (1.01%), ergost-5-en-3β-ol (1.27%), norolean-12-ene (1.11%) and 1,4-epoxynaphthalene-1(2H-

methanol,4,5,7-tris(1,1-dimethylethyl)-3,4-dihydro-(1.75%). Forty one compounds were present in trace amounts.

Maximum percentage of compounds present in the bark extract was of pentacyclic triterpenes norolean-12-ene, urs-12-ene, 12-oleanen-3α-yl acetate, methyl commate A, lupeol and lup-20(29)-en-3β-ol acetate (18.92%). 9-Octadecenamide, *n*-octadecenamide, N, N-dipropyl- 1-buten-1-amine (14.65 %) were the aliphatic amides. Fatty esters detected in the extract were 1-methyl ethyl *n*-dodecanoate, methyl pamitate (methyl *n*-hexadecanoate), ethyl *n*-hexadecanoate (ethyl palmitate), (Z,Z)- methyl 9,12-octadecadienoate (methyl linolieate), methyl 9-octadecadienoate (methyl oleate), methyl *n*-octadecanoate (methyl stearate), (E,E)-methyl 9,12-octadecadienoate, ethyl oleate, 2-[bis(hydroxyethyl) amino] ethyl octadecadienoate, (Z,Z,Z)-ethyl 9,12,15-octadecatrienoate (ethyl linoleniate), trimethyl silyl octadecadienoate (trimethyl silyl linoleiate), 2-hydroxy-1-(hydroxymethyl)ethyl palmitate, 2-hydroxy-3-methyl-,diethyl succinate and 2,3-dihydroxypropyl octadecanoate (14.29%). *n*-Tetradecane, *n*-tetratriacontane, *n*-heneicosane, *n*-docosane, *n*-tetracosane, *n*-octadecane, *n*-eicosane and *n*-dotriacontane were categorized as the aliphatic alkanes (5.1%). The phytosterols characterized in the stem bark extract included were cholest-5-en-3β-ol, 22, 23-methylenecholestene-3β-ol, ergost-5-en-3β-ol, stigmasta-5, 22(E)-dien-3β-ol and stigmasta-5-en-3β-ol (4.96%). The heterocyclic compounds were identified as 1-allyl-2,8,9-trioxa-5-aza-1-silabicyclo[3.3.3] undecane, 1H-indole-3-ethanamine and 1-hexadecyl-2,3-dihydro-1H-indene (3.25%). The aromatic phenol was distinguished as 2, 4-bis (1, 1-dimethylethyl)-phenol (1.68%). The only acid amide present in the extract was hexadecanamide (2.17%). 1, 2-Benzenedicarboxylic acid (phthalic acid) was detected in 1.98% yields. (3aS, 9aS, 9bR)-6,6,9aa-Trimethyl-*cis*-perhydronaphtho[2,1-b]furan, 1,4-epoxynaphthalene-1(2H-methanol,4,5,7-tris(1,1-dimethylethyl)-3,4-dihydro- (2.01%) was an aromatic compound. The tetracyclic triterpenes were specified as 9, 19-cyclolanost-24-en-3β-ol and lanosta-8, 24-dien-3β-ol acetate (1.69%). 7,9-Tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione was a cyclic ketone occurring in 1.4% yield.

The reported GC-MS analysis of the leaf extracts revealed the presence of ergost-5-en-3-ol (25.2%), (Z)-9-octadecenoic acid (21.3%) and 2, 6, 10-trimethyl, 14-ethylene-14- pentadecne (33.1%) [20][21].

The ethanolic extract of the stem bark was examined for antibacterial activity against *E. coli*, *S. aureus*, *B. subtilis* and *S. pyogenes.* The alcoholic extract showed mild antimicrobial activity against clinically isolated pathogenic microbial strains in comparison to standard, tetracycline. The observations were recorded in Table 2. The antimicrobial activity was reported due to the presence of cardiac glycosides, phenols, alkaloids, tannin and quinines[16]. On the basis of GC-MS analysis of *C. procera* grown in Jazan region of Saudi Arabia was devoid of these compounds.

CONCLUSION

The phytochemical analysis indicated that phenolic compounds were present in small quantity. The bark of *C. procera* cannot be useful for treatment of skin disorder and/or in aromatherapy. The presence of phytosterols (4.96%) and triterpenes (20.96%) in maximum quantity could show good inhibitory activity against three human cancer cell lines including lung cancer, glioblastoma and prostate cancer. Further research is required for this part of the plant of this region.

Acknowledgement

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Table no 1. Chemical constituents of the ethanolic extract of the bark of *C. procera*.

S.No	Retention time	Component	% Area
1	7.9	*n*-Tetradecane	0.4
2	8.1	2,4-Bis (1,1-dimethylethyl)-phenol	1.68
3	8.2	*n*-Tetratriacontane	0.52
4	8.9	1-Methylethyl dodecanoate	0.3
5	9.5	*n*-Heneicosane	0.38
6	9.8	*n*-Docosane	1.14

7	11.2	Methyl hexadecanoate	0.63
8	11.2	7,9-Tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	1.4
9	11.3	*n*-Tetracosane	1.46
10	11.5	Dibutyl phthalate	0.39
11	11.6	Ethyl hexadecanoate	0.32
12	12.3	(Z,Z)-Methyl 9,12-octadecadienoate	0.49
13	12.4	Methyl 9-octadecadienoate	0.98
14	12.4	3,7,11,15-Tetramethyl-,[R-[R,R-(E)]]-2-hexadecen-1-ol	0.63
15	12.5	Methyl octadecanoate	1.71
16	12.6	*n*-Octadecane	1.0
17	12.7	(E,E)-Methyl 9,12-Octadecadienoate	0.45
18	12.8	Ethyl oleate	0.35
19	12.8	2-[Bis(hydroxyethyl) amino] ethyl octadecadienoate	0.74
20	12.8	(Z,Z,Z)-Ethyl 9,12,15-octadecatrienoate	0.4
21	12.9	*n*-Eicosane	0.99
22	12.9	*n*-Hexadecanamide	2.17
23	13.1	Trimethyl silyl Octadecadienoate	0.51
24	13.2	1-Allyl-2,8,9-trioxa-5-aza-1-silabicyclo[3.3.3] undecane	0.65
25	13.2	3-Decanethiol	0.31
26	13.3	Cycloglycylvaline	0.33
27	13.5	Hexadecyl heptafluorobutyrate	0.48
28	13.7	1,8-Diazacyclotetradecane-2,7-dione	0.77
29	13.9	p-Menthan-2-one-1,3,3-d3	0.71
30	14.0	9-Octadecenamide	12.84
31	14.1	*n*-Octadecenamide	1.49
32	14.3	(3aS,9aS,9bR)-6,6,9aa-Trimethyl-cis-	2.01

		perhydronaphtho[2,1-b]furan	
33	14.5	1,3,5-Trisilacyclohexane	0.51
34	14.6	2-Deoxy-bis(thioheptyl)-D-ribose dithioacetal	0.46
35	14.7	Glycidol stearate	0.4
36	14.8	2-Hydroxy-1-(hydroxymethyl)ethyl hexadecanoate	1.29
37	14.9	1,2-Benzenedicarboxylic acid	1.98
38	15.0	*n*-Dotriacontane	0.67
39	15.0	N,N-Dipropyl-1-buten-1-amine	0.32
40	15.2	*n*-Hexadecanal oxime	0.32
41	15.3	Octadecamethyl-cyclononasiloxane	0.43
42	15.4	Decyltetraglycol	0.34
43	15.5	2-Hydroxy-3-methyl-diethyl succinate	0.3
44	15.7	1H-Indole-3-ethanamine	1.59
45	15.8	1-Hexadecyl-2,3-dihydro- 1H-indene	1.01
46	16.0	1,3,5-Trisilacyclohexane	0.49
47	16.0	8S,13-Cedran-diol	0.36
48	16.1	2,3-Dihydroxypropyl octadecanoate	5.82
49	16.6	(all-E)-2,6,10,15,19,23-Hexamethyl 2,6,10,14,18,22-tetracosahexaene	0.35
50	20.2	dl-α-Tocopherol	0.89
51	20.5	Cholest-5-en-3β-ol	0.37
52	22.1	22,23-Methylenecholestene-3β-ol	0.33
53	22.2	Ergost-5-en-3β-ol	1.27
54	22.6	Stigmasta-5,22 (E)-dien-3β-ol	0.91
55	23.8	Stigmasta-5-en-3β-ol	2.08
56	24.5	Dimethyl(docosyloxy)butoxy-silane	3.61
57	25.0	Norolean-12-ene	1.11
58	25.8	9,19-Cyclolanost-24-en-3β-ol	0.77

59	26.2	Urs-12-ene	2.05
60	26.9	12-Oleanen-3β-yl acetate	6.56
61	28.0	Lanosta-8,24-dien-3β-ol acetate	0.92
62	28.2	Methyl commate A	6.38
63	28.6	Lupeol	0.49
64	28.8	1,4-epoxynaphthalene-1(2H-methanol,4,5,7-tris(1,1-dimethylethyl)-3,4-dihydro-	1.75
65	28.9	Lup-20(29)-en-3β-ol acetate	2.33

Table no 2. Antimicrobial activity of the bark ethanolic extract of *C. procera* on tested pathogenic microbes

Sample conc. (µl/ml)	Zone of inhibition (mm) *E. coli*	Zone of inhibition (mm) *S. aureus*	Zone of inhibition (mm) *B. subtilis*	Zone of inhibition (mm) *S. pyogenes*
3 (A)	00	00	00	00
5 (B)	00	00	00	00
7 (C)	00	00	00	00
9 (D)	05	02	07	04
50 (Co)	---	---	---	---
50 (S)	20	16	34	24

CONFLICT OF INTEREST: None.

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