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Research Article

Phytochemical and Biological Studies on *Crotalaria madurensis* (Family Fabaceae)

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ABSTRACT

Two new triterpenoid saponins have been isolated from leaves and flowers of *Crotalaria madurensis* Wight&Arn named; sophradiol 3-*O*-β-D-$^4C_1$-glucopyranosyl-(1"→4')-*O*-β-D-$^4C_1$ glucuronopyranoside (**3**) and sophradiol 3-*O*- α-L-$^1C_4$-rhamnopyranosyl (1'"→4")-*O*-ß-D-$^4C_1$ glucopranosyl-(1"→6')-*O*-ß-D-$^4C_1$-glucopyranoside (**4**) beside myo-inositol (**1**) and three other triterpene saponin their structures were established as sophradiol 3-*O*-ß-D-$^4C_1$-glucuronopyranoside (**2**) 3, 23-di- hydroxyl olean 12-en 28-oic acid 3-*O*- α-L-$^1C_4$- rhamnopyranoside (1"→6')- *O*-β-D-$^4C_1$- glucopyranoside (**5**) 23-hydroxy-3*α*-[*O*-*α*-L-$^1C_4$-rhamnopyranosyl-(1"→4') - *O*-*α*-L-$^4C_1$-arabinopyranosyl-oxy] olean-12-en-28-oic acid *O*-*α*-L-$^1C_4$-rhamnopyranosyl- (1""→4"")-*O*-*β*-D-$^4C_1$-glucopyranosyl-(1""→6"')-*O*-*β*-D-$^4C_1$-glucopyranosyl ester (**6**). In addition to six flavonoids identified as Quercetin 3-*O*-α-L-$^1C_4$-rhamnopyranosyl-(1'"→2")-*O*-ß-D-$^4C_1$-glucopyranoside (**7**), rutin (**8**), isoquercitrin (**9**), quercetrin (**10**), 8-Hydroxy quercetin 3-*O*-ß-D-arabinopyranoside (**11**), and quercetin (**12**). The structures elucidated by chemical evidences and spectroscopic analysis (1 & 2D-NMR, ESI-MS/MS and UV)**.** Aqueous alcoholic extract; total saponins fraction and certain pure compounds have significant antischistosomal activity, analgesic, anti-inflammatory and improve the liver function. Anti-bacterial studies of different extracts on both gram negative and gram positive bacteria showed inhibition of the growth of certain microorganism.

Keywords: *Crotalaria madurensis*, antischistosomal**,** anti-bacterial**,** antinociceptive**,** sophradiol**,** quercetin, myo-inositol.

INTRODUCTION

Genus *Crotalaria* L. (Leguminosae) includes about 600 species distributed throughout the tropics and subtropics[1]; with only 5 species represented in Egypt (*C. thebaica, C. impressa, C. senegalensis, C. egytiacae, C. microphylla*)[2]. *Crotalaria* species are known as a rich source of non toxic pyrrolizidine alkaloids (PAs)[3-5]. Beside PAs, some flavonoid glycosides have been reported[6-8], prenylatedchalcone[9], dihydrochalcones[10], chalcones[11], flavanones[12], triterpenes[7,13-15] and isoflavones[16]. Moreover, different *Crotalaria* species have long been used as medicinal Folkloric remedies especially in India for treatment of different aliments[17,18]. Some species have wide use as diuretic, in treatment of sore throat, inflammation of mouth and to produce cooling sensation[19], as well as used as purgative[18] for purifying blood in impetigo and psoriasis[20], also as anti-inflammatory, anti-hepatotoxicity[15]. Also, large numbers of these plants have been investigated for their antifertility activity in laboratory animals[21-22] but only few of them have shown a significant effect. Also, *Crotalaria* inhibits *in vitro* growth of the human malaria parasite *Plasmodium falciparum*[23]. As a continuation to the study on *C. madurensis*[7], the present paper aimed at the isolation of further saponins and flavonoid glycosides of new structural features on the basis of their wide-range of chemical structure variation and biological importance, also evaluate antischistosomal, analgesic, anti-inflammatory anti-bacterial activities and also, evaluate the effect of the aqueous alcoholic extract on the liver function.

Equipments

The NMR spectra were recorded at 300, 500 (^{1}H) and 75, 125 (^{13}C) MHz, respectively, on a Varian Mercury 300, and JEOL GX- NMR spectrometer, and δ values are reported in ppm relative to TMS in the convenient solvent. ESI-MS analyses were measured on a Finnigan LCQ deca LC/ MS and double focusing sector field MAT 90 MS spectrometer (Finnigan, Bremen, Germany). UV spectra of pure samples were recorded, separately, in MeOH using different diagnostic UV shift reagents using a Shimadzu UV 240 spectrophotometer[24]. For column chromatography (CC), Sephadex LH-20 (Pharmacia, Uppsala, Sweden), microcrystalline cellulose (Merck, Darmstadt, Germany), polyamide 6S (Riedel de Haën AG, Seelze, Germany) were used. For paper chromatography Whatman No. 1 sheets (England) were used while silica gel (Sigma, 28-200 mesh) was used for saponin CC, and F254 for TLC (Merck, Germany).

MATERIALS

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Plant Collection

The fresh leaves, stems and flowers of *Crotalaria madurensis* Wight&Arn were collected from plants cultivated in Al-Kanater Al-Khiraya, during March (2008). The identity of the plant was established by Prof. Dr. Wafaa M. Amer Department of Botany, Faculty of Science, Cairo University. Voucher specimens (C-15) are kept in the Department of Pharmacognosy, Faculty of Pharmacy, Al-Azhar University. The plant material was air-dried and kept in tightly closed container**.**

Animals and chemicals for biological studies

Animals: Male Swiss Albino mice (18-20 g) and rats (100-150 g) of CD-1 strain bread and maintained at the Schistosoma Biology Supply Center (SBSC), Theodor Bilharz Research Institute (TBRI), Giza, Egypt were infected with the Egyptian strain of *S. mansoni* (80±10 *cercaria*/mouse).

Chemicals and kits for biological studies

Commercial pellet diet, (El-Kahira Company for Oil and Soap), Praziquantel (Distocide®), (EPICO, Cairo, Egypt), Cremophor; (Sigma Chemical Company St. Louis Missouri), 2,2-Diphenyl-1-Picryl-Hydrazyl (**DPPH**) free radical (0.1Mm), (Fluka Company, Cairo, Egypt), Methanol (Merck Company, Cairo, Egypt), Ascorbic acid (1%)**,** Green tea**,** Aspirin**,** Acetic acid (0.6% v/v) and Morphine sulphate (10 mg/kg *i.p.*).

Methods of Chemical Investigation

Quantitative estimation of phenolic and flavonoid contents

Total phenolic content of different organs of *C. madurensis* leaves, stems and flowers were determined by the Folin-Ciocalteus reagent method[25], it was expressed as GAE (Gallic Acid Equivalents) while, their total flavonoid content was determined by the aluminium chloride colorimetric methods, it was expressed as quercetin equivalents[26].

Extraction and isolation

The air-dried powdered leaves stem and flowers of *C. madurensis* (1kg) were extracted under reflux with hot 70% MeOH (7 X 4L). After evaporation of the solvent, the obtained dry residue was defatted with $CHCl_3$ under reflux (7 × 1 L). The resulting residue (180 g) was suspended in water (900 ml) and extracted with ethyl acetate (5 × 500 ml), followed by *n*-butanol (7 × 300 ml). The *n*-butanol extract concentrated to dryness by removing the solvent in a rotary evaporator and was washed with 100 ml distilled water (X 3). 2D-PC analysis proved that the chloroform extract is free from polyphenols. Flavonoids were found in the ethyl acetate fraction, whereas saponins were concentrated in the *n*-butanol extract. The *n*-butanol extract gave negative reaction with $FeCl_3$ but pink color with sulphuric acid spray reagent on TLC when heated at 120ºC for three min. *n*-butanol extract (70 g) was subjected to column chromatography over silica gel column (Ø 5.0 x 300 cm,) using a gradient of $CHCl_3$-MeOH (8:2, 7:3, 6:4 1:1, 3:7) and 0:1, (each 75 ml) to give six main fractions (A-F) according to the differences in composition indicated by TLC analyses. Fr. A (13g) eluted with $CHCl_3$–MeOH (8:2), was purified by silica gel column, eluted with $CHCl_3$- EtOAc (8:2) to give pure (**1)** (18 mg). Fr. B (10g) eluted with $CHCl_3$–MeOH (7:3) and purified by silica gel column, eluted with $CHCl_3$-EtOH (4:7) to give pure (**2)** (15 mg). Crude (**3)** was crystallized from fr. C (15g) $CHCl_3$–MeOH (6:4) and purified by repeated crystallization from MeOH to yield pure (**3)** (40 mg). Fr. D (7g) $CHCl_3$–MeOH (1:1), was chromatographed on a silica gel column eluted with $CHCl_3$-MeOH-H_2O (3:7: 0.1) to give pure (**4**) (42 mg). Eraction E (11 g) $CHCl_3$–MeOH (3:7) was chromatographed on a silica gel column eluted with $CHCl_3$-MeOH-H_2O (3:9: 0.1) to give pure (**5**) (35 mg)**.** Crude (**6**) was crystallized from fr. F (9g) $CHCl_3$–MeOH (0:1) and purified by repeated crystallization from MeOH to yield pure (**6**). All separation processes were followed up by Co-TLC using solvent systems: $CHCl_3$- EtOAc (8:2), MeOH-$CHCl_3$ (3:7) and MeOH- $CHCl_3$-H_2O (35:60:5).

The ethyl acetate extract (50g) fractionated on a polyamide column (Ø 3.0 x 120 cm) using a stepwise gradient from H_2O, H_2O/MeOH mixtures up to pure MeOH for elution. Based on comparative paper chromatography (Co-PC) with the use of UV light, 1% $FeCI_3$, or Naturstoff spray reagent for detection[27], the individual 35 fractions (each 1 L) were pooled into 6 collective fractions (G – L). Fraction G (H_2O, 3 g) was found to be a dark brown material with no phenolic character. Fraction H (10 – 40% MeOH, 5 g) was fractionated on cellulose C with 40% EtOH as an eluent, followed by a Sephadex LH- 20 column using BIW (*n*-BuOH/2-propanoI/H_2O, 4:1:5 v/v/v, organic layer) to afford pure (**7**) (20 mg). Fraction I (40 – 60% MeOH, 5 g) was subjected to repeated CC on cellulose and Sephadex LH-20 with 20 – 60% aqueous MeOH as an eluent, resulting in pure samples of (**8**) (32 mg). Fraction J (70%, 4 g) was chromatographed on Sephadex with MeOH to give **(9)** (28 mg). Fraction K (60 – 80% MeOH, 3 g), was fractionated Sephadex with MeOH to give (**10**) (22 mg). Fraction L (80 – 100% MeOH, (7 g) was subjected CC on Sephadex LH-20 with 40 – 60% aqueous MeOH as an eluent, resulting in pure samples of (**11**) (21 mg) and **(12**) (19 mg). All separation processes were followed by 2D-PC and CoPC using Whatman No. 1 paper with *n*-BuOH/ AcOH/H_2O (4:1:5, top layer) (S1) and 15% aqueous AcOH (S2) as solvent systems.

Experimental data of isolated compounds

Sophradiol 3-*O*-β-D-$^4C_1$-glucopyranosyl-(1"→4')-*O*-β-D-$^4C_1$ glucuronopyranoside (**3**) It is isolated as off white amorphous powder, R_f = o.55 and 0.59 in solvent systems MeOH-$CHCl_3$ (3:7) and MeOH- $CHCl_3$-H_2O (35:60:5), respectively. Negative ESI-MS: *m/z* 769.5 $[M-H]^-$ ^1H and ^{13}C NMR spectral data (500/125MHz, DMSO-d_6) (Table 1).

Sophradiol 3-*O*- α-L-$^1C_4$- rhamnopyranosyl (1'"→4")-*O*-ß-D-$^4C_1$ glucopranosyl-(1"→6')-*O*-ß-D-$^4C_1$-glucopyranoside (**4**) is obtained as off white amorphous powder, R_f = 0.51 and 0.61 in solvent systems MeOH-$CHCl_3$ (3:7) and MeOH- $CHCl_3$-H_2O (35:60:5), respectively. – Negative ESI-MS: *m/z* 911.5 $[M-H]^-$, ^1H and ^{13}C NMR spectral data (500/125MHz, DMSO-d_6) (Table 1)

Methodology of Biological Studies

Each aqueous alcoholic extract of *C. madurensis* leaves (200 mg / kg b. wt), total saponins fraction(each 200 mg / kg b. wt) and some isolated compounds (**1, 3** and **8**) were administrated orally to the animals as prepared in distilled H_2O by the aid of tween 80 and subjected to the following evaluations:

Toxicological study

The LD_{50} of each aqueous alcoholic extracts and total saponins were determined using method described by Litchfield and Wilcoxon 1949[28]. The therapeutic dose used in this study was 200mg/kg p.o. which was 20-fold less than the dose used in acute toxicity.

Antischistosmal activity[29]

Antischistosomal activity was determined in mice infected with *Schistosoma mansoni* [(100±10) *cercaria* /mouse] in terms of worm load and distribution in both hepatic and portomesenteric vessels, oogram pattern (percentage of the different egg developmental stages in small intestine) and ova count in liver and intestine[29].

Worm load and distribution in hepatic perfusion and portomesenteric perfusion[30]

Hepatic perfusion

Portal vein was ligatured and a needle connected to an automatic pipetting machine was inserted in the inferior vena cava. Hepatic perfusion was done by using ice-cold normal saline solution and the worms were collected and counted.

Portomesenteric perfusion

The previous ligature of the portal vein was removed. A needle was inserted into the thoracic aorta downward to perfuse the mesenteric vessels and the perfusate fellow out to the portal vein. The perfusate was left to settle and then the worms were collected, counted and classified.

Oogram pattern[31]

After animal perfusion, small intestine was removed and three fragments (each 1cm) were cut off, opened, dried gently on a filter paper and examined microscopically. One hundred of *S. mansonai* ova were counted in each fragment and classified according to their developmental stages as the following:

Immature eggs that were classified into four stages according to the size of the embryo.

Mature eggs that containing fully developed miracidium.

Dead eggs, which appeared as semitransparent, granulated, darkened with retracted embryos.

Ova count[32]

Pieces of liver and intestine were weighted (**W**) and placed in 5 ml of 4 % KOH solution, then incubated at 37 ºC for 24 hr, to reach complete digestion. Three samples 0.1 ml each, were taken, on a counting slide and counted under low power of the microscope; the mean number of ova was calculated ($\mathbf{X}^{'}$). The rest of the digest was poured in a graduated cylinder and measured (**V**). The number of egg /g liver = $\mathbf{X}^{'}$ **x V/W.**

Evaluation of antinociceptive activity

Acetic acid-induced writhing in mice

The animals were divided into four groups each of six (n=6 per group). The first group received saline and was consider as control, while the second group received orally dose of aspirin (100 mg/kg b.wt.). The remaining two groups received orally **1** and **3** (400 mg/kg). After 30 min. interval, the mice received *i.p*. injection of 0.6% v/v, 10 mg/kg acetic acid[33]. The animals were placed in a large glass cylinder and the intensity of nociceptive behavior was quantified by counting the total number of writhes occurring between 0-20 min after stimulus injection, as described earlier.

Hot-plate test (Thermal test)

The hot-plate test was performed on mice by using an electronically hot-plate (Ugo Basile, Model-DS37) was maintained at 55±0.2°C. The animals were divided as in case of acetic acid method and received saline, morphine sulphate (10 mg/kg *i.p.*) as reference drug[34] and isolated compounds **1** & **3** (400 mg/kg) 30 min prior to testing[35]. The reaction time was noted by observing either the licking of the hind paws or jumping movements before and after drug administration. The cut-off time was 20 sec**.**

Tail immersion test

The lower two-thirds of the tail were immersed in beaker containing water kept at 50±0.5˚C. The mice were exposed to hot water for no longer than 20 sec. to ovoid tissue injury[36]. The reaction time is defined as the time in seconds until the tail was withdrawn from the water, the reaction time was the measured at 0, 60, 120 min. after the oral administration of saline, isolated compounds **1** & **3** (400 mg/kg) and morphine (10 mg/kg *i.p.*), with the reaction time of 0 min. being the start of test.

Evaluation of anti-oxidant activity

Evaluation of anti-oxidant activity by DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay *in vitro* using method reported by **Braca** *et al*. 2001[37]. The antioxidant activity of the alcoholic extracts and isolated compounds **(8** and **10)** is expressed comparing with standard[38]; green tea and ascorbic acid at various concentrations (1 to18 µg/ml). Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The percentage of the radical scavenging activity (RSA) was calculated by the following equation:

$$RSA\% = [A_{control} - A_{test}]/ A_{control} \times 100$$

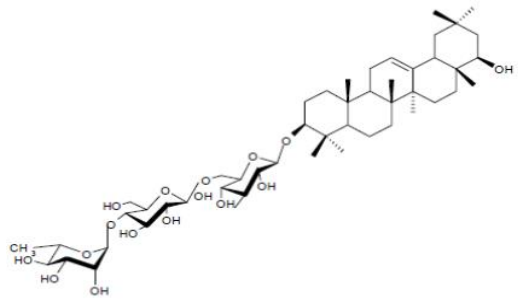
Where $A_{control}$ is the absorbance of the control reaction and A_{test} is the absorbance in the presence of the sample of the extracts. Triplicate measurements were taken and mean values calculated. The IC_{50} value for each sample, defined as the concentration of the test sample leading to 50% reduction of the initial DPPH concentration, was calculated from the nonlinear regression curve of Log concentration of the test extract (µg/ml) against the mean percentage of the radical scavenging activity.

Antimicrobial study

Test organisms

The different alcoholic extracts of stem, flower, leaves and total crude saponin fraction of different organs of *Crotalaria madurensis* were screened for their antibacterial activity against *Escherichia coli* (ATCC-25922), *Klebsiella pneumonia* (as representative of Gram-negative bacteria), *Staphylococcus aureus* (ATCC-25923) and *Pseudomonas aeruginosa.* (as representative of Gram –positive bacteria).

Inoculum Preparation



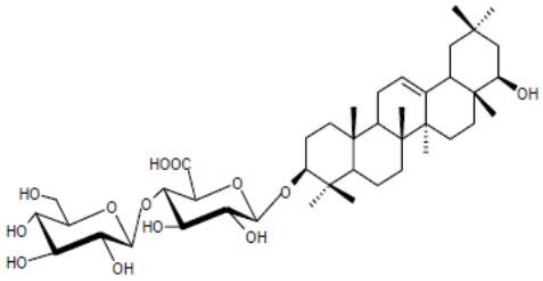


Table 1: ^{1}H and ^{13}C NMR spectral data of compounds 3 and 4 (500/125MHz, DMSO-d_6).

C	$3\delta_C$	3 δ_H	δ_C 4	$4\delta_H$
1	39.9		40.0	
2	26.0		25.6	
3	89.7	3.65, br d	89.1	3.39 , br d,
4	39.7		39.5	
5	55.6		55.0	
6	18.5		18.9	
7	33.2		32.7	
8	40.1		40.1	
9	47.6		47.1	
10	36.7		36.3	
11	23.5		23.1	
12	122.5	5.13, br s,	121.6	5.51, br s,
13	144.6		144.2	
14	42.2		41.7	
15	26.6		25.6	
16	28.8		28.4	
17	37.5		37.1	
18	45.1	2. 85, dd (9.15 Hz)	44.6	
19	46.5		45.1	
20	30.7		30.3	
21	42.2		41.7	
22	75.6	4.98 (1H, brs aliphatic-OH), 4.95 (1H, br s, OH-22	76.1	5.16 , br s, OH-22
23	28.8	0.81 s	27.8	0.84 s
24	15.8	0.81 s	15.4	0.84 s
25	16.6	0.96 s	16.2	1.00 s
26	17.2	1.02 s	16.8	1.05 s
27	25.5	1.27 s	25.2	1.06 s
28	28.4	0.72 s	28.4	0.76 s
29	33.2	0.85 s	33.8	0.90 s
30	20.9	s 0.87	20.5	0.97 s

Table 1 (cont.): ^{1}H and ^{13}C NMR spectral data for sugar units of compounds 3 and 4 (500/125MHz, DMSO-d_6).

No.	3		4	
	δ_C	δ_H	δ_C	δ_H
	ß-Dglucuronopyranosyl		ß-D-glupyranoside	
1'	100.0	4.69 d, (6.8 Hz),	99.7	4.74, d, (7.5 Hz),
2'	72.5	3.50-3.10	70.5	3.64-3.10 (m,
3'	74.6	(5 H, m,	74.6	remaining sugar
4'	78.5	remaining	74.1	protons)
5'a	74.9	sugar	77.7	
6'a	173.3	pprotons)	75.3	
6'b			61.9	
ß-D-glupyranoside			ß-D-glupyranoside	
1"	100.5	4.76, d, (6.9 Hz),	**99.9**	4.81d,(7.5 Hz)
2"	74.2	3.50-3.10	**71.1**	3.64-3.10 (m,
3"	76.9	(5 H, m,	**76.6**	remaining sugar
4"	70.8	remaining	**70.0**	protons)
5"	76.2	sugar	**76.8**	
6"	68.4	pprotons)	**68.1**	
			α-L-rhamnoside	
1"'			**103.9**	5.02, br s
2"'			**72.4**	3.64-3.10 (m,
3"'			**70.6**	remaining sugar
4"'			**72.0**	protons)
5"'			**63.0**	
6"'			**18.2**	0.88(3H, br d,

Inoculum was prepared as saline suspension of isolated colonies selected from 18 to 24 hour agar plate. The suspension is adjusted to match the 0.5 McFarland turbidity standards, using a vortex mixer and using saline as standard.

Cups plate method

A lawn culture was produced on Müeller-Hinton agar plates. Four cubes were made on the Petri dishes and 50 µl aliquots of extracts were pipette on. The plates were left for 1 hr at room temperature and then incubated at 37°C for 48 hours. Plates were examined for inhibition zones of the growth of bacteria around the extracts. The averages of those zones were recorded in millimeters. Each experiment was repeated three times and the results are the average of the three runs.

Minimum Inhibitory Concentration (MIC)

The broth micro dilution procedure recommended by the Clinical and Laboratory Standards Institute (the National Committee for Clinical Laboratory Standards) was used for determination of the MICs[39]. *Escherichia coli* (ATTC-25922), *P. aeruginosa* and *Staphylococcus aureus* (ATTC-25923) were used. Double-strength Müeller-Hinton broth (MHB), 4X strength extracts solutions prepared as serial two-fold dilutions and the test organism at a concentration of $2x10^6$/ml were used. Minimum inhibitory concentrations

Table 2: Effect of treatment with aqueous alcoholic extract, total saponins & compound 8 of *C. madurensis* (200 mg/kg for 2weeks) on worm in *S. mansoni* infected mice compared to praziquantel (500mg/kg for 2 days).
**Significant difference from infected control at P<0.05.*

Animal groups	Total no. of worms in hepatic	Total no. of worms in portomesenteric	Total no. of males	Total no. of females	Total no. of couples	Total no. of worms	% Worm reduction
Infected Control	4.00 ± 0.57	21.83 ± 0.90	13.33 ± 0.59	12.50 ± 0.84	9.00 ± 0.40	25.83±0.13	----
Praziquantel	1.17 ± 0.33*	0.00 ± 0.00 *	0.00 ± 0.00 *	0.00 ± 0.00 *	0.0 ± 0.0*	0.00 ± 0.00 *	93.52%
Total saponins	1.50 ± 0.52*	15.50 ± 0.17*	7.50 ± 052*	9.50 ± 0.17*	4.50 ± 0.52*	17.00±0.35 *	34.18%
Alcoholic extract	4.00 ± 0.28	13.00 ± 0.28*	7.00 ± 0.57*	10.00 ± 0.05*	2.00 ± 0.01*	17.00±0.57 *	34.18%
Compound 8 (Rutin)	1.67 ± 0.16*	23.00 ± 0.57	11.00 ± 0.28	13.67 ± 0.16	8.00 ± 0.02	24.67±0.43 *	4.49%

Values given are means ± SE.

Table 3: Effect of treatment with aqueous alcoholic extract, total saponins and compound 8 of *C. madurensis* (200mg/kg for 2weeks) on oogram pattern egg/gm tissue in liver and intestine in *S. mansoni* infected mice compared to praziquantel (500mg/kg for 2 days).

Animal groups	(Oogram pattern) % Egg developmental stages Immature stage						
	First	Second	Third	Fourth	Total immature ova	Mature ova	Dead ova
Infected control	4.17±1.17	12.83±0.58	15.67±0.82	6.67±0.55	39.33 ± 2.49	44.00±2.63	16.67±1.47
Praziquantel (500mg/kg for 2days)	0.0± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00*	0.00±0.00*	100± 0.00*
Total saponins	2.00±0.57	4.33 ± 0.71	3.17 ± 0.88	2.17± 0.47	18.33±2.61	40.17±3.59 *	41.5±4.76*
Aqueous alcoholic extract.	2.83±0.58	5.00±1.17	6.67±2.34	3.50±1.37	18.00±5.06	40.17±3.59	41.83±7.54 *
Compound 8 (Rutin)	4.17±1.17	11.00±1.23	14.67±1.47	6.67±0.55	36.50±3.13	44.00±2.63	19.50±1.45

*Significant difference from infected control at P<0.05

Table 4: Effect of treatment with aqueous alcoholic extract, total saponins and compound 8 of *C. madurensis* (400mg/kg for 2weeks) on the number of ova/gm liver and intestine in *S. mansoni* infected mice compared to praziquantel (500mg/kg for 2 days).

Animal groups	Number of ova per gram tissue (X 10^3)			
	Liver	Intestine	Total number of ova	%Ova Reduction
Infected control	15.87 ± 1.58	20.75 ± 1.85	36.62 ± 3.00	--
Praziquantel (500mg/kg for 2days)	2.10 ± 0.72*	1.49 ± 0.62*	3.58 ± 1.33*	90.22%
Total saponins	10.67 ± 0.52	12.22 ± 1.49	22.89 ± 1.59	37.49%
Alcoholic extract	11.00±0.40	12.22±1.49	23.22±1.62	36.59%
compound 8 (Rutin)	14.62±1.70	19.08±1.17	33.70±2.65	7.97%

*Significant difference from infected control at P<0.05

(MICs) which inhibited bacterial growth were established by a serial dilution technique using 96-well microliter plates. 100 µl of double-strength MHB, 50 µl each of the extracts and the organism suspension are mixed and incubated at 35°C for 24 hours. MIC is expressed as the highest dilution which inhibited growth judged by lack of turbidity in the well.

RESULT AND DISCUSSION

Quantitative estimation of total phenolic contents of *C. madurensis* leaves, stems and flowers were determined as 5.27, 2.52 and 2.7, while those of total flavonoids contents

Table 5: Antinociceptive activity of compounds 3 & 6 were isolated from *C. madurensis* leaves compared with reference drug (aspirin) using acetic acid-induced writhing test.

Animal Groups	Dose of drug	Writhing number (Count/20min) Mean± S.E	% Inhibition	% Analgesia
Control (Acetic acid 0.6% saline)	0.01ml/g	64.67 ± 2.15	-------	--------
Aspirin	100 mg/kg	15.33 ± 1.31*	76.29%	100%
Compound 1	400 mg/kg	37.00±1.44*	42.78%	56.08%
Compound 3	400 mg/kg	8.83 ± 1.62*	86.34%	113.17%

**Significant difference from control group at P< 0.0 Values given are means ± SE*

Table 6: Antinociceptive activity of isolated compounds 3 & 6 from *C. madurensis* leaves compared with reference drug (morphine) using hot plate test.

Animal Groups	Dose of drug	Reaction time (Sec) Mean± S.E	% Increase	% Analgesia
Normal Control	Saline	10.83± 0.51	-------	--------
Morphine	10 mg/kg	20.00 ± 1.37*	84.67%	100%
Compound 3	400 mg/kg	17.67 ± 0.65*	63.15%	74.59%
Compound 6	400 mg/kg	18.33±0.62	69.25%	81.78%

*Significant difference from control group at P< 0.05.
Values given are means ± SE.

were yielded 3.72, 1.38 and 1.56 g/100 ml extract respectively.

The defatted total aqueous methanol extract of *C. madurensis* leaves and flowers was fractionated by repeated column chromatographic separations to obtain compounds **1** – **12**. Based on chemical , physicochemical analyses and spectral data which compared with published data, known compounds **1**, **2** and **5**–**12** were identified as Myo-inositol (**1**)[40], Sophradiol 3-*O*-ß-D-$^4C_1$-glucouronopyranoside (**2**)[41], 3, 23-di- hydroxyl olean 12-en 28-oic acid 3-*O*- α-L-$^1C_4$- rhamnopyranoside (1"→6')-*O*-β-D-$^4C_1$- glucopyranoside (**5**)[42] 23-hydroxy-3*α*-[*O*-*α*-L-$^1C_4$-rhamnopyranosyl-(1"→4') - *O*-*α*-L-$^4C_1$-arabinopyranosyl-oxy] olean-12-en-28-oic acid *O*-*α*-L-$^1C_4$-rhamnopyranosyl- (1""→4"")-*O*-*β*-D-$^4C_1$-glucopyranosyl-(1""→6"")-*O*-*β*-D-$^4C_1$-glucopyranosyl ester. (**6**)[42] Quercetin 3-*O*-α-L-$^1C_4$-rhamnopyranosyl-(1'"→2")-*O*-ß-**D**-$^4C_1$-glucopyranoside (**7**), Rutin (**8**), Isoquercitrin (**9**), Quercetrin (**10**), 8-Hydroxy quercetin 3-*O*-ß-D-arabinopyranoside (**11**) and Quercetin (**12**)[43-44].

Compound **3**: Complete acid hydrolysis of **3** afforded ß-D-glucose and ß-D-glucouronic acid in aqueous phase and sophradiol in organic phase (Co-PC, with authentic sample). The resonances due to eight sp3 methyl carbons at δ 25.5 (C-27), 17.2 (C-26), 16.6 (C-25), 20.9 (C-30), 33.2 (C-29), 28.8 (C-23), 15.5 (C-24), 28.4 (C-28), a secondary carbinol at 75.6 and two sp^2 carbons at δ 122.5 and 144.6 in the ^{13}C NMR of **3** coupled with the corresponding information from the ^1H NMR [8 tertiary methyl proton singnals, methine protons linked to oxygen bearing carbon at δ 4.95 (H-22), hydroxymethine proton at δ 3.65 for H-3, a proton attributed to H-18 at δ 2.85 (dd, *J* = 14.3Hz) and a broad singlet vinyl proton at δ 5.13 of H-12 confirmed the aglycone moiety as 3, 22-dihydroxy-olean-12-en skeleton (sophradiol). All assigned ^1H and ^{13}C-resonances of the aglycone moiety were confirmed by comparison with published data[41,45]. Two anomeric proton signals were assigned at 4.76 (d, *J* = 6.9Hz, glucose) and 4.69 (d, *J* = 6.8 Hz, glucuronic acid), in the ^1H NMR spectrum. This evidence was confirmed by the presence of two anomeric carbon signals at δ 100.5 and 100.0 in the ^{13}C spectrum belonging to glucose and glucuronic acid moieties, respectively. The two sugar moieties were deduced to adopt *β*-D-$^4C_1$ pyranosyl stereo structures, on the basis of *J*-value of their anomeric protons and δ-values of their ^{13}C-resonance (Table 1). Glycosidation at C-3 was concluded from the deshielded shift of C- 3 to δ 89.7 (≈ + Δ 8 ppm) and shielded effect of both C-2 and C-1 relative to those of unsubstituted moiety[44]. Similarly, C-4' of the glucuronic acid moiety was strongly deshielded at δ 78.5 (≈ + Δ7-8 ppm) compared to the chemical shift of the analogous carbon resonance of a non-substituted moiety (δ 70.8), led us to identify the glycoside moiety as 3-*O*-β–D-glucopyranosyl-(1"→4')-*O*-β-D-glucuronopyranoside. All assigned ^1H and ^{13}C- resonances of **3** were confirmed by comparison of related published data[45]. Hence, compound **3** was identified as Sophradiol 3-*O*-β-D-$^4C_1$-glucopyranosyl-(1"→4')-*O*-β-D-$^4C_1$ glucuronopyranoside.

Table 7: Evaluation of anti-oxidant activity of *C. madurensis* extract and isolated compounds 8 &10 by DPPH assay *in vitro* compared to Ascorbic acid & Green tea:

Sample	DPPH
Ascorbic acid (1%)	8.9 ± 0.63
Green tea	6.7 ± 0.10
Alcoholic extracts	47.06 ± 0.63
Compound 8	29.88 ± 0.23
Compound 10	50.53 ± 0.34

Compound **4**: Negative ESI-MS of **4** showed molecular ion peak at *m/z* 911.5 $[M-H]^-$. Complete acid hydrolysis of **4** afforded glucose and rhamnose in aqueous phase and

Table 8: Inhibition zones (mm) and Minimum Inhibitory concentrations (µg/ml) of different extracts against mostly encountered microorganisms.

Microorganisms	C*eftriaxon* Inhibition zones	CMSE Inhibition zones	MIC	CMFE Inhibition zones	MIC	CMLE Inhibition zones	MIC	CMTS Inhibition zones	MIC
S. aureus	7	12	75	14	37.5	9	500	8	300
P. aeruginosa	9	8	150	11	75	8	500	13	150
K. pneumonia	7	7	-	10	-	8	-	12	-
E. coli	8	10	75	8	75	11	500	11	300

CMSE: *C. madurensis* stems extracts, CMFE: *C. madurensis* flowers extracts, CMLS: *C. madurensis* leaves extracts, CMTS: *C. madurensis* total saponins.

sophradiol in organic phase (Co-PC, with authentic sample). The resonances due to eight SP^3 methyl carbon were observed at δ 32.5 (C-29), 28.4(C-28), 27.7 (C-23), 25.2(C-27), 20.5 (C-30),16.8 (C-26), 16.2 (C-25) and 15.4(C-24) and two SP^2 carbons at δ 121.6 and 144.2 in ^{13}C NMR of **4** coupled with the corresponding information from ^1H NMR [8 tertiary methyl proton singals], methine protons linked to oxygen bearing carbon at δ 5.16 (H-22) and a broad singlet vinyl proton at 5.51of H-12 confirmative the aglycone moiety as 3, 22-dihydroxy-olean-12-en skeleton (sophradiol). All assigned ^1H and ^{13}C-resonance of aglycone moiety were confirmed by comparison with **3** and previously published data[41]. All assigned ^1H and ^{13}C- resonance of the aglycone moiety were also confirmed by HSQC and HMBC correlation spectroscopy. Glycosidation at C-3 was concluded from the deshielded shift of C-3 to δ 89.1 signal (~ +Δ8 ppm) and shielded effect of both C-2 and C-1 relative to those of unsubstituted moiety[46]. Three anomeric proton signals were assigned at 5.01 (br s, α-rhamnose), 4.81 (d, *J* = 7.5Hz, ß-glucose) and 4.74 (d, *J* = 7.5Hz, ß- inner glucose) in ^1H NMR spectrum through direct one bond coupling in HSQC with their own anomeric carbon signals at 103.9, 99.9 and 99.7 respectively, (Table 1). The sugars moieties were deduced to adopt α-L-$^1C_4$ and ß-D-$^4C_1$-pyranosyl stereostructure for rhamnose and glucose moities respectively; on the basis of *J* and δ values of their ^1H and ^{13}C-resonance (Table 1).

The interglycosidic and sugars-aglycone linkages were deduced from the long range three bond HMBC correlations. The HMBC exhibited correlation between H-1′ (δ 4.74) inner glucosyl and C-3 (δ 89.1) aglycone, H-1″ (δ 4.81) glucosyl and C-6′(δ 68.0) inner glucosyl and H-1‴ [(δ 5.01)- rhamnosyl and C-4″(δ 77.7) glucosyl to establish triglycoside moiety at C-3 of aglycone as 3-*O*- α-L rhamnopyranosyl -(1‴→4″)-*O*-ß-D-glucopranosyl (1″ →6′)-*O*-ß-D-glucopyranoside. All ^1H and ^{13}C resonaces assigned by the aid of HMBC and HSQC correlation peaks and by previously related published data[46].

Hence compound **4** identified as Sophradiol 3-*O*- α-L-$^1C_4$-rhamnopyranosyl (1‴→4″)-*O*-ß-D-$^4C_1$ glucopranosyl-(1″→6′)-*O*-ß-D-$^4C_1$-glucopyranoside.

Determination LD_{50} of each aqueous alcoholic extracts and total saponins; no mortality was observed up to seven days of monitoring. It was found that each of investigated extracts were non toxic up to 4g/Kg b.wt., which was the maximum soluble dose.

Antischistosmal activity

animals treated with aqueous alcoholic extract, total saponins at dose (200 mg/kg for 7 days) reduced total number of worms (34.18%) which were higher than **8** (4.49%). This reduction was significantly different from infected control group in comparison to group treated with praziquantel **(**antischistosmal reference drug) (Table 2). Also significant changes were observed in worm hepatic shift tissue egg load either in liver or intestine, and percent egg developmental stages (Table 3, 4).

Acetic acid-induced writhing in mice

on treatment with compound **3** (400 mg/kg) in case of, acetic acid induce writhing (contraction of abdominal muscle together with a stretching of the hind limbs) in mice was high significantly reduced (86.34%) more than aspirin (76.29%) and compound **1** (42.78%) (Table5). On treatment with compounds **1** & **3** at dose 400 mg/kg in case of thermal test**,** a significant prolongation in reaction time to thermal stimulus after administration in comparison to reference drug (Table 6).

Tail immersion test

isolated compounds **1& 3** have a significant prolongation in time of tail withdrawal after 0, 60, 120 min. comparing to control (Table 6).

Recent work high lights the potentional health promoting properties of flavonoids because of their broad pharmacological effects such as vasoprotective, anti-inflammatory…..etc. Many of these effects are due to their antioxidant properties [scavenge free radicals] and to the synergistic effects with other antioxidants[47].

Total alcoholic extract, compounds **8** and **10** showed marked activity compared to reference drugs but the most effective is compound **8** (Table 7) in terms of their antioxidant activity. The % DPPH radical scavenging power of alcoholic extract and tested compounds 8, 10 was represented in (Table 7) followed this order green tea › ascorbic › compound **8** › alcoholic extract › compound **10**. The IC_{50} value for alcoholic, compounds **8** and **10** were found to be 47.06±0.63, 29.88 ± 0.23 and 50.53 ± 0.34 respectively in comparison with reference drugs [green tea and ascorbic acid]. ***Anti-oxidant activity*;** The antioxidant activity of them like other natural phenolic antioxidants is a consequence of the presence of phenolic moieties in the structures. The antioxidants activity of phenolic natural product is predominantly owing to their redox properties (the ability to act as reducing agent, hydrogen donors and singlet oxygen quenchers and to some extent could also be due to their metal chelation potential[47]. Antioxidant tests could be based on the measurement of free radical

scavenging potency (hydrogen-donating ability) (Table7). The radical scavenging donate hydrogen to free radicals, leading to non toxic species. The use of DPPH radical provides an easy, rapid and convenient method to evaluate the antioxidants and radical scavengers[48]. Therefore, in this study, the selected alcoholic extract of *Crotalaria madurensis*, compounds **8** and **10** which are isolated from this species of *Crotalaria,* were investigated for their possible antioxidant and radical scavenging activity by DPPH assay and their IC_{50} values were calculated for further comparisons.

Alcoholic extracts [CMLE (leaves), CMSE (stems), CMFE (flowers) and CMSE (total saponins fraction) of different organs of *C. madurensis*, & respectively)] were found to be active on tested organisms. Two gram negative bacteria (*K. pneumonia & E. coli*) *and* two gram –positive bacteria (*S. aureus & P. aeruginosa*), were observed to be sensitive to the tested extracts in comparison to reference drugs Ceftriaxon (broad spectrum antibiotic), showed inhibitory activity by observation of inhibition zones which were recorded in mm. (Table 8) **.** Two fold micro dilution broth method used in studying the influence of concentration of different extracts of *C. madurensis* on the antimicrobial activities against bacterial strains. The MIC data of different extracts were presented in (Table 8). The results showed both CMSE and CMFE higher antimicrobial activity against *S. aureus, P. aeruginosa* and *E. coli* than CMLE and CMTS. The difference in antibacterial activity of the extracts may be due to the variation in the composition of extracts, structure of bioactive constituents, their interactions with bacterial cell wall components[49,50].

CONCLUSION

These results lend support the usage of *C. madurensis* by local tribal population in using for wounds and skin diseases against bacteria. Significant all biological activities may be linked to its higher phenolic contents and/or constituents of different organs. However, further studies are required to know the mechanism of action of these compounds using suitable animal models to confirm this attribution.

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