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Phytochemical investigation and histopathological evaluation of antiulcerogenic activity of *Cassia roxburghii* DC. leaves in rats

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ABSTRACT

The petroleum ether, chloroform, and 70% ethanol extracts from leaves of Cassia roxburghii DC., Were investigated for their antiulcerogenic activity using indomethacin as non steroidal – anti-inflammatory drug induced –ulcer in animal model at a dose 300mg/kg. The histopathological examination of gastric mucosa and duodenum showed potent antiulcerogenic effect for the petroleum ether extract followed by ethanol extract. In contrast the chloroform extract showed no gastroduodenal protection. In order to determine the biologically active compounds, phytochemical study of the bioactive petroleum ether extract was achieved by column chromatography, which allowed isolation of betulin, emodin and aloemodin. Structures of the isolated compounds were elucidated by spectroscopic analysis. GC/Mass of fatty acids and unsaponifiable matters lead to the identification of thirteen fatty acids, the unsaturated fatty acids represent (47%) of the total fatty acids. Sterols represent 16% of the unsaponifiable mater. The monounsaturated diterpene alcohol phytol was the most dominant (40%).

Keywords: *Cassia roxburghii* DC., antiulcerogenic, anthraquinons, phytosterols.

INTRODUCTION

Cassia species, represent a large genus of the Fabaceae (Leguminoseae) trees, they belong to subfamily Caesalpinioideae.The species are well known for their high medicinal activities [1-3] *C. fistula* pods were reported to have very low level of toxicity (50% lethal dose (LD_{50}) = 6600 mg/kg b.w) and no pathological effects were seen on liver, kidney and rat's testis [4]. Whereas the LD_{50} of the methanol extract of leaves was found to be 3500 mg/Kg b.w. [3]. It was found that different extracts of leaves of *C. auriculata* were safe up to a maximum oral dose of 2000 mg/kg b.w. There were no changes in normal behavioral pattern and no signs and symptoms of toxicity and mortality were observed [5].

Cassia roxburghii DC. (syn: *C. marginata* Roxb.) known as Red Shower tree is a beautiful tree with bright pink flowers with shades of orange and red. The name refers to the thickened margins of the leaves. Red cassia is native to Sri Lanka and southern India. *C. Marginata* is rich source of flavonoids and anthraquinones [6-12]. The seeds are rich in oil [13]**.** The seed protein of *Cassia marginata* was reported to exhibit a marked lowering effect on blood and liver cholesterol levels [14]. Besides, the antimicrobial activity and hepatoprotective effect of *C.roxburghii* seed extracts have been reported [15].The reducing power , H_2O_2 scavenging assay and hepatoprotective activity of *Cassia roxburghii* leaf were evaluated [16].

Peptic ulcer is a chronic disease which impairs the quality of life and is associated with increased morbidity and mortality. Peptic ulcer disease is a world wide problem. Statistics from all sources indicate 10% or more of adult population are affected within their life time and 50% of healthy individuals complain of dyspepsia [17]. Peptic

ulcer affects individuals from 20 to 60 years of age with males being predominantly affected. The incidence of duodenal ulcer is more frequent than gastriculcer (ratio 4:1). Although there are many products used for the treatment of gastric ulcers, including antacids and antihistaminics, most of these drugs produce several adverse reactions, such as arrhythmias, impotence, gynacomastia and hematopoeitic changes. Thus there is a need for more effective and less toxic anti-ulcer agents. Plant extracts are some of the most attractive sources of new drugs, and has been shown to produce promising results for the treatment of gastric ulcers.[18-20]. Reviewing the literature for antiulcerogenic activity of Cassia species , studies were carried for extracts from *Cassia auriculata , Cassia singueana* leaves at a dose 250mg/Kg and 750 mg/kg against ethanol induced mucosal damage in rats. [5, 21]. The authors recommended further studies to determine the active principles, that was considered with interest in this study. Therefore, this work deal with the investigation of the antiulcerogenic activity of different extracts from leaves of *Cassia roxburghii* DC., determination of the promising fraction and identification of the bioactive constituents.

EXPERIMENTAL SECTION

General experimental procedures

Mass spectrometric analyses were performed on a Finnigan MAT 112, electron impact ionization at 70 eV.^{1}H- and ^{13}C-NMR spectra were done on a Varian Mercury VX 300 NMR spectrometer at 300 MHz and 75.46 MHz, respectively, using CDCl3 as solvents. The chemical shifts were reported in ppm values using CDCl3 as the internal standard.

Condition of GC/MS analysis of Fatty acids methyl ester

Gas chromatography mass spectrophotometer HP6890Series (Agilent). Capillary column TR-5 MS [(5% phenyl) Polysil Phenylene siloxane], thermo scientific. 30 m length, 0.25 mm ID and 0.25µm thickness was used, temperature programming; 140^{o}C, increase temperature from 50 – 200^{o}C at a rate of 5^{o}C /min, maintained at 200^{o}C for 3 min, injector temperature 200^{o}C, flow rate1ml /min, duration 34 min. Detector MSD.

Condition of GC/MS analysis unsaponnifiable matter

The instrument used was GC / Mass Finnigan mat SSQ 7000 GC system. Capillary column DB-5 Fused silica [(5% phenyl) methyl polysiloxane], 30 m length, 0.25 mm ID and 0.25µm thickness was used, temperature programming; 150^{o}C for 3min, increase temperature from 50 – 300^{o}C at a rate of 3^{o}C /min, maintained at 300^{o}C for 5 min, injector mode, splitless detector MS flame ionization EI, EV 270, injector temperature 250^{o}C, flow rate 1ml/min, duration 53min.

Plant material and phytochemical analysis

Plant material

Cassia roxburghii DC., family *Fabaceae* leaves were collected in December 2006 from the Orman garden, Giza, Egypt. The plant was identified by Mrs. Terase Labib, Taxonomist of Orman

Preparation of extracts

The air dried powdered leaves (250g) were, extracted successively with petroleum ether (60-80) (SDFCL, India), CP, chloroform (RFCL, India), CC and 80% methanol (Fisher Scientific, UK), CE at room temperature. Each extract was evaporated separately under vacuum to yield [17, 7and 50g] respectively. From each extract an oral dose of 300mg /kg b.w. was prepared

Botanical Garden. A voucher specimen C-5-63 was kept in Herbarium of Orman garden.

Saponification of the petroleum ether fraction

The petroleum ether fraction (1g) was saponified according to method reported by [22] to separate the unsaponifiable matter from fatty acids followed by methylation of fatty acids.The unsaponifiable matter (0.75g) and the fatty acids (0.1g) were analyzed by Gas/Mass adopting the previously mentioned conditions. Peak assignments were carried out by comparing the mass fragmentation patterns and relative retention times with those of reference compounds, literature sources, and the Wiley MS database of the equipment.

TLC examination of the petroleum ether extract

TLC Examination of the petroleum ether fraction on pre-coated silica gel plates 60 F 254 (Merck) .the plate were developed with toleuene: ethyl acetate [8:2] showed two yellow spots, the fraction gave positive born trager"s test.

Column chromatography of the petroleum ether extract

Petroleum ether extract (10g) was chromatographed on silica gel column (Merck 200 mesh), eluted with petroleum ether (60-80) with gradient increase of polarity with ethyl acetate (0.5, 1, 2, 3 and 5%). Fractions each of 200 ml were collected, examined by TLC under UV(240-360nm), and sprayed with vanillin sulfuric acid, similar fractions were poured together, fraction 1 eluted with petroleum ether gave waxy hydrocarbons, fractions from 2-8 eluted with 0.5 % ethyl acetate contained fatty acids, fractions from 9--18 eluted with 1 % ethyl acetate contained mixtures of steroidal and triterpenoidal compounds, fractions 14,15 showed major triterpenoidal compound ,which was isolated by preparative thin layer chromatography using toulene ethyl acetate (9:1) as developing system. fractions from 20-22 eluted with 2 % ethyl acetate offered two compounds: i) as white needle from ethanol. ii) orange powder from methanol. Fractions 29, 30 eluted with 3% ethyl acetate gave compound iii) as orange needle from ethanol.

2.3. Antiulcer bioassay

2.3.1. Animals

Adult male albino rats weighing 180-200 g purchased from Rats were obtained from the Laboratory Animal Colonies, National Research Centre, Cairo, Egypt. They were acclimatized to the new laboratory conditions before using for experimentation for a period of 15 days and kept in quite room at temperature of 22-2^{o}C, relative humidity (70 ± 4%), and a 12 h light/dark cycle. Rats were provided with food and water *ad libitum*.Animals studies were carried out in accordance with the institutional guidelines and adhered to internationally accepted principles for laboratory animal use and care as found in European Community Guidelines (EEC,Strasbourg ,18III.1986,test amended according to provision o the protocol , ETS N^{o} 170 in 2005).

2.3.2. Animal groups for Antiulcer Assay

Total of 80 rats were divided into 10 groups of eight animals. Group I was kept as control without any treatment and all other groups were fasted for 24 h and administered 80% ethanol. Group II, received 60 mg/kg, orally of indomethacin alone. Groups III, IV, V and VI were treated with *Cassia* (300 mg/kg from extract CP.CC.CE) and ranitidine (50 mg/kg), respectively, 1 h prior to the administration of 60 mg/kg, orally of indomethacin by gastric instillation. The animals were sacrificed under ether anesthesia 1 h after ethanol administration. The stomach and duodenum were removed and fixed for histological examination.

2.3.3. Histological studies

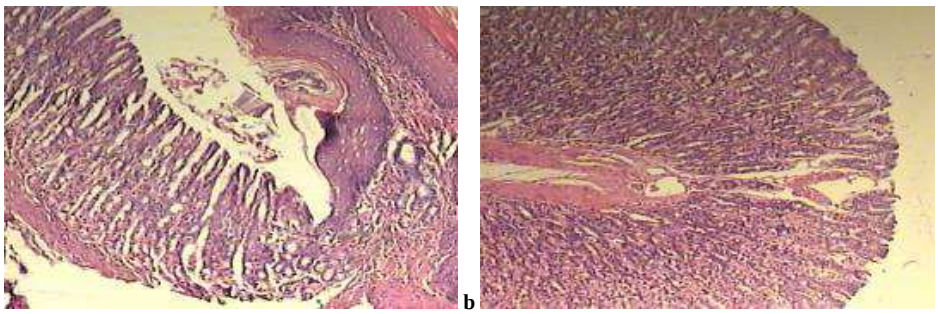
After tissues were immersed for 48 h at 4 ◦C in the fixative solution (4% formaldehyde, in phosphate buffer, pH 7.6). Paraffin sections, 5µm thick, were made and stained with hematoxylin–eosin solutions (H&E). Tissue preparations were observed and micro-photographed under a light Olympus microscope.

RESULTS AND DISCUSSION

3.1. Histopathological examination

Stomach

The normal gastric mucosa showed intact surface epithelium with underlying uniform rounded gastric glands lined by secretory cells. The lamina propria is composed of loose connective tissue with lymphoid cells and blood vessels. The muscularis mucosa is composed of inner circular and outer longitudinal layers of smooth muscles (Figure 1-a). Microscopic examination of stomach of rat given indomethacin showed deep narrow ulcers with vascular congestion and areas of surface erosions in the gastric mucosa (Figure 1-b). In some rats deep ulcer next to an area of surface erosion was seen (Figure 1-c). Histopathological of the stomach of rat treated with petroleum extract of cassia and indomethacin showed the gastric mucosa of intact surface epithelium, the thickness of the mucosa is within normal with uniform gastric glands (Figure 1-d).



a **b**

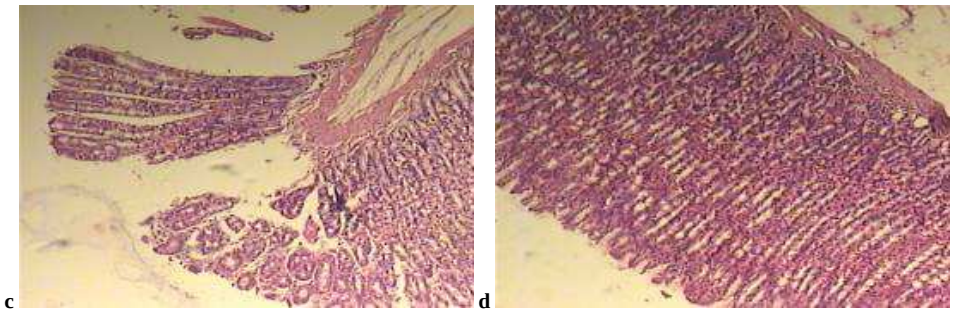


Figure 1: Sections of stomach of (a): control the junction between the esophageal mucosa (squamous epithelium) and the normal gastric mucosa, (b) (Indo) rat treated with indomethacin showing deep narrow ulcer with vascular congestion, (c) rat treated with indomethacin showing deep ulcer next to an area of surface erosion, (d) rat treated with petroleum ether extract of cassia and indomethacin showing intact surface epithelium, the thickness of the mucosa is within normal (H&E, X 100)

Examination of rat stomachs given chloroform extract of cassia and indomethacin showed multiple deep ulcers up to the muscularis mucosa with cell debris and mucous (Figure 2-a). In some rats infiltration of the lamina propria by mixed inflammatory cells and congested blood vessels which indicate sever irritation (Figure 2-b). Histopathological investigation of rat stomachs treated with the ethanolic extract of cassia and indomethacin showed superficial erosions and infiltration of the lamina propcria by mixed inflammatory cells (Figure 2-c). Examination of rat stomachs treated with ranitidine and indomethacin showed intact surface epithelium with blood vessel congestion and few inflammatory cells in the lamina propria. The gastric glands were within normal (Figure 2-d).

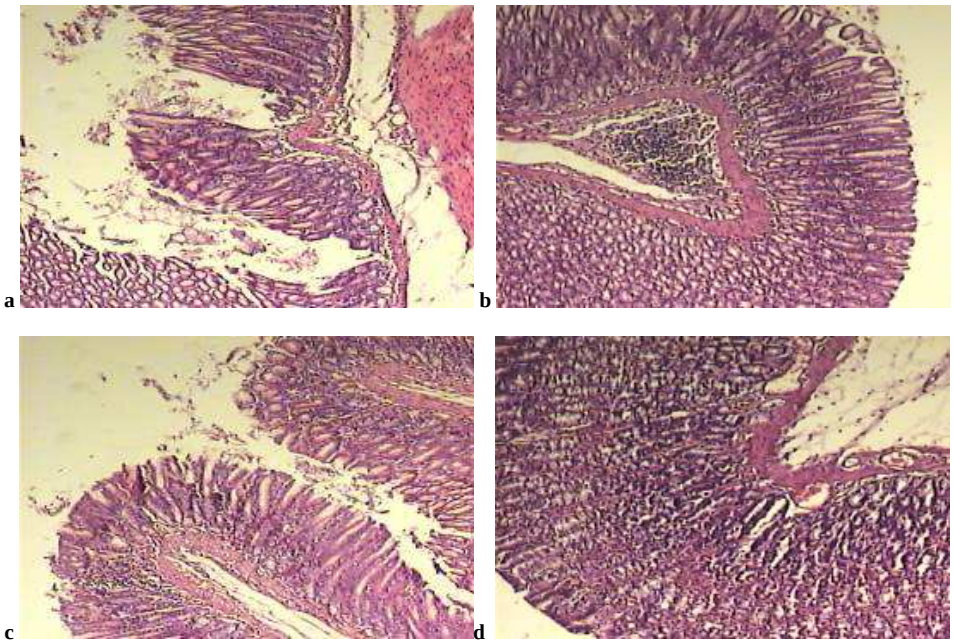


Figure 2: Sections of stomach of (a) rat treated with chloroform extract of cassia and indomethacin showing two deep ulcers up to the muscularis mucosa with cell debris and mucous, (b) rat treated with chloroform extract of cassia and indomethacin showing infiltration by mixed inflammatory cells, (c) rat treated with the ethanolic extract of Cassia and indomethacin showing superficial erosion and infiltration by mixed inflammatory cells, (d) rat treated with ranitidine and indomethacin showing intact surface epithelium but there is blood vessel congestion (H&E, X 100)

Small intestine

The normal intestinal mucosa showed tall (finger like) villi covered with absorptive cells, goblet cells and few endocrine cells. The connective tissue core of the villi contains blood and lymph vessels (Figure 3-a). Microscopic examination of the intestinal mucosa of rats treated with indomethacin showed short broad villi with increased number of goblet cells and infiltration of its cores by mixed inflammatory cells. Other areas showed sever atrophy of the villi and the glands with no goblet cells. The covering epithelium of the villi is totally sloughed in some areas (Figure 3-b). Histopatholgical investigation of the intestinal mucosa of rats treated with the petroleum extract of Cassia and indomethacin showed increased infiltration of the villous cores with mixed inflammatory cells, the covering epithelium showed increased number of goblet cells which indicate some irritation (Figure 3-c). Examination of then intestinal mucosa of rats treated with the chloroform extract of Cassia and indomethacin showed showing short, broad villi with infiltration of its cores by mixed inflammatory cells. There was an increase

in the number of goblet cells (Figure 3-d). Microscopic investigation of the small intestinal mucosa of rats treated with the ethanolic extract of Cassia and indomethacin showed short, broad villi with dilated lymph vessels and a decrease in the number of goblet cells which indicate moderate irritation (Figure 3-e). Examination of the small intestinal mucosa of rats treated with ranitidine and indomethacin showed tall (finger like) villi with normal number of the goblet cells (Figure 3-f).

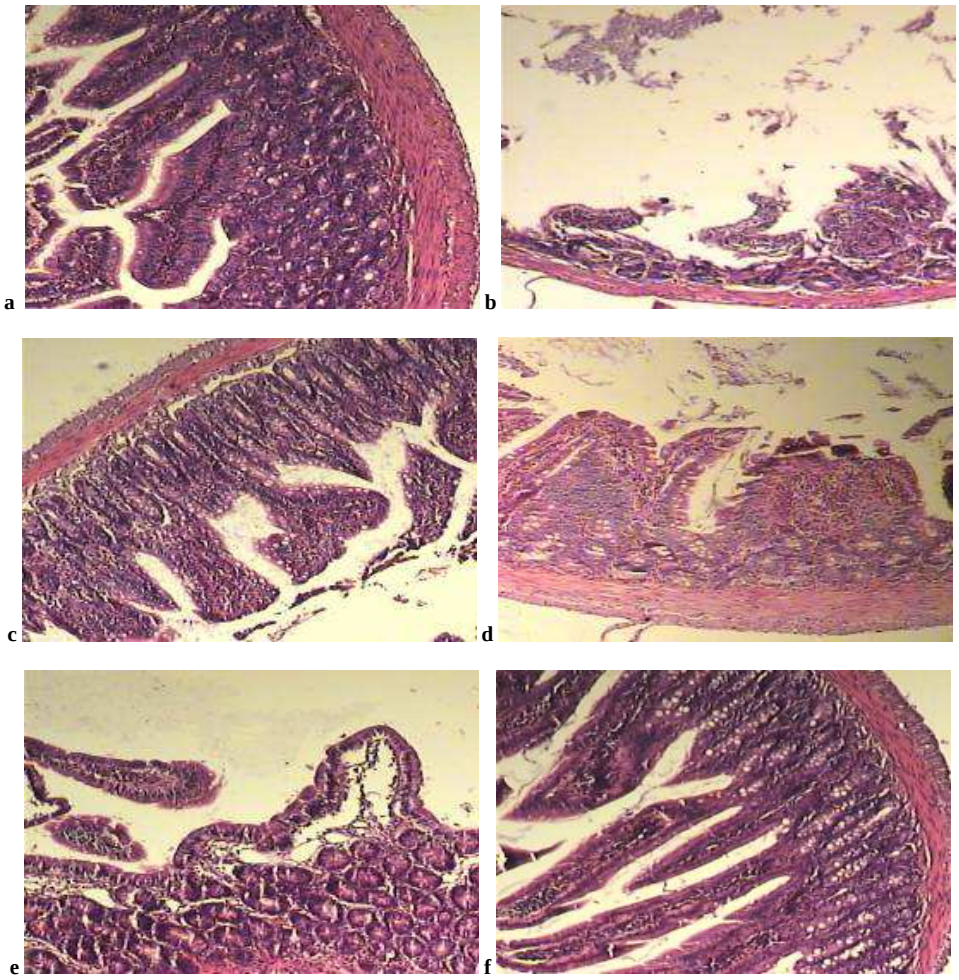


Figure 3: Sections of small intestine of (a) control rat shows tall intestinal mucosa (finger like) villi covered with absorptive cells, goblet cells and few endocrine cells, (b) rat treated with indomethacin showing sever atrophy of both the villi and the glands. The covering epithelium of the villi is totally sloughed. The intestinal glands are atrophic with no goblet cells, (c) rat treated with petroleum Intestinal villi showing increased infiltration of its cores with mixed inflammatory cells, the covering epithelium snowing increased number of goblet cells, (d) rats treated with the chloroform extract of Cassia and indomethacin showing short, broad villi with infiltration of its cores by mixed inflammatory cells. There is increased number of goblet cells, (e) rat treated with the ethanolic extract of Cassia and indomethacin showing short, broad villi with dilated lymph vessels and decreased number of goblet cells, (f) rat treated with ranitidine and indomethacin showing tall (finger like) villi, the number of the goblet cells is within normal (H&E, X 100).

Table 1: GC/Mass analysis of Fatty acid methyl esters

Fatty acid methyl esters	Rt[a]	M^+ m/z	Base peak	Relative percent
Decanedioic acid dimethyl ester.	0.62	230	199,125,74,55.	0.31
Tetradecanoic acid methyl ester.	0.70	242	199,143,87,74	0.04
Undecanedioic acid dimethyl ester.	0.77	244	213,139,98,55	0.87
9-Dodecenoic acid methyl ester.	0.84	212	138,96,74	1.28.
Tetradecanoic acid 9-methyl-methyl ester.	0.86	256	213,143,74	1.15
9-hexadecenoic acid methyl ester.	0.98	268	236,194,152	1.89
Hexadecanoic acid methyl ester.	1	270	227,143,74,43	31.47
12-oxo-octadecanoic acid methyl ester.	1.09	312	281,285,98,55	2.30
Heptadecadienoic acid methyl ester	1.15	280	123,109,81,67	0.59
Heptadecanoic acid methyl ester.	1.16	284	253,241,143,74	0.80
8,11-octadecadienoic acid methyl ester.	1.27	294	263,95,81,67,41	29.67
9,12,15-octadecatrienoic acid methyl ester.	1.29	292	108,79,67,55,41	21.28
Octadecanoic acid methyl ester.	1.33	298	255,199,143, 74	8.3

a Rt= relative retention time

Phytochemical results

The active ingredients present in the Petroleum ether extract can contribute for the gastroprotective action of *C. roxburghii* DC. The GC /Mass analysis of the fatty acids allow identification of thirteen major components**.** The unsaturated fatty acids represent (53 %) while saturated fatty acids represent (47%) of the total fatty acids. Also, oxygenated fatty acids represent (1%) (Table1).

Analysis of the unsaponifiable matter (unsap) (Table 2**)** revealed that the monounsaturated diterpene alcohol phytol as major components which represent 40%., the sterols account for 16 % of the total unsap. pregnane constitutes 6 % .

Table 2: GC/Mass analysis of unsaponifiable matter

Compound	Rt[a]	Base peak	M^+ M/z	Relative percent
Butyl hydroxyl Toleuene	0.62	205	205	0.45
6,10,14-trimethylpentadecane ($C_{18}H_{36}$)	0.84	56	252	2.07
Octadecene ($C_{18}H_{36}$)	0.85	57	252	0.57
6,10,14-trimethylpentadecanone($C_{18}H_{36}O$)	0.88	58	268	0.52
Phytol($C_{20}H_{40}O$)	1	71	296	40.06
Eicosane ($C_{21}H_{44}$)	1.10	57	282	0.48
5-hydroxy-dodecanoate -∂-lactone ($C_{12}H_{22}O_2$)	1.11	99	198	2.86
cholesterol ($C_{27}H_{46}O$)	1.13	57	386	3.53
pentacosane($C_{25}H_{52}$)	1.18	57	352	0.53
hexacosane ($C_{26}H_{54}$)	1.21	56	366	13.32
cyclohexylheneicosane($C_{27}H_{54}$)	1.24	82	378	0.65
heptacosane ($C_{27}H_{56}$)	1.26	57	380	0.57
brassicasterol ($C_{28}H_{46}O$)	1.31	57	398	1.34
squalene $C_{30}H_{50}$	1.32	55	410	1.54
nonacosane ($C_{29}H_{60}$)	1.35	57	408	9.35
cyclohexyldocosane($C_{28}H_{56}$)	1.38	82	392	1.09
5α - Pregnane-3,20-dione ($C_{21}H_{32}O_2$)	1.41	316.	316.41	6.21
Ergosterol diol ($C_{28}H_{46}O_3$)	1.43	57-97	430	0.53
Hentriacontane ($C_{31}H_{64}$)	1.44	57	436	5.62
Cyclohexyltricosane ($C_{29}H_{58}$)	1.46	82	406	0.59
stigma-5,22-dien-3-ol ($C_{29}H_{48}O$)	1.47	83	412	1.29
stigma-5-ene-3-ol ($C_{29}H_{50}O$)	1.50	414	414	2.44
sitosterol ($C_{29}H_{50}O$)	1.51	87	414	1.13

a Rt= relative retention time

Column chromatography allow isolation of betulin [23] and two anthraquinones compounds, identified as emodin (20mg) aloemodin (11mg) and acetyl aloeemodin (15 mg) by NMR, Mass and UV, the data match those previously reported [24,25].

Concerning the methanol extract the author repoted in a previous study the diglucosides of emodin , aloemodin , aloemodin glucoside and quercitrin as major components[12].

The steroidal compounds are known for their antioxidant and anti-inflammatory effect (26,27). Synthetic esters of phytol with fatty acids have been patented as antiulcerogenic and anti-inflammatory agents [28]. The antiulcer effect of the phytosterol fraction was reported [29,30].

The anti-inflammatory activity of anthraquinones were reported [31].The anti-inflammatory and antiulcerogenic effects of emodin were reported at a dose 15 mg/kg (Ip), it decreased acid and pepsin output and augmented mucus secretion in terms of total carbohydrate: protein ratio in the gastric juice of aspirin treated pylorus-ligated rats, indicating that the antiulcerogenic effect of emodin may be due to its effect on gastric secretion [32-34].

CONCLUSION

The present study reveal that petroleum ether extract (300 mg/kg) treated groups showed gastroduodenal protection against Indomethacin induced ulcer similar to standard drug Ranitidine (50 mg/kg). The phytosterols and anthraquinons present in the petroleum extract were reported for their anti-inflammatory and antiulcer activity.The methanol extract (300 mg/kg) showed significant gastroduodenal protection against Indomethacin induced ulcer when compared to standard drug Ranitidine (50 mg/kg). But to elucidate the exact mechanism of this modulatory effect, and to examine its potential therapeutic effects further studies are essential.

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