Journal of Materials and Environmental Sciences
ISSN : 2028-2508
CODEN : JMESCN

J. Mater. Environ. Sci., 2018, Volume 9, Issue 10, Page 2768-2778

http://www.jmaterenvironsci.com

Phytochemical and Biological Studies on *Enterolobium contortisiliquum* (Vell.) Morong Pericarps

A.A. Matloub[1]*, R.S. Mohammed[1]*, S.S. El Souda[2], S.M. El-Hallouty[1], E.Z. Gomaa[3], A.A. Hassan[4]

[1]*Pharmacognosy Dept. National Research Centre, 33 El Bohouth St, Dokki, P.O.12622, Giza, Egypt.*
[2] *Chemistry of Natural Compounds Dept., National Research Centre, 33 El Bohouth St, Dokki, P.O.12622, Giza, Egypt.*
[3]*Department of Biological and Geological Sciences, Faculty of Education, Ain Shams University, Cairo, Egypt.* [4]*Department of Chemistry of Natural and Microbial Products, National Research Centre, 33 El Bohouth St, Dokki, Giza, Egypt*

Received 23 Dec 2017,
Revised 31 Mar 2018,
Accepted 04 Apr 2018

Keywords
- ✓ *Enterolobium contortisiliquum,*
- ✓ *triterpene saponin,*
- ✓ *phenolic compounds,*
- ✓ *cytotoxicity,*
- ✓ *antimicrobial*

Matlouba2015@gmail.com
Phone: +201001405293
reda_sayed2008@yahoo.com
Phone: +201227636237

Abstract

Phytochemical study of *Enterolobium contortisiliquum* led to identify α, β-Amyrin and 4-methyl-2,6-di-tert-butylphenol as well as hexadecanoic and 9,12-octadecadienoic acids as main components of unsaponifiable matter and fatty acids, respectively, by GC/MS analysis. Glucose, xylose and rhamnose were found as major polysaccharide hydrolysate. Sixteen amino acids have been identified in protein fraction (PF). One bisdesmosidic and two monodesmosidic triterpene saponins of acacic acid were identified by ESI-MS for the first time. Gallic, protocatechuic, syringic, *p*-coumaric acids, pyrogallol, quercetin-7-*O*-rutinoside, catechin, isovitexin and quercetin were isolated from phenolic fraction (PhF). Polysaccharide (PSF), saponin (SF), the crude extract (CE), and petroleum ether (PE) exhibited potent cytotoxic activity on HepG2 with IC_{50} 15.7, 22.8, 43.5 and 56.7 µg/ml whereas SF, CE, PhF, PE and PSF exhibited IC_{50} 12.3, 32.1, 34.6, 64.6 and 78.3 µg/ml against MCF7 cell line, respectively. The maximum antibacterial inhibition was observed with catechin and protocatechuic acid against *Pseudomonas aeruginosa* as well as PSF and PF against *Klebsiella pneumonia*.

1. Introduction

Enterolobium contortisiliquum (Vell.) Morong (Synonym: *Enterolobium timbouva* Mart). commonly known as the ear pod tree) is a tree belonging to Family Leguminosae (Subfamily Mimosoideae) and widely populated in Egypt. *E. contortisiliquum* is a herbal medicine traditionally used to treat parasitism and gonorrhea in Brazil [1]. The phytochemistry of *E. contortisiquum* pods revealed the isolation of bisdesmosidic triterpene saponins contortisiliosides A-G [2], among them contortisiliosides A and C showed *in vitro* moderate cytotoxic activity against BAC1.2F5 mouse macrophages and EL-4 mouse lymphoma cells. The essential oil of *E. contortisiliquum* seeds had been reported to have an antimicrobial activity [3]. In the present study, phytochemical investigations as well as the cytotoxic and antimicrobial activities of the crude extract (80% alcohol), fractions, and the isolated compounds were carried out for the first time on the dried pericarps of *E. contortisiliquum*.

2. Material and Methods

2.1. Experimental

GC/MS analysis of the lipoidal contents was adopted using a Thermo Scientific (Waltham, MA), Trace GC Ultra & ISQ Single Quadrupole MS, TG-5MS fused silica capillary column (30 m x 0.25mm x 0.1mm film thickness). For GC/MS detection, an electron ionization system with ionization energy of 70 eV was used. The inert gas helium was used as a carrier gas, at a flow rate of 1 ml/min. The injector and MS transfer line temperature were set at 280 ºC. Temperature was programmed for unsaponifiable matter (USM) analysis at an initial temperature 50 ºC (2 min), 50–150 ºC at a rate of 7 ºC/min, 150–270 ºC at a rate 5 ºC/min, 270–310 ºC as a final temperature at an increasing rate of 3.5 ºC/min. While temperature programmed of fatty acid methyl esters (FAME) was 150–280 ºC at a rate of 5 ºC/min. The amino acid contents were determined by using an LC 3000 amino acid analyzer (Eppendorf-Biotronik, Maintal, Germany). Relative molecular weight of isolated protein was estimated using Mini-gel electrophoresis (BioRad, USA). The crude saponin was determined using

an API 4000 Q trap mass spectrometer (Applied Biosystems/MDS Sciex, Concord, ON, Canada) via a Turbo Ion Spray ionization interface, and performed in positive mode. The full-scan mass covered the range from *m/z* 100-1500. The instrument was controlled and data acquisition was carried out using analyst software (version1.5.1) from Applied Biosystems Sciex. Polyamide S6 (Riedel-De-Haen AG, Seelze Haen AG, D-30926 Seelze Hanver, Germany) was used for column chromatography of phenolic compounds, purification of compounds was performed in Sephadex LH-20 (Pharmazia Sweden). Ultraviolet and visible absorption spectrometer (UV–VIS, 200–500 nm, Labomed Inc.) was for measuring UV absorbance of the isolated compounds in methanol and with different diagnostic shift reagents. NMR measurements were carried out using Jeol EX-500 spectroscopy; 500 MHz (^{1}H NMR) and 125 MHz (^{13}C NMR) using DMSO-d_6 as solvent. The aglycone and sugar moieties were identified by complete acid hydrolysis for *O*-glycosides followed by co-chromatography with authentic samples (Sigma) using solvent system (4). The sugar unit of compound 8 was determined using ferric chloride degradation followed by identification of sugar in system 4.
Materials for biological activities, cytotoxic activity; MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide); antimicrobial study the following strains were used: *Bacillus subtilis* (NCTC-10400), *Bacillus cereus (*ATCC- 9634*), Staphylococcus aureus* (ATCC-29737), *Staphylococcus epidermidis* (ATCC-12228), *Streptococcus mutans* (ATCC-25175), *Streptococcus pneumoniae* (ATCC-6303), *Micrococcus luteus* (ATCC-9341), *Klebsiella pneumonia* (ATCC-10031), *Proteus vulgaris (*ATCC-13315), *Salmonella Typhi* (NCIMB-9331), *Escherichia coli* (NCTC-10418), *Pseudomonas aeruginosa* (ATCC-10145) and *Bordetella pertussis* (ATCC-9797) were obtained from Fermentation Biotechnology and Applied Microbiology (FERM-BAM) Centre, Al-Azhar University, Cairo, Egypt. While the antifungal activity of the samples were evaluated against the yeast *Candida albicans* NRRL YB-242 using nutrient agar media, and fungi *Aspergillus flavus* NRRL 3357, *Aspergillus niger* 200, *Aspergillus ochraceus* NRRL 3174, and *Fusarium solani* NRRL 22661, they were provided from the Center of Culture Collection of the National Research Centre, Dokki, Cairo, Egypt. Standard antibacterial (Doxycycline HCl, 30 µg/ml) and standard antifungal (Flucanazole, 50 µg/ml, Pfizer) were simultaneously used as positive controls. Potato-dextrose-agar (PDA) and Luria-Bertani (LB) medium were used as culture media for inoculation bacteria and fungi, respectively.

2.2. Plant
Dried pods of *Enterolobium contortisiliquum* (Vell.) Morong were collected from the tree in March 2014 from Orman Garden, Giza, Egypt. The pods were kindly authenticated by Madam Tressa Labib Professional Taxonomist, specialist in identification of plants, to whom the author is deeply indebted. A voucher specimen (No. 102) was kept in the Herbarium of NRC Cairo, Egypt. The seeds were manually separated from pericarps and the pericarps were milled.

2.3. Phytochemical screening
The preliminary phytochemical screening tests (color reaction) were conducted on *E. contortisiliquum* pericarps according to standard methods [4].

2.4. Preparation of the extracts and fractions
2.4.1. Crude extract (CE)
The dried powdered pericarps of *E. contortisiliquum* was macerated with 80% ethanol (24 h) for extraction. The extract was filtered and dried in vacuum at 45°C in a rotary evaporator and stored at 4°C until further use.

2.4.2. Isolation of lipoidal matter
Dried powdered of *E. contortisiliquum* pericarps was defatted with petroleum ether (40-60°C) using Soxhlet, filtered and evaporated to dryness under vacuum. The unsaponifiable matter and fatty acids were prepared from petroleum ether residue (PE) as described in Hassan et al., (2015) [5]. The USM and FAME were subjected to gas chromatography/mass spectrometry (GC/MS) analysis. The identification of the compounds was accomplished by comparing their retention times and mass spectral data with those of the library (Wiley Int. USA) and NIST (Nat. Inst. St. Technol., USA) and / or published data [6].

2.4.3. Polysaccharide fraction (PSF)
The defatted powdered of *E. contortisiliquum* pericarps was soaked in 30 volume (w/v) of distilled water and kept overnight at 4 °C, stirred well and allowed to return to room temperature. The slurry was first filtered through muslin cloth and then with Whatman no.1 filter paper (particle retention 11µm). Extract was concentrated under reduced pressure, and precipitated by the addition of 4-fold volume of 95% (v/v) ethanol (Doummar & Sons Co., Syria), centrifuged at 3000 rpm for ten min. The precipitate was washed twice with acetone, diethyl ether and absolute ethanol then freeze dried (Virtis, Gardiner, USA), to obtain a crude

polysaccharide. The polysaccharide extract (1gm) was hydrolyzed with 4N hydrochloric acid and hydrolysate was analysed by GLC analyses (HP 6890, USA) as mentioned in Matloub et al.,(2013) [7], using arabinose, xylose, ribose, rhamnose, glucose, galactose, fructose, glucuronic acid, galacturonic acid, manitol and sorbitol (Sigma) as reference sugars.

2.4.4. Protein fraction (PF)

The water soluble protein was prepared from the defatted powder as mentioned in Aly *et al*., (2016) [8] using trichloroacetic acid (TCA) for induce precipitation protein. The amino acid composition of isolated protein was determined as described by [7]. Relative molecular weight of isolated protein was estimated as described by Darwesh et al., (2015) [9] using protein markers (Mwt 11-245 kDa; Sigma, USA).

2.4.5. Saponin fraction (SF)

The defatted powder was extracted with methanol using Soxhlet, filtered and the extract was dried under vacuum. The dried methanol extract was dissolved in water then subjected to liquid/ liquid fractionation using water/ n-butanol. The n-butanol fraction was dried and dissolved in methanol, and then diethyl ether was gradually added till complete precipitation of saponin. The precipitate was separated by centrifuge (saponin fraction SF) washed several time with diethyl ether and dried in vacuum desiccators. For qualitative identification saponin composition, the SF was subjected to ESI/MS.

2.5. Isolation and identification of phenolic compounds

The supernatant containing phenolic compounds was dried under vacuum and subjected to chromatoghraphic isolation using polyamide S6 column. PhF (10 gm) was dissolved in mixture of methanol: water (1:3) and subjected to reversed phase chromatography polyamide S6 column using water (W) and methanol (M) as eluent. Fractions of 500 ml each were collected inspected on Whatman No.1 descending paper chromatography (PC) using different solvent systems 6% HOAc (1), 15% HOAc (H_2O: HOAc 85:15) (2), 30% HOAc (3), and BAW (n-BuOH: HOAc: H_2O 4:1:5, upper layer) (4). Four main fractions (A –D) were obtained by combining similar fractions according to their PC properties. Fraction A eluted with 10% M/W and subjected to PC (3MM) using system 1 led to the isolation of compounds gallic acid (1), protocatechuic (2), pyrogallol (3), syringic (4) and p-coumaric acid (5). Fraction B eluted with 20% M/W and subjected to PC fractionation using system 2 give compounds querecetin-7-*O*- rutenoside(6), catechin (7), which was purified on a Sephadex LH-20 column using H_2O/MeOH (7:3) as eluent. Fraction C eluted with 40% methanol/water was subjected to PC fractionation using system 2 which was further chromatoghraphed on Sephadex LH-20 column using M/W (10%) afforded compound isovitexin (8). Fraction D eluted with 60% M/W was subjected to Sephadex LH-20 using M/W (1:1) to yield compound quercetin (9).

2.6. Biological Activity

2.6.1. Cytotoxic evaluation

Cytotoxic effect of the CE, PE, PSF, PF, SF, PhF and compounds 2, 6 & 7 isolated from *E. contortisiliquum* pericarps as well as doxorubicin (reference drug) was accomplished on HepG2 and MCF7 human cancer cell lines. Cell viability was assessed by the mitochondrial dependent reduction of yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to purple formazan. Data were subjected to paired-samples SPSS Statistical Software Package (version 8.0). $P<0.005$ was regarded as significant. Also, a probit analysis was carried for IC_{50} and IC_{90} determination.

2.6.2. The antimicrobial activity

The antimicrobial activity of CE, PE, PSF, PF, and PhF fractions as well as isolated compounds 2, 6 & 7 was determined against several bacterial (Gram-positive and gram- negative bacterial strains) and fungal pathogenic strains using the agar well diffusion assay method as described in Balouiri *et al*., (2016) [10]. Bacterial and fungal strains were inoculated into Luria-Bertani (LB) broth and potato dextrose agar (PDA), respectively. Wells of 6 mm diameter (antibacterial activity) and 12 mm diameter (antifungal activity) was punched over the agar plates using a sterile cork borer and filled with the samples at concentration 1mg/ml. The plates were incubated at 37 ^{o}C for 24h for bacterial growth and at 30 ^{o}C for 48 h for fungal growth. The antibacterial activity was estimated by measuring the diameter of the clear zone of growth inhibition in mm.

3. Results and discussion

3.1. Phytochemical study

Phytochemical screening of *E. contortisiliquum* pericarps revealed the presence of various classes of biologically active compounds (saponins, triterpenes and/ or sterols, fatty acids, carbohydrates, proteins and

phenolic compounds). The yields of crude, successive extracts and fractions were presented in Table 1. Saponification of petroleum ether extract (PEE) of *E. contortisiliquum* pericarps afforded 74.80% of unsaponifiable matter (USM) and 18.36% of the fatty acids. GC/MS analysis of USM led to identification of 36 compounds for the first time accounting for 96.93% which were summarized in Table 2. Among them α-amyrin (31.75%), 4-methyl-2,6-di-tert-butylphenol (21.55%) and β-amyrin (10.19%) represented as predominant compounds. While GC/MS analysis of fatty acid methyl ester (FAME) allowed identification of 29 fatty acids recorded in Table 3. The saturated and unsaturated fatty acids represented 43.10 % & 56.90 % respectively, where palmitic acid constitute 23.68% and linoleic acid constitute 53.80% of total identified fatty acids.

Table 1: The yields of successive extracts and fractions from of *E. contortisiliquum* pericarps

Extracts and Fractions	Yields (w/w% of dried powder)
Crude extract	71.46
Petroleum ether extract	1.00
Methanol extract	35.00
Saponin fraction	3.50
Phenolic fraction	11.00
Polysaccharide fraction	7.49
Protein fraction	4.50

Table 2: Unsaponifiable compounds isolated from *E. contortisiliquum* pericarps

Compounds	**R_t**	**%**	**Mwt** *m/z*	**BP** *m/z*
Limonene	7.83	0.39	136	68
2-Ethylhexanol	8.18	0.42	112	57
Glyceraldehyde diethylacetal	12.35	0.47	164	103
1,1,3,3- Tetraethoxypropane	18.64	1.25	220	103
2,6-Di (t-butyl)-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one	20.63	0.34	236	165
4-Methyl,2,6-di-tert-butylphenol	21.66	21.55	220	205
14-Hexadecenal	25.85	0.21	238	43
Heptadecane	26.00	0.20	240	57
1-Heptadecene	28.09	0.07	238	57
Octadecane	28.22	0.11	254	57
6,10,14-Trimethyl-2-pentadecanone	29.30	0.50	268	43
Nonadecane	30.33	0.12	268	57
Eicosane	32.35	0.26	282	57
Heneicosene	34.09	0.11	294	43
Heneicosane	34.29	0.40	296	57
Phytol	34.68	0.32	296	71
Docosane	36.14	0.57	310	57
1,19-Eicosadiene	37.37	0.03	278	55
Tricosene	37.82	0.05	322	55
Tricosane	37.92	0.54	324	57
Tetracosane	39.63	0.99	338	57
1,21-Docosadiene	40.86	0.21	306	55
Pentacosane	41.27	1.60	352	57
Hexacosane	42.85	1.65	366	57
9-Hexacosene	44.19	0.09	364	43
Heptacosane	44.38	2.25	380	57
Octacosane	45.84	0.81	394	57
Squalene	46.36	1.44	410	69
Nonacosane	47.27	2.00	408	57
2,3-Epoxy squalene	49.64	0.58	426	69
1-Octacosanol	50.10	4.04	410	57
Lanosterol	52.65	1.80	426	411
Stigmasterol-7,22-dien-3β-ol (Chondrillasterol)	53.44	6.78	412	271
Friedoolean-14-en-3β-ol (Taraxerol)	53.76	2.72	426	204
β-Amyrin	54.09	10.19	426	218

α-Amyrin	54.97	31.75	426	218

Table 3: Methyl ester of fatty acids isolated from *E. contortisiliquum* pericarps

Compounds	**R_t**	**Relative %**	**Mwt** *m/z*	**BP** *m/z*
Methyl caprate	16.67	0.05	186	74
Dimethyl subenic (dimethyl octanedioate)	20.16	0.03	202	74
Methyl laurate	21.89	0.34	214	74
Dimethyl azelate (Dimethyl Nonanedioate)	22.60	0.02	216	55
Dimethyl sebacate (dimethyl decadanedioate)	25.10	0.02	230	55
Methyl tertradecanoate	26.70	1.66	242	74
Dimethyl undecadanedioate	27.36	0.03	244	55
Methyl 9-dodecenoate	28.51	0.05	254	74
Methyl pentadecanoate	28.85	0.22	256	74
Methyl 9-hexadecenoate	30.57	1.46	268	55
Methyl palmitate	31.44	23.68	270	74
Methyl heptadecanoate	33.00	0.05	284	74
Methyl 9,12,15-octadecatrienoate (Methyl linolenate)	35.08	1.35	292	79
Methyl 9,12-Octadecadienoate (Methyl linoleate)	35.35	53.80	294	67
Methyl stearate	36.82	3.27	298	74
Methyl nonadecanoate	37.35	0.17	312	74
Methyl 11-eicosenoate	38.18	1.74	324	55
Methyl eicosanoate	38.67	2.74	326	74
Methyl 9-octadecen-13-hydroxy-12-methoxy-oate	39.60	0.03	342	145
Methyl heneicosanoate	40.30	0.71	340	74
Methyl 13-docosenoate	41.41	0.21	352	55
Methyl docosanoate	41.98	3.40	354	74
Methyl tricosanoate	43.45	0.70	368	74
Methyl tetracosanoate	45.04	2.58	382	74
Methyl pentacosanoate	46.41	0.38	396	74
Methyl hexacosanoate	47.84	0.70	410	74
Methyl heptacosanoate	49.17	0.12	424	74
Methyl octacosanoate	50.51	0.38	438	74
Methyl triacontanoate	53.26	0.11	466	74

GLC analysis of PSF hydrolysate derivative led to identify 9 sugars (Table 4). Glucose (59.17%), xylose (11.52%) and rhamnose (10.15%) were the dominant from total identified mucilage saccharides. Regard to PF, sixteen amino acids were identified in hydrolyzed protein using Amino Acid Analyzer (Table 5), where glutamic acid (0.614 g/ 100 g protein), leucine (0.404 g/100 g protein) and alanine (0.376 g/100 g protein) were represented as predominant amino acids in addition, ammonium salts gave 0.384 g/ 100 g protein. The sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) showed a single band having molecular weight of approximately 48 kDa. The isolated protein differed from the previously reported [11], they isolated enterolobin, a hemolytic protein, from *E. contortisililiqumm* seeds which was characterized by high levels of aspartic, glutamic acids, serine and theronine in addition to cystiene and methionine at lower levels and its molecular weights was 55 kDa (by SDS-PAGE).

Concerning to SF, the positive ESI/MS detected three protonated compounds at *m/z* 1136.60 (S1), 1107.00 (S2) and 1118.60 (S3) illustrating the molecular mass of three saponins from the crude saponin fraction (Figure 1). The MS/MS^2 spectrum of molecular ion 1136.60 (S1) showed product ion at *m/z* 1119.70 $[M–H_2O]^+$ as base peak losing 18 u designated as liberation of one molecule of water followed by the sequential loss of two 162 u corresponding to two hexose moieties, afforded two product ions at *m/z* 957.30 $[M + H\text{-}162]^+$ and 795.00 $[M + H\text{-}162\text{-}162]^+$, respectively.

Table 4: GLC of polysaccharide hydrolysate from *E. contortisiliquum* pericarps

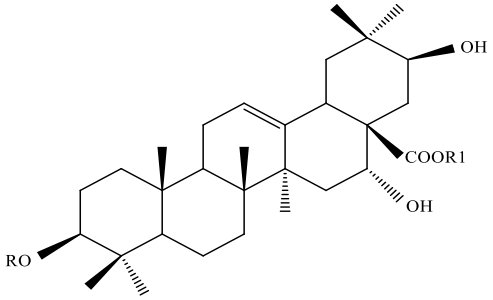
Sugars	**R_t**	**Relative %**
Arabinose	7.454	11.52
Xylose	7.562	7.38
Ribose	7.817	0.62
Rhamnose	8.355	10.15
Mannitol	9.690	1.15
Sorbitol	9.812	0.60
Fructose	10.00	0.56
Galactose	10.77	8.84
Glucose	10.927	59.17
Galactouronic acid	11.791	-
Glucouronic acid	14.469	-

Table 5: Amino acid composition of protein isolated from *E. contortisiliquum* pericarps

Amino Acids		g/ 100 g of isolated protein
Essential Amino Acids	Threonine	0.148
	Valine	0.187
	Methionine	0.004
	Isoleucine	0.160
	Leucine	0.404
	Phenylalanine	0.354
	Lysine	0.252
	Total	1.509
Non- essential Amino Acids	Aspartic acid	0.338
	Glutamic acid	0.614
	Serine	0.309
	Glycine	0.100
	Histidine	0.163
	Arginine	0.160
	Alanine	0.376
	Proline	0.321
	Tyrosine	0.250
	Total	2.631
Total contents of amino acids		**4.140**

According to Garai and Mahato, (1997) [12], the molecular ion *m/z* 795 was identified as acacic acid lactone-3-*O*-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranoside isolated previously from *Acacia auriculiformis* (subfamily: Mimosoideae). As aforementioned data, the compound 1 possessed two additional hexose moieties than acacic acid lactone-3-*O*-*β*-D-glucopyranosyl(1→6)-*β*-D-glucopyranoside implying a tetrasaccharide saponin. The initial loss of 18 u indicated that the carboxylic acid (C28) was free (non esterified) forming lactone. This provides important information on the linkage of oligosaccharides at C3. Regards to MS/MS^2 spectrum of *m/z* 1107 $[M + H]^+$ (S **2**) revealed the molecular ions at *m/z* 1089.10 $[M - OH]^+$, 957 [M-OH-

pentose]$^+$ and 795.30 [M-OH-pentose-hexose]$^+$ indicating sequential loss of 18u, 132u and 162u representing the loss of a molecule of water, a pentose and a hexose residue, respectively. Also, the spectrum showed fragment at *m/z* 633.60 corresponding to loss of a hexose moiety. The fragmentation pattern of compound S2 is analogous with that of compound S1 except for the presence of one pentose sugar instead of a hexose. Furthermore, acid hydrolysis of crude saponin revealed the presence of arabinose and glucose. Hence, compounds S1 and S2 were suggested to be 3-*O*-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranosyl glucopyranosyl glucopyranosyl acacic acid and 3-*O*-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranosyl glucosoylarabinosoyl acacic acid, respectively. The MS/MS^2 spectrum of compound S3 showed [M+H]$^+$ ion at *m/z* 1118.60 and daughter fragments at *m/z* 986.60 and 824.70 produced by loss of pentose moiety [M+H-132]$^+$ followed by loss of hexose [M+H-132-162]$^+$. The molecular ion at *m/z* 824.70 was identified previously [13] as prosapogenin; acacic acid 3-*O*-α-L-arabinopyranosyl(1→6)-2-acetamido-2-deoxy-*β*-D-glucopyranoside produced from crude saponin *of Acacia concinna*. Also, subsequent appearance of fragment ion at *m/z* 806 losing 18 u designated the loss of water molecule indicated that the carboxylic acid (C28) was esterified by oligosaccharide (hexose-pentose moieties). So, the compound S3 was suggested to be 3-*O*-α-L-arabinopyranosyl-(1→6)-2-acetamido-2-deoxy-*β*-D-glucopyranoside acacic acid 28-*O*-glucopyranosyl-arabinofuranosyl ester. *E. contortisiliquum* was belonged to Leguminosae-Mimosoideae which was known as rich with acacic acid (3*β*, 16*α*, 21*β*-trihydroxyolean-12-en-28 oic acid)-type saponins [14]. Worth mention that bisdesmosidic triterpene saponins; enterolosaponins A & B as well as contortisiliosides A-G were isolated previously from pericarps of *E. contortisiliquum* pods [15, 2].



Compound	R	R1
S1	-Glu-glu-glu-glu	H
S2	-Glu-glu-glu-arab	H
S3	-GluNAc-glu	-glu-arab

Figure 1: Structure of compounds S1-S3

On the other hand, reversed phase column chromatograph of PhF on polyamide S6 led to isolation of 9 phenolic compounds for the first time from *E. contortisiliquum* pericarps using water and methanol as eluent, they were identified as: gallic (1), protocatechuic (2), syringic (3), pyrogallol (4), *p*-coumaric acids (5), quercetin-7-*O*-α–rhamnopyranosyl-(1'''→6'')-*β*–glucopyranoside (6), catechin (7), isovitexin (8) and quercetin (9). All isolated compounds were elucidated by comparing their spectral data with those previously published data [16]. In previous study, caffeic acid, isoquercitrin, hyperin, astragalin, hesperidin, rutin, quercetin, kaempferol, herniarin, and chrysin were isolated from *E. timbouva* leaves [17].

3.2. Biological activities:

Cytotoxic activity against HepG2 and MCF7 cultured *in vitro* was examined for CE and other isolated fractions (Figures 2 & 3, respectively). The CE, PE, SF and PSF showed cytotoxicity with IC_{50} 43.5, 56.7, 22.8 and 15.7 µg/ml, respectively against HepG2 cell line whereas CE, PE, SF, PSF and PhF exhibited cytotoxicity with IC_{50} 32.1, 64.6, 12.3, 78.3 and 34.6 µg/ml, respectively, against MCF7 cancer cell line. On the hand, doxorubicin gave IC_{50} 36.9 & 26.3 µg/ml against HepG2 and MCF7 cancer cell lines, respectively. Moreover, the compounds; 2, 6 and 7 isolated from the bioactive PhF, were re-evaluated on MCF7 cell line which did not show any activity on MCF7 cell line (IC_{50} > 100 µg/ml). According to U.S. National Cancer Institute (NCI) and Geran protocol [18], the observed cytotoxicity can be categorized as follow: SF and PSF exhibited high

cytotoxic activity on HepG2 and MCF7 cell lines, respectively. Whereas CE, PE and PhF showed moderate cytotoxicity towards HepG2 and MCF7 cells in addition SF showed moderate cytotoxicity on MCF7. Saponin is a chemical class of plant derived glycosides, composed of lipophilic aglycone and hydrophilic sugar moieties [19] some important biological activities are connected with saponines; antimicrobial, anti-inflammatory, antitumor, anti diabetic and anti fungal [20]. As aforementioned, Leguminosae-Mimosoideae was rich with acacic acid-type saponins which possessed cancer related activities including cytotoxic/antitumor, immunomodulatory, antimutagenic, and apoptosis inducing properties. Further, their activities depend on the acylation and esterification by different moieties at C-21 and C-28 of the acacic acid-type aglycone [14]. Mimaki and coauthors [15, 2], reported that enterolosaponin A showed *in vitro* highly selective cytotoxic activity against BAC1.2F5 mouse macrophages while contortisiliosides A and C exhibited *in vitro* moderate selective cytotoxic activity against BAC1.2F5 mouse macrophages and EL-4 mouse lymphoma cells. PSF was reported to have cytotoxic activity through direct effect on the inhibition of the proliferation of cancer cell *in vitro* [21].

Regard to the antibacterial activity, it was evaluated according to the inhibition of growth related to Doxycycline Hydrchloride (Dox HCl) as reference drug and the result was recorded in Table **6**. The PSF and PF exhibited good antimicrobial activity against *Klebsiella pneumonia* (-ve). While, the CE, PE and PhF showed good antimicrobial activity against *Micrococcus luteus* (+ve). Furthermore, catechin and Protocachuic acid showed good activity against *Pseudomonas aeruginosa* (-ve) at concentration 1 mg/ml.

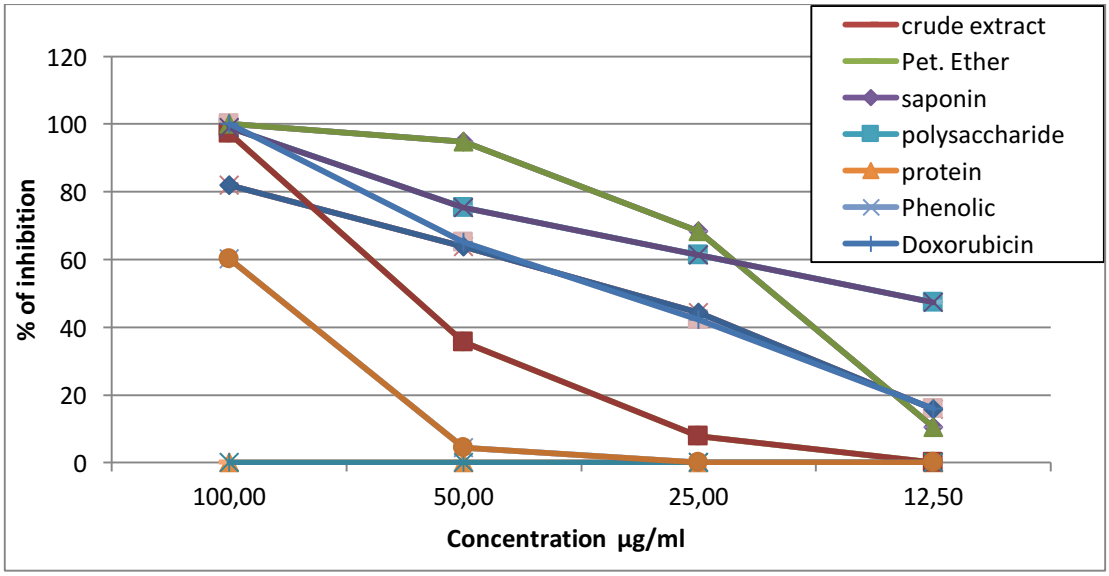


Figure 2: Cytotoxic activity of crude extract and fractions isolated from *Enterolobium contortisiliquum* pericarps against HepG2 human cell line in *vitro*

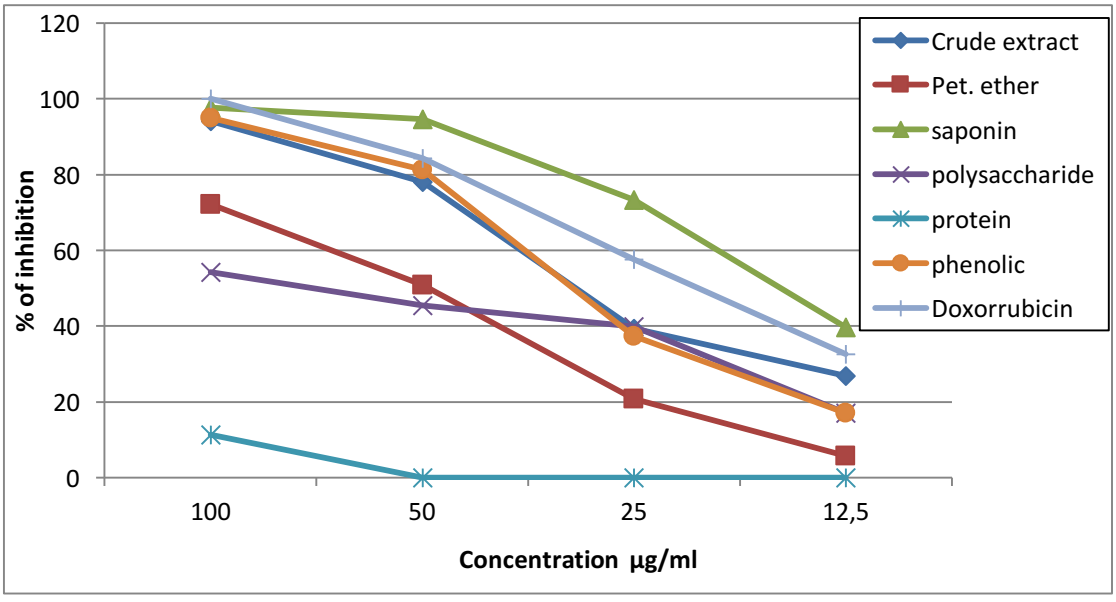


Figure 3: Cytotoxic activity of crude extract and fractions isolated from *Enterolobium contortisiliquum* pericarps against MCF-7 human cell line in *vitro*

Table 6: The antibacterial activity of CE, PE, PSF, PF, PhF and isolated compounds from *E. contortisiliquum* pericarps

Bacterial strains	**Relative Inhibition %**							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Bacillus subtilis	25.95	14.81	33.33	14.81	14.81	-	22.22	-
Bacillus cereus	13.33	13.33	23.33	13.33	33.33	-	-	-
Staphylococcus aureus	9.09	12.12	12.12	15.15	12.12	18.18	18.18	15.15
Staphylococcus epidermidis	10.00	16.66	16.66	13.33	-	-	-	-
Streptococcus mutans	40.00	-	23.33	-	30.00	-	-	-
Streptococcus pneumonia	-	20.00	16.66	30.00	20.00	30.00	-	-
Micrococcus luteus	40.00	33.33	10.00	-	30.00	-	-	-
Klebsiella pneumonia	16.66	33.33	**56.66**	**46.66**	13.33	16.66	-	-
Proteus vulgaris	29.41	41.17	29.41	41.17	29.41	41.17	29.41	35.29
Salmonella Typhi	14.28	19.04	19.04	-	19.04	-	28.57	-
Escherichia coli	20.00	20.00	20.00	36.00	20.00	36.00	36.00	-
Pseudomonas aeruginosa	-	-	-	-	-	**48.38**	-	**54.83**
Bordetella pertussis	-	11.42	-	-	-	-	17.14	-

1-CE, **2-**PE, **3**-PSF, **4**-PF, **5-**PhF, **6-**catechin, **7-** Quercetin-7-rutenoside, **8**- Protocachuic acid. The antibacterial activity was evaluated according to the inhibition growth related to Doxycycline Hydrchloride. Activity> 75%, high; 75-50%, good; 50-25%, normal; < 25%, weak activity.

On the other hand, antifungal activity of The CE, PE, PSF, PF and PhF as well as compounds quercetin 7-rutinoside, catechin and protocachuic acid showed slight activity on *A. niger* and *C. albicans* (Table **7**). Polysaccharides (PS) belong to class of macromolecules chemically, they are polymer of monosaccharide joined to each other by glycosidic linkages. The monosaccharide units have many interconnection points so they can form a wide variety of branched or linear structures [22]. PSF of *E. contortisiliquum* pericarps exhibit good antimicrobial activity against *Klebsiella pneumonia, Bacillus subtilis* and *Proteus vulgaris*. PE exhibited moderate cytotoxic and antimicrobial activities. It was mainly composed of sterol (8.58% USM) and pentacyclic triterpenoid compounds (44.66% of USM), which were reported to possess several medicinal properties including anticancer and anti-HIV activities [23]. *β*-Amyrin, one of the major compound in the USM of PE, was reported to have antimicrobial, antifungal and cytotoxic properties [24]. In addition, it exhibited some cytotoxic activity against A549 and HL-60 cancer cell line [25]. Stigmast-4-en-3-one also displayed high antitumor-promoting activity [26]. Linoleic acid (C18:2, 53.80%) and palmitic acid (C16:0, 23.68%) constitute the major identified fatty acids in the PE of *E. contortisiliquum* pericarps which were known to possess cytotoxic activity [27].

Table 7: The antifungal activity of CE, PE, PSF, PF, PhF and isolated compounds from *E. contortisiliquum* pericarps

Extract (1mg/ml)	Inhibition zone mm				
	A. flavus	*A.ochraceus*	*A. nige*r	*C. albicans*	*F. solani*
CEF	-	-	1	1	-
PEF	-	-	1	1	-
PhF	-	-	1	1	-
PSF	-	-	2	2	-
PF	-	-	1	2	-
Catechin	-	-	2	2	-
Quercetin 7-rutinoside	-	-	2	2	-
Protocachuic acid	-	-	2	2	-
Fluconazole (reference drug)	17	3	7	14	7

Linoleic acid was reported to possess antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli, Mycobacterium smegmatis*, *M. fortuitum*, *M. aurum* and *M. phlei* [28, 29]. Lauric, palmitic (C16), linolenic, linoleic, oleic, stearic (C18) and myristic acids are known as potential antibacterial and antifungal agents [28, 30]. The previous reported results are consistent with the obtained antimicrobial activity of the PE against *Proteus vulgaris, Micrococcus luteus* and *Klebsiella pneumonia* which can be contributed to the terpenoidal and fatty acids contents according to the previously reported studies. The PhF exhibited moderate cytotoxic, depends on their lipophilic nature which is essential for phenolic compound to penetrate into the cell, the lipoprotein nature of cell membrane facilitate the solubility of polyphenols [31]. According to results of column chromatography analysis, the PhF constituted high percentage of phenolic acids and flavonoids. It exhibited good antimicrobial activity against *Bacillus cereus, Streptococcus mutans, Micrococcus luteus* and *Proteus vulgaris*. Catechin showed antibacterial activity against *Pseudomonas aeruginosa & Proteus vulgaris* whilst quercetin-7-rutenoside possessed antibacterial activity against *E. coli*, *Salmonella Typhi* and *Proteus vulgaris*. However, protocachuic acid showed activity against *Pseudomonas aeruginosa, Proteus vulgaris E. coli* and *Streptococcus pneumonia*. In previous studies, catechin and many polyphenols were reported to possess antibacterial action against *E. coli, Enterococcus faecalis, Staphylococcus aureus, Candida albicans* and *Pseudomonas aeruginosa* [32]. Protocatechuic acid was reported to have antibacterial and antimutagenic activity [33-35]. Quercetin a free radical scavenger [36] was reported for its bacteriostatic activity [37]. Phenolics act as antimicrobial through their abilities to bind with proteins or glycoproteins of the cell membrane which is the adhesion site in which bacteria tend to bind before it invade the human cell, this will lead to disturbances in the exposed surface contains the receptors [38].

Conclusion

The isolation and chemical characterization of lipoidal matter, polysaccharide, protein, saponin and phenolic fractions isolated from *E. contortisiliquum* pericarps as well as their biological activity were studied for the first time. The current study indicated that the fractions isolated from *E. contortisiliquum* pericarps contain promising molecules with potentially useful cytotoxic and antibacterial activities profiles, has to be considered of great value as a source of lead compounds with pharmaceutical applications.

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