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HPLC Investigation of Carbohydrates and Phenolic Constituents of *Livistona decipiens* and *Livistona australis* Leaves and Assessment of their Protective Activity against Ulcerative Colitis

Fathia S. Elshaarawy[1], Suzan A. Mina[1], Nagwan M. Gabr[1], Soad M. Abdelkhalik[1,2], Rehab.Kamel[3], Haitham A. Ibrahim[1], Eman G. Haggag[1]*

[1]*Department of Pharmacognosy, Faculty of Pharmacy, Helwan University. Cairo, Egypt, 11795.*
[2]*Department of Pharmacognosy, Faculty of Pharmacy, Nahda University, BeniSuef, Egypt*
[3]*Department of Pharmacology, Faculty of Pharmacy, Helwan University. Cairo, Egypt, 11795*

***Corresponding author:** Eman G. Haggag, Department of Pharmacognosy, Faculty of Pharmacy, Helwan University, Cairo, Egypt, 11795. Tel.: +201000023022*
E-mail address: Eman.G.Haggag@pharm.helwan.edu.eg

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ABSTRACT

Objectives: This study aimed to compare two *Livistona* species; *Livistona decipiens* Becc and *Livistona australis* Mart for their phenolic and carbohydrate contents and for their protective activity against ulcerative colitis. **Methods:** A high performance liquid chromatographic (HPLC) technique with inline connected to photo diode array detector (DAD) and electro array (EA) used for detection of the polyphenolic contents. Also HPLC with refractive index detection (HPLC-RI) was used to determine and to quantify carbohydrate contents before and after partial acid hydrolysis, using 0.2 N H_2SO_4, for 3 h at 100°C, for each of the defatted methanol extract concentrate. Protective activity against ulcerative colitis was evaluated by acetic acid inducing-ulcers method for both methanol extracts of both species. **Results**: Both investigated *Livistona* species are rich in polyphenolic constituents, showing a great similarity. The major flavonoid compound in both species was luteolin-6-*C*-arabinoside-8-*C*-glucoside and the major aglycone was acacetin, while the major phenolic acid in both species was ellagic acid. However there was a difference in the carbohydrate content between the two species, the main sugars, before hydrolysis, in *L. decipiens* were arabinose, mannose and sucrose, while the main sugars in *L. australis* were glucose, fructose and maltose. However, stachyose was the major polysaccharide obtained after partial acid hydrolysis. *L. australis* showed protective activity against ulcerative colitis in lower dose 500 mg/kg when compared to *L. decipiens* that only showed effectiveness at a doubled dose 1000 mg/kg. **Conclusion:** Both *Livistona* species have potential medicinal value being rich in polyphenolic and polysaccharide contents and having protective activity against ulcerative colitis.

***Keywords:**, HPLC; Livistona australis; Livistona decipiens; Polysaccharides, Polyphenolic, Ulcerative colitis;*

INTRODUCTION

Palms are the princess of the plant kingdom, representing the third most important plant family with respect to human use[1]. Oils obtained from the palm nut, coconut and palm kernels were recognized as health oils in Ayurvedic medicine almost 4000 years ago. Numerous edible products are obtained from palms, including familiar date palm fruits, coconut palm nuts and various palm oils[2]. More than 800 uses have been recorded for the date palm alone, for it is the foundation of life for several cultures. Genus *Livistona* "Fountain Palm" is a genus of 36 species of hermaphrodite, shrubby palms, comprising the most common fan-palms of greenhouses and decorative use, native to East Asia and Australia[3].

Certain flavonoids (luteolin, tricin, quercetin and myricetin) and phenolic acids (gallic, chlorogenic, *ρ*-hydroxy benzoic, *ρ*-coumaric, ferulic, caffeic acids) were considered as markers in earlier studied palm

species[4-6] Consequently, it was found essential to examine the two palm species under investigation for presence of palm flavonoids and phenolic acids markers.

Carbohydrates are widely distributed in biological systems providing various functions and because of their structural diversity and the quite varied analytical method for their determination, research for their medicinal uses and methods for their determination has significantly increased over years[7]. Reviewing literature, there were no reports concerning the investigation of the carbohydrates of *Livistona decipiens* Becc *and Livistona australis* Mart. Also, since there was only one reported work on the phenolic constituents of *Livistona australis*[8] in addition to the preliminary phytochemical screening of both *Livistona* species revealing the presence of carbohydrates and phenolic constituents in both leaves, it was deemed of interest to carry out this comparative study.

MATERIAL AND METHODS

Plant material

The leaves of both *L decipiens* and *L. australis* were collected from Al-Orman garden, Giza, Egypt on 22 April 2014. They were identified by Dr. Terase Labib, Department of Flora and Taxonomy, El-Orman Botanical Garden, Giza, Egypt. A voucher specimens 01Lde/2014 and 02Lau/2014 for both *L decipiens* and *L australis*, respectively were kept in the Herbarium in the Department of Pharmacognosy, Faculty of Pharmacy, Helwan University, Egypt.

Animals

Sixty six female Wistar rats weighing 180-200 g were obtained from the breeding unit of the Egyptian Organization of Biological Products and Vaccines (Helwan, Egypt). The rats were housed in cages at a temperature-controlled (22±2°C) environment and provided free access to pelleted food and purified drinking water *ad-libitum*. The animal experiments described later were approved by the Ethics Committee, Faculty of Pharmacy, Helwan University (011 A 2018).

Chemicals

For biological study

Acetic acid was purchased from El Nasr Pharmaceutical chemicals Co, DMSO was purchased from Sigma-Aldrich Co., and Sulphasalazine 500 mg/kg was purchased from Pfizer Company.

Standard materials

Authentic flavonoids

The standard flavonoids used; luteolin-6-*C*-arabinoside-8-*C*-glucoside, luteolin-6-*C*-glucoside-8-*C*-arabinoside, apigenin-6-*C*-arabinoside-8-*C*-galactoside, apigenin-6-*C*-rhamnoside-8-*C*-glucoside, apigenin-6-*C*-glucoside-8-*C*-rhamnoside, luteolin-7-*O*-glucoside, naringen, rutin, hesperidin, apigenin-7-*O*-neohespiroside, kaempferol-3,7-*O*-dirhamnoside, apigenin-7-*C*-glucoside, quercetrin, quercetin, naringenin, hispertin, kampferol, rhamnetin, apigenin, acacetin, were supplied by HPLC – Lab, Food-Tech Research Institute, Cairo, Egypt.

Authentic phenolic compounds

Standard phenolic acids used; gallic, ellagic, benzoic, 4-amino-benzoic, caffeic, vanillic, *ρ*-coumaric ferulic, iso-ferulic, salicylic, cinnamic, 3,4,5-methoxy-cinnamic protocatchuic, chlorogenic, *α*-coumaric, and *e*-vanillic, while the standard poylphenolics used were catechol, catechin, epicatechein and resveratrol, which were all supplied by HPLC – Lab, Food-Tech Research Institute, Cairo, Egypt.

Authentic carbohydrates

Standards carbohydrates used; galactouronic, glucuronic, rhamnose, mannose, arabinose, xylose, stachyose, sucrose, galactose, maltose, fructose, glucose, were supplied by HPLC – Lab, Food-Tech. Research Institute, Cairo, Egypt.

Apparatus

HPLC Agillant 1200 equipped with auto sampling injector, solvent degasser, (UV) detector set at 280 nm for the phenolic acids and phenols, while it was set for 330 nm for the flavonoids, a quaternary HP pump (series 1100), a column Agillant C18 ZORBAX-ODS 5µm (4.6 × 250 Id, mm), the column temperature was maintained at 35°C for phenolic analysis. The chromatographic system coupled to the refractive index detector (HPLC-RI) was equipped with a quaternary pump (water 2695 alliance, Milford MA, USA), degasser, auto injector, and water RI2414 refractive index detector (Milford MA,USA), under isocratic condition with type l water (Milli-Q integral*, millipore*, saopaulo SP, Brazil) an BIO RAD Aminex-carbohydrates HPX- 87 Ca (300 mm × 7.8 mm), were used for carbohydrate analysis.

Methods

Preparation of the plant extract

The air dried powder of each of *L. decipiens and L. australis* leaves (300 g) was soaked in methanol (1L × 3 times) and allowed to stand at room temperature for three days then filtered. The filtrates were evaporated under reduced pressure and temperature. The dried methanol extracts of *L. decipiens* (46.7 g) and *L. australis* (54.5 g), each was defatted with petroleum ether to yield (43.7 g) and (53.0 g) of defatted methanol extract concentrates, respectively, which were stored at low temperature[9].

Table 1. HPLC analysis of flavonoid content in *L. decipiens* and *L. australis* leaves

Flavonoid	Retention time (min)	*L. decipiens* Weight(µg/g)	*L. australis* Weight(µg/g)
Luteolin-6-*C*-arabinoside-8-*C*-glucoside	9.369	15073.09	36538.55
Luteolin-6-*C*-glucoside-8-*C*-arabinoside	10.544	2790.09	2688.03
Apigenin-6-*C*-arabinoside-8-C-galactoside	11.443	3241.61	4019.47
Apigenin-6-*C*-rhamnoside-8-C-glucoside	11.789	1824.27	2017.00
Apigenin-6-*C*-glucoide-8-C-rhamnoside	11.981	9965.32	12726.65
Luteolin-7-*O*-glucoside	12.079	1671.70	1099.22
Naringen	12.150	2711.78	2023.43
Rutin	12.310	382.81	542.76
Hesperidin	12.345	4952.17	21569.60
Apigenin-7-*O*-neohespiroside	12.825	573.39	2041.88
Kaempferol-3,7-*O*-dirhamnoside	12.874	--------	1883.46
Apigenin-7-C-glucoside	13.094	168.90	431.81
Quercetrin	13.232	386.73	623.31
Quercetin	14.696	593.26	733.83
Naringenin	14.918	307.50	235.69
Hispertin	15.211	1380.80	1131.18
Kampferol	15.964	411.43	212.38
Rhamnetin	16.090	332.32	242.00
Apigenin	16.223	357.86	185.46
Acacetin	18.395	8770.87	3646.44

Table 2. HPLC analysis of phenolic content in *L. decipiens* and *L. australis* leaves

Phenolic compound	Retention time (min)	*L. decipiens* Weight (µg/g)	*L. australis* Weight (µg/g)
Gallic acid	7.321	82.62	158.84
Pyrogallol	7.345	5935.44	19661.25
4-amino-benzoic acid	7.920	33.93	80.38
Protocatechuic acid	8.624	673.00	951.23
Catechins	8.704	256.28	559.77
Chlorogenic acid	9.226	2030.16	4179.35
Catechol	9.544	383.34	450.56
Epicatechins	9.700	415.62	591.49
Caffiec acid	10.278	160.44	99.05
Vanillic acid	10.389	1873.13	2373.70
***ρ*-coumaric acid**	11.682	344.34	377.80
Ferulic acid	11.852	206.23	424.99
Iso-ferulic acid	12.172	279.75	437.91
Resveratrol	12.647	121.91	174.85
Ellagic acid	13.012	10962.88	9484.42
***e*-vanillic acid**	13.061	4541 .34	-------
***α*-coumaric acid**	13.299	588.84	698.15
Benzoic acid	13.456	3882.15	60.38
3,4,5-methoxy-cinnamic acid	13.923	189.21	346.76
Salicylic acid	14.330	1438.21	2171.50
Cinnamic acid	15.160	112.12	107.15

Preparation of flavonoid and phenolic acid samples

Phenolic compounds were determined as follows[10]; five g of each sample was mixed with methanol and centrifuged at 10000 rpm for 10 min and the supernatant was filtered through a 0.2µm Millipore membrane filter then 1-3 ml was collected in vials for injection into HPLC Agillant 1200 equipped with auto sampling injector, solvent degasser, ultra violet (U.V) detector set at 280 nm for the phenolic acids and 330 nm for the flavonoids and quaternary HP pump (series 1100) the column temperature was maintained at 35°C.

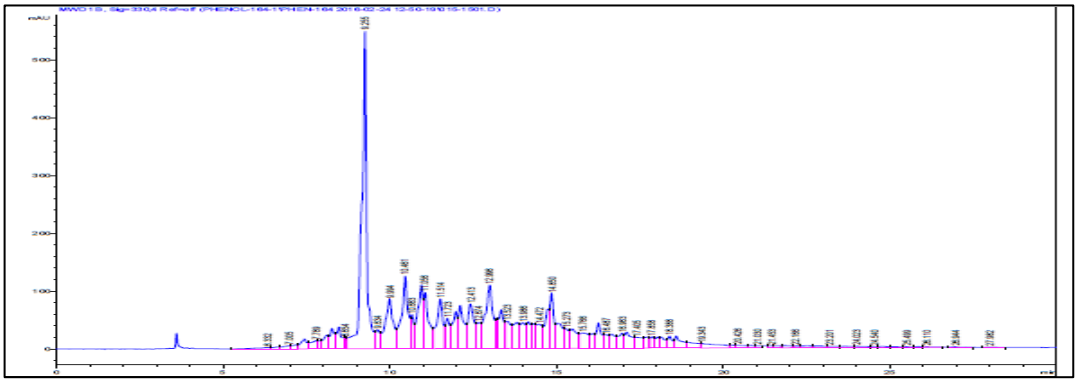

Figure 1. HPLC analysis of flavonoid content in *L. decipiens* leaves

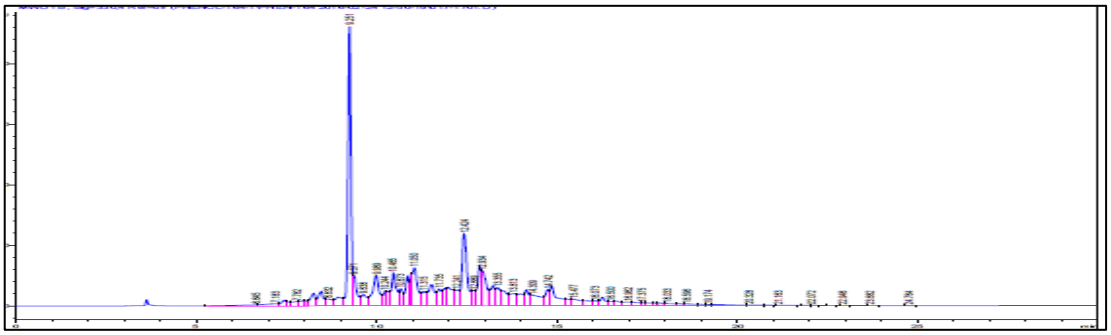

Figure 2. HPLC analysis of flavonoid content in *L. australis* leaves

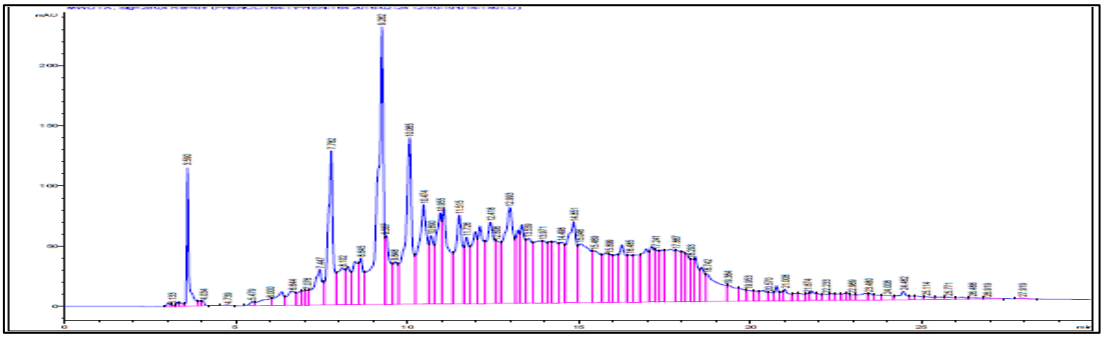

Figure 3. HPLC analysis of phenolic content in *L. decipiens* leaves

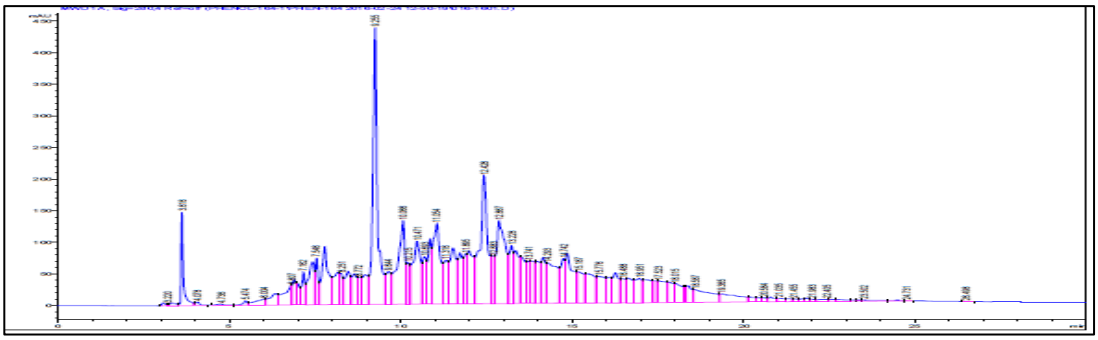

Figure 4. HPLC analysis of phenolic content in *L. australis* leaves

Table 3. HPLC analysis of carbohydrate content before partial acid hydrolysis in *L. decipiens* and *L. australis* leaves

Carbohydrate	Retention time (min)	*L. decipiens* % g /100 g	*L. australis* % g /100 g
Glucuronic	4.081	0.71	-------
Stachyose	4.285	1.88	0.52
Galactouronic	4.553	0.40	0.57
Sucrose	5.007	1.99	----------
Maltose	5.075	--------	2.7
Glucose	6.111	0.52	3.61
Xylose	6.822	0.53	0.33
Galactose	7.053	--------	0.33
Rhamnose	7.152	--------	0.24
Mannose	7.265	2.11	-------
Arabinose	8.371	2.63	------
Fructose	8.562	0.38	3.20
Mannitol	11.465	0.009	0.03
Sorbitol	15.055	0.013	0.02

HPLC analysis of flavonoid content

Flavonoid separation was carried out by reversed phase HPLC (RP-HPLC) equipped with diode array (DAD) detector; the column temperature was maintained at 35°C, gradient elution was employed for flavonoids, a mobile phase consisting of 50 Mm H_3PO_4/H_2O, pH 2.5 (solution A) and acetonitrile (solution B); as follow; isocratic elution 95% A, 5% B 0 - 5 min, linear gradient from 95% A, 5% B to 50% A, 50% B 5 - 55 min, isocratic elution 50% A, 50% B 55 - 65 min, linear gradient from 50% A, 50% B to 5% A, 95% B 65 - 100 min the flow rate of the mobile phase was 0.7 ml/min and the injection volume was 10 µl of the standards and extracts. Flavonoids were quantifying by external standard method, quantification was based on peak area (DAD)[11].

HPLC analysis of phenolic content

Separation and determination of phenolic acids were performed by RP-HPLC - DAD, the solvent systems used were gradient of A (8% CH_3COOH/H_2O), and B (acetonitrile). The best separation was obtained with the following gradient at 0 - 20 min 5% B, 95% A, at 20 - 50 min,10% B, 90% A, at 50 - 55 min, 30% B, 70% A, at 55 - 100 min, 50% B, 50% A, at 100 - 120 min, 100% B the solvent flow rate was 1 ml/min and the separation was performed at 35°C, and the injection volume was 10 µl of the standards and extracts. Phenolic compounds were assayed by external standard calibration at 280 nm and expressed in µg/g[10].

HPLC analysis of carbohydrate content

Each residue (0.1 g) as well as of individual authentic reference sugars were separately diluted 1:10(v/v) with Milli Q water (type I) and then filtered through 0.22 um filter membrane (water Milford, MA, USA). Aliquots of 1.5 ml of each of these solutions were placed in vials for analysis of the free sugars and were carried out by comparison of the retention times of the peaks with those of authentic compounds. The retention times of the separated sugar residues were detected by water –millipore differential refract-meter R401 and the data collected and integrated using Perkin Elmer chromatography data station in order to quantify the amount of each sugar present in samples[12].

Table 4. HPLC analysis of carbohydrate content after partial acid hydrolysis in *L. decipiens* and *L. australis* leaves

Hydroylsate	Retention time (min)	*L. decipiens* % (g/100g)	*L. australis* % (g /100 g)
Glucuronic	4.081	0.80	-------
Stachyose	4.285	2.05	2.56
Galactouronic	4.553	0.53	0.61
Sucrose	5.007	0.24	0.18
Maltose	5.075	-------	-------
Glucose	6.111	1.72	0.24
Xylose	6.822	------	-------
Galactose	7.053	-------	1.01
Rhamnose	7.152	0.87	--------
Mannose	7.265	-------	--------
Arabinose	8.371	-------	---------
Fructose	8.562	0.79	0.89
Mannitol	11.465	0.76	0.04
Sorbitol	15.055	0.20	0.04

Partial acid hydrolysis of carbohydrates

Partial acid hydrolysis was done using 0.2 N H_2SO_4 by boiling solutions containing 0.1 g of defatted methanol extract in 12.5 ml of 4% w/w (4 g conc.H_2SO_4 + 96 ml distilled water), for a period of 3 hours at 100°C[13]. The hydrolyzed solution was neutralized by the addition of 2 g of barium carbonate to each sample. The neutralized hydrolysates were filtered to remove insoluble barium salts and the clear supernatants were analyzed for neutral sugar residues using HPLC.

HPLC separation conditions for carbohydrates

The chromatographic system coupled to the refractive index detector (HPLC-RI) was equipped with a quaternary pump (waters 2695 alliance, Milford MA, USA), degasser, auto injector, and waters RI-2414 refractive index detector (Milford MA, USA), and chromatographic data were acquired using the Empower*2 software. The samples were analyzed using an BIO RAD Aminex-carbohydrates HPX-87 Ca (300 mm × 7.8 mm) under isocratic condition with type l water (Milli-Q integral*, millipore*, saopaulo SP, Brazil), the injection volume was 5 µl, and the flow rate was 0.5 ml/min, the column temperature was maintained at 80°C, and the detector at 50°C, the samples detection were performed by comparing with retention times of standards[12].

Toxicological study:

Twelve female Wistar rats were divided into two groups, each of six rats were used for evaluation of acute toxicity of *L. decipiens* and *L. australis* leaf methanol extracts. LD_{50} of both methanol extracts was determined using limit test[14] at one dose level of 2000 mg/kg of rat body weight administered orally by gavages.

Both leaf methanol extracts of *L. decepiens and L. australis* failed to produce any mortality in rats. So LD_{50} of both methanol extracts is considered to be more than 2000 mg/ kg.

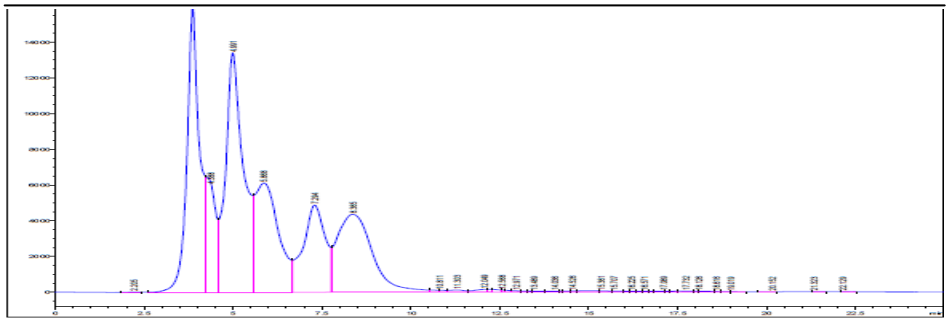

Figure 5. HPLC analysis of carbohydrate content before hydrolysis in *L. decipiens* leaves

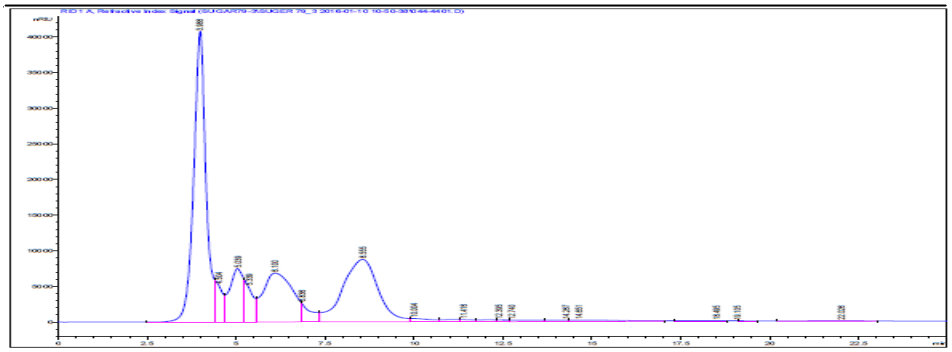

Figure 6. HPLC analysis of carbohydrate content before hydrolysis in *L. australis* leaves

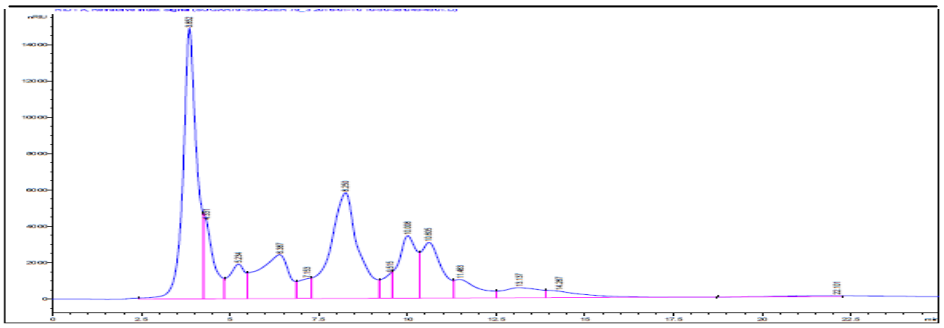

Figure7. HPLC analysis of carbohydrate content after hydrolysis in *L. decipiens* leaves

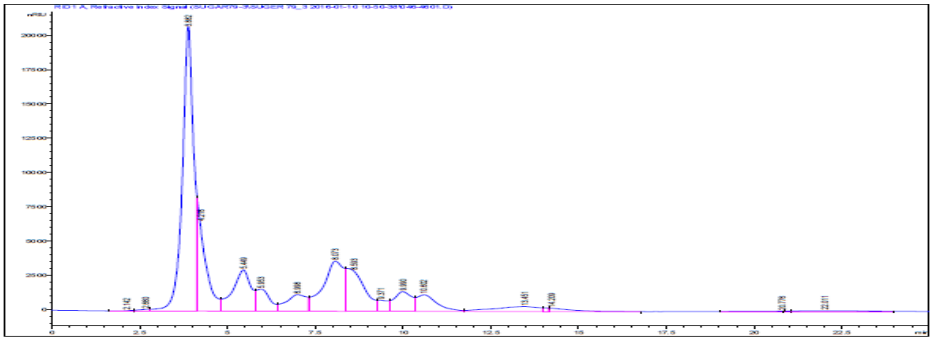

Figure 8. HPLC analysis of carbohydrate content after hydrolysis in *L. australis* leaves

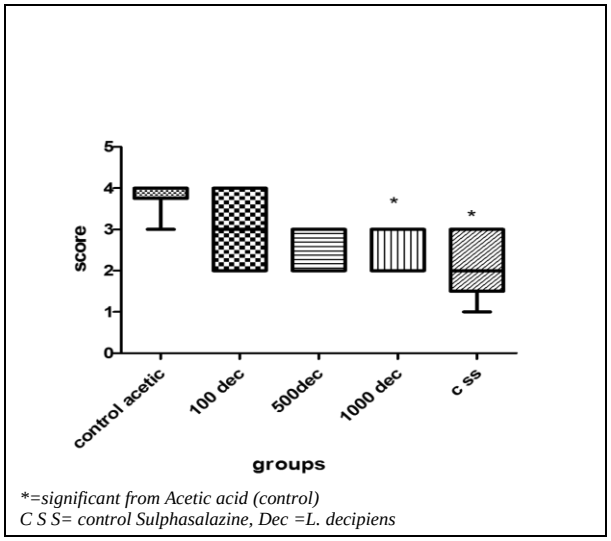

Figure 9. Effect of methanol extracts of leaves of *L. decipiens* on acetic acid-induced ulcerative colitis.

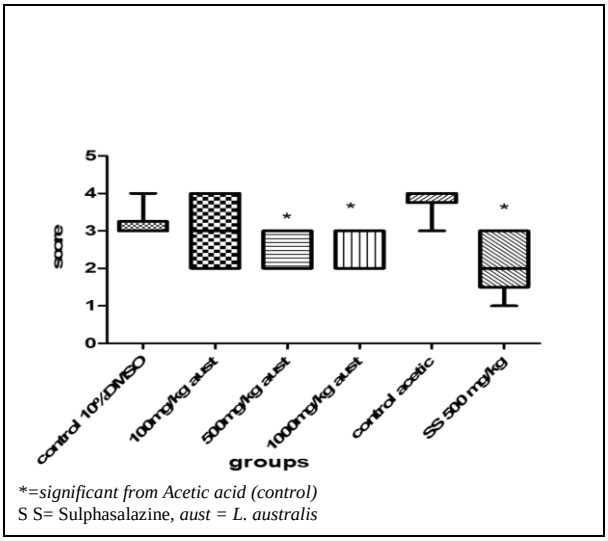

Figure 10. Effect of methanol extracts of leaves of *L. australis* on acetic acid-induced ulcerative colitis.

Evaluation of protective activity against ulcerative colitis using *Acetic acid-induced ulcerative colitis method*

Fifty four female Wistar rats were divided into nine groups, each of six rats as follows; the first group was a control group for rats given *L. decipiens* extract. They were administered 10% tween 80 in normal saline. The second group was a control group for rats given *L. australis* extract. They were administered 10% DMSO in normal saline. The third, fourth and fifth groups; rats were given 100, 500, 1000 mg/kg of *L. decepiens* methanol extract (p.o.), respectively. The sixth, seventh and eighth groups; rats were given 100, 500, 1000 mg/kg of *L. australis* methanol extract (p.o.), respectively. The ninth group; rats were administered sulphasalazine 500 mg/kg, (p.o.).

Animals received the respective drug treatment daily, for four days. On the third day, the animals were fasted for 24 h. colitis was induced by rectal instillation of acetic acid (2 ml of 3% solution in normal saline) on the fourth day, one hour after the above mentioned treatments. On the fifth day, the animals were sacrificed by cervical dislocation and their colons were isolated for the assessment of ulcer lesions[15].

Macroscopic scoring

The macroscopic evaluation of colon mucosal damage has been done according to the scale ranging from 0 to 4[15]; as follows; *(0 = No macroscopic changes, 1 = Mucosal erythema only, 2 = Mild mucosal edema, slight bleeding or small erosions, 3 = Moderate edema, bleeding ulcers or erosions, 4 = Severe ulceration, erosions, edema and tissue necrosis)*.

Statistical analysis

Statistical comparison between groups was carried out using Kruskal-Wallis test followed by Dunn's multiple comparisons test by means of GraphPad prism software (Version 6). Results were expressed as median and interquartile range. The statistical significance of difference was considered at $P<0.05$.

RESULTS AND DISCUSSION

HPLC analysis of flavonoids and phenolic compounds in *L. decipiens* and *L. australis* leaves

Detection of individual flavonoid compounds by HPLC methods has mostly been based on UV/VIS absorption. Use of photo diode array detector enables collection of on line spectra and simultaneous quantification at several wavelengths[16-18].

Figures 1, 2, 3 and 4 as well as tables 1 and 2 illustrate the chromatographic detection of flavonoids and phenolic compounds from the alcoholic extract of the *L. decipiens* and *L. australis* leaves, respectively. Total of twenty flavonoid compounds were tentatively determined in *L. australis*, while only nineteen flavonoid compounds were detected in *L. decipiens.* The major flavonoid glycoside tentatively determined in both species was luteolin-6-*C*-arabinoside-8-*C*-glucoside and the major flavonoid aglycone determined was acacetin, while the major phenolic acid in both species was ellagic acid. However, some flavonoids were previously reported[8] isolated from *L. austarlis* leaves, could not be detected here as the authentic standards were not available for HPLC analysis.

HPLC analysis of carbohydrates

Most applied methods for the quantification of carbohydrate is HPLC analysis. HPLC has been extensively used for polysaccharide analysis due to its separation capabilities, for its different separation modes, coupled with different detection methods[7]. In this study, partial acid hydrolysis was done using 0.2 N H_2SO_4 for 3 hours at 100°C, which has allowed maintaining tetrasaccharides as stachyose intact. However, it was reported that[19] partial acid hydrolysis of polysaccharides for obtaining high yield of oligosaccharides was done using 0.5 N H_2SO_4 for 3 hours at 100°C, yielding 50% polysaccharides and 30-40% of the free sugars of the original percent present before partial acid hydrolysis[19]. Polysaccharides hydrolyzed with 0.2 M triflouroacetic acid (TFA) has yield oligosaccharides with different molecular weights[20].

Complete acid hydrolysis of polysaccharides into monosaccharide (quantitative saccharification) by 77% H_2SO_4[21] has the disadvantage of causing degradation of monosaccharide with sequential formation of toxic substances[20]. However, the peculiarities inherent to each hydrolysis method proposed in the literature can yield different quantitative and productive efficiences[20].

Glycosidic compounds such as glycoproteins, glycolipids and flavonoid glycosides present in plant extract, may lid to increase the amount of polysaccharides detected by HPLC analysis after partial acid hydrolysis[7] as shown in table 4, where the percent of stachyose has been increased after partial acid hydrolysis (2.05% and 2.56% for *L. decipiens* and *L. australis*, respectively), when compared to its percent before hydrolysis (1.88% and 0.52% for *L. decipiens* and *L. australis*, respectively).

The main sugars present in *L. decipiens* before hydrolysis were arabinose, mannose and sucrose, while the main sugars in *L. australis* were glucose, fructose and maltose. Xylose could not be detected after hydrolysis for both *Livistona* species. Results are presented in tables 3 and 4 as well as illustrated in figures 5, 6, 7 and 8.

Protective activity against ulcerative colitis

Table 5 as well as figures 9 and 10 illustrates the effect of methanol extracts of leaves of *L. decipiens* and *L. australis* on acetic acid induced ulcerative colitis. The two methanol extracts had anti-inflammatory effect on acetic acid induced ulcerative colitis. However, *L. decipiens* fruit pulp was reported having anti-ulcer activity[22].

However, *L. australis* had protective activity against ulcerative colitis in low dose of 500 mg/kg when compared to *L. decipiens*, which only showed effectiveness at a doubled dose of 1000 mg/kg.

The reported anti-inflammatory effect of flavonoids[23] as well as of polysaccharides isolated from some plant organs[24] in addition to the reported nondigestible carbohydrates including some polysaccharides and oligosaccharides, that delivered intact to the large intestine, exerting prebiotic properties[19], may all enhance and synergist the protective activity against ulcerative colitis.

Table 5. Protective effect of methanol extracts of leaves of *L. decipiens* and *L. australis*on on acetic acid-induced ulcerative colitis

Group	*Median (IQR)*
Acetic acid (control)	*4 (3.75-4)*
L. decipiens 100 mg/kg	*3 (2-4)*
L. decipiens 500 mg/kg	*3 (2-3)*
L. decipiens 1000 mg/kg	*2* (2-3)*
Sulphasalazine 500 mg/kg	*2* (1.5-3)*
L. australis 100 mg/kg	*3 (2-4)*
L. australis 500 mg/kg	*2* (2-3)*
L. australis 1000 mg/kg	*2* (2-3)*
10% DMSO	*3 (3-3.25)*

Macroscopic and morphological scoring of the colon appearance
**=significant from acetic acid, (IQR) = (Inter quartile range)*

CONCLUSION

From this comparative study it could be concluded that both investigated *Livistona* species are rich in phenolic contents with great similarity in their constituents, while they showed difference in their carbohydrate content. However, *L. australis* had protective activity against ulcerative colitis in lower dose 500 mg/kg when compared to *L. decipiens*, which only showed effectiveness at a doubled dose 1000 mg/kg.

Conflict of Interest

The authors declare that they don't have any conflict of interest.

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