Original Research Paper

Characterization of Natural Products from the Peel of *Lagenaria siceraria* Fruit Using Chromatographic Techniques

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Herbal products, which comprise a wide variety of bioactive molecules, have been used as remedies for different diseases throughout history. *Lagenaria siceraria*, a fruit vegetable, is employed in folk medicine as a treatment for various disorders including diabetes, hyperlipidemia, and heart and liver ailments. In the present work, a number of compounds were isolated and characterized from the ethyl acetate fraction of the methanolic extract of its peel, including β-sitosterol, vanillin, quercetin, rutin, 3-*tert*-butyl-4-hydroxyanisole, stearic acid, 2,4-bis(1,1-dimethylethyl)phenol, 2,2′-methylene-bis[6-(1,1-dimethylethyl)-4-methylphenol], 1,2-benzenedicarboxylic acid mono(2-ethylhexyl) ester, hexadecanoic acid and its methyl ester, (*Z*,*Z*)-9,12-ocatdecadienoic acid and its ester, and (*Z*,*Z*,*Z*)-9,12,15-ocatdecatrienoic acid methyl ester. Separation of the phytochemicals was done using column and thin-layer chromatography, while gas chromatography–mass spectrometry (GC–MS), liquid chromatography-mass spectrometry (LC–MS), and high-performance liquid chromatography (HPLC) were employed for their identification. These compounds are being reported for the first time from the peel of the fruit of *L. siceraria*. The results provide a possible chemical rationale for the medicinal applications of this fruit.

Keywords: *Lagenaria siceraria*, peel, bioactive compounds

Introduction

Lagenaria siceraria (bottle gourd) is an herbaceous plant that grows as a vine and is cultivated around the globe, in various types of climatic environments, specifically in subtropical and tropical regions [1]. With the growing global trend towards herbal medicines, the plant has obtained a lot of folkloric attraction due to the beneficial effects it has. It is a popular vegetable with many other applications and been reported to be one of the first plants to be domesticated on earth [2].

L. siceraria is utilized for the treatment of many ailments. The pulp of the fruit is used both as an emetic and purgative, and is well-known for its cooling, diuretic, anti-bilious, and pectoral properties [1, 2]. The fruit of *L. siceraria* is also used for the treatment of rheumatism, insomnia, jaundice, diabetes, hypertension, congestive cardiac failure (CCF), ulcer, piles, insanity, colitis, and skin disease [3]. The fractions obtained from the methanolic extract of *L. siceraria* have been found to be pharmacologically active [4].

Natural products are diverse and, therefore, can prove to offer chemical diversity in tackling drug resistance. Moreover, these diverse molecules that have an activity against microbes might at the same time have a completely novel biochemical mechanism of action. *L. siceraria* contains high level of choline, polyphenols, fibers, and other nutritionally significant compounds. The literature points out that *L. siceraria* has been widely employed in research for medical purposes during the past few decades [6–9]. The plant has been well investigated for its phytochemicals, and a number of flavonoids, triterpenoids, polyphenols, fatty acids, and their esters, and a host of other compounds have been reported [5, 10–12]. Identification of bioactive compounds present in medicinal herbs is important in order to understand their mode of action as well as to predict new applications for them. In many cases, the chemical constituents in a plant are not in quantities sufficient for extensive spectroscopic characterisations required for identification. Chromatographic techniques as such (column, thin-layer chromatography [TLC], high-performance liquid chromatography [HPLC], etc.) or hyphenated with spectroscopic equipment (gas chromatography–mass spectrometry [GC–MS], LC–MS, etc.) offer a viable solution. Since its first discovery in Russia by Tswett in 1900 [17], chromatography has enjoyed supremacy in separation and isolation of phytochemicals over other techniques. In the present work, we used column chromatography (CC), TLC, HPLC, GC–MS, and LC–MS for isolation and identification of phytochemicals from the peel of *L. siceraria* fruit. After sub-fractionation by CC, TLC was used to isolate pure compounds. HPLC served to compare retention times of the isolated compounds with those of the standards leading to identification of the formers. GC–MS is a technique of choice for volatile substances, while LC–MS is useful to identify less volatile polar compounds such as flavonoids [18].

Materials and Methods

Chemicals and Reagents. Silica gel (100-200 mesh) used for column chromatography was from Sigma-Aldrich, St. Louis (Missouri, USA). Precoated TLC (silica gel 60 F254) and quercetin were purchased from Merck (Darmstadt, Germany). Rutin and β-sitosterol were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). The solvents used for extraction and fractionation were of HPLC grade.

Extraction and isolation. Fresh fruit of *L. siceraria* was obtained from an agricultural farm of Pattoki (Punjab, Pakistan). The plant was identified by Dr. Khalid Rasib, associate professor of Biological Sciences, FC College University, Lahore. Fresh peel (7.85 kg) of the fruit was separated, ground, and macerated in methanol for 20 days (3 × 15 L) in a glass flask, at room

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temperature with regular shaking. The methanolic extract was filtered and pooled. The solvent was evaporated in vacuo using vacuum-assisted rotary evaporator (Buchi, Flawil, Switzerland) to obtain the methanolic extract as a gummy material. It was dried in an incubator at 40 °C for 2 days. The methanolic extract (458 g) was subjected to liquid–liquid extraction using solvents of increasing polarity using separating funnel. The extract was suspended in distilled water and exhaustively extracted by partitioning with hexane, chloroform, ethyl acetate, and *n*-butanolic successively leaving behind residual aqueous fraction. As the ethyl acetate fraction proved to be most active in our previous studies [4], it was selected for isolation and identification of chemical compounds using column and thin-layer chromatography.

Column chromatography. The ethyl acetate fraction (11.2 g) was subjected to CC using silica gel as the stationary phase. The dimensions of the column used were 57.96 cm × 1.54 cm. Wet slurry method was used for column packing. Weighed silica (24.8 g) was immersed in hexane overnight and was sonicated for 1 h to remove trapped air. A small piece of glass wool was deployed at its base to prevent silica from contaminating the eluents. The column was filled with hexane, and the slurry was poured from above leaving the tap open. Constant tapping by the side aided proper setting and packing of the column. Hexane was eluted 3 times before loading the ethyl acetate fraction, which was covered with a small amount of the silica gel slurry. Elution started with pure hexane and then gradually increasing ratio of ethyl acetate, followed by combinations of ethyl acetate and methanol with increasing polarity (Table 1).

Thin-Layer Chromatography (TLC). Precoated TLC plates were used for further separation of the sub-fractions obtained from the column. The spots were scratched from the TLC plates and were dissolved in appropriate solvents and filtered. The filtrates were collected and analyzed using HPLC, GC–MS, and LC–MS. Various solvent systems were found appropriate for separation. The outcomes are shown in Table 2.

Locating agents used for TLC examination: (a) Folin–Ciocalteu reagent, (b) vanillin HCl (1 g vanillin + 10 mL conc. HCl), (c) vanillin H_2SO_4 (3 g vanillin + 95% CH_3OH [50 mL] + 2 M H_2SO_4 [15 mL]), and (d) 5% NaOH followed by drying for 1–2 min.

HPLC analysis. The compounds isolated from TLC were further purified and compared with their standards on HPLC. A flow rate of 1 mL/min was set on HPLC (Shimadzu [Kyoto, Japan] SPD-M10AVP) with an ultraviolet (UV) detector. Elution was done with mobile phase methanol–acetonitrile (90:10). The λ_{max} of the standard compounds was determined beforehand in a double beam UV spectrophotometer (Labomed Inc., Los Angeles, California, USA).

The λ_{max} of the standards was as follows: vanillin (250 nm), 3-*tert*-butyl-4-hydroxyanisole (290 nm), quercetin (254 nm), rutin (260 nm), β-sitosterol (206 nm), and stearic acid (245 nm).

LC–MS analysis. Liquid chromatography (LC) Agilent 1100 series (Bremen, Germany) fitted with binary pump, an auto-sampler with a 100 μL loop, a diode array detector set for recording at 254, 280, and 320 nm, and scanning from 200 to 600 nm was employed for the analysis. Ion-trap MS, fitted with an electrospray ionization (ESI) source, Bruker Daltonics HCT Ultra (Bremen, Germany) operating in full-scan with source temperature of 550 °C, spray voltage of −4500 V, declustering potential tuned to −255 eV, collision energy of −44 eV, entrance potential of −10 eV, cell exit potential of −35 eV, collision activated dissociation of 5 psi, curtain gas at 15 psi, mass dwell time of 400 ms, unit mass resolution of 0.1 *m*/*z*, operated in a quadrupole analyzer at <400 *m*/*z*, scan speed to 1000 *m*/*z* per second, and auto-MSn mode resulted in a fragment ion *m*/*z* pattern. The maximum fragmentation amplitude was set at 1 V, starting at 30% and ending at 200%. MS operating conditions were in negative mode and were optimized using 5-ocaffeoylquinic acid with a capillary temperature of 365 °C and a dry gas (nitrogen) flow rate of 10 L/min. The nebulizer pressure was set at 10 psi. Internal calibration was done with 10 mL of 0.1 M sodium formate solution injected through a six-port valve prior to each chromatographic run. Calibration was done using the enhanced quadratic mode. Solvent system used for elution was methanol–acetonitrile (90:10).

GC–MS analysis. GC–MS analysis of the isolated compounds was done on GC–MS Varian (Palo Alto, California, USA). Column specification was the following: 30 m × 0.25 mm and 0.25 μm thickness packed with 5% diphenyl–95% dimethyl polysilosane cross bonded liquid phase (DB-5) in a capillary column. The carrier gas was research grade helium (99.999%) sent with a flow rate of 28.6 cm/s (1 mL/min). GC–MS was run in splitless mode with a run time of 47 min and a restabilization time of 5 min. The samples were kept at room temperature before analysis. They were heated in the initial compartment at 80 °C for 5 min. The MS scan mode was set at *m*/*z* 40 to 650, and the GC injector temperature was 260 °C. Main oven temperature was 50 °C (1 min), and it was programmed to increase to 260 °C at a ramping rate of 5 °C/min.

Table 1. Solvent systems used as mobile phase for column chromatography (200 mL of each combination was used)

Mobile phase			Sub-fraction obtained
S. no.	Hexane (%)	Ethyl acetate (%)	
1	100	0	Frac1
2	90	10	Frac2
3	80	20	Frac3
4	70	30	Frac4
5	60	40	Frac5

Frac, fraction.

Results and Discussion

The present study was carried out as part of our endeavours to discover new therapeutic agents that are more effective, safer, and readily available to common people [2, 4, 5]. The

Table 2. TLC analysis of sub-fractions obtained from column chromatography of ethyl acetate fractions *L. siceraria* fruit peel

Eluate from column	Mobile phase for TLC	R_F value	Compound isolated
Frac1	BAW (4:1:5)	0.48	β-Sitosterol
Frac2	BAW (4:1:5)	0.69	Stearic acid ester
Frac3	EA–Hex (3:2)	0.46	Rutin
Frac4	BAW (4:1:5)[a]	0.51	Vanillin, obtained as needle like crystals
	EA (100%)	0.43	3-*tert*-Butyl-4-hydroxyanisole
Frac5	EA–MeOH (4:1)	0.53	Quercetin

[a]Appeared as crystals in the eluate, which were collected by removing the solvent.
BAW, 1-butanol–acetic acid–water; EA, ethyl acetate; MeOH, methanol; Hex, hexane; Frac, fraction.

Table 3. Identification of four isolated compounds from ethyl acetate fraction of *L. siceraria* fruit peel by GC–MS analysis

Retention time (min)	Percentage (%)	Compound	Molecular formula	Molecular mass
10.985	66.359	Vanillin	$C_8H_8O_3$	152
11.724	31.836	3,4-Dimethoxybenzaldehyde methylmonoacetal	$C_{10}H_{14}O_4$	198
17.671	24.521	Stearic acid, methyl ester	$C_{19}H_{38}O_2$	298
11.580	95.068	3-*tert*-Butyl-4-hydroxyanisole	$C_{11}H_{16}O_2$	180

methanolic extract of the ground dried peel of the fruit of *L. siceraria* was subjected to fractionation into solvents of increasing polarity. The ethyl acetate fraction so obtained was subjected to column chromatography, and the sub-fractions so obtained were then analyzed and separated by TLC. Characterization of the isolated compounds was carried out based on GC–MS, HPLC, and LC–MS.

The findings are displayed in Tables 1–5. The sub-fraction Frac1 obtained from the column (Table 1) showed a number of spots on TLC, a major one at R_F 0.48 with solvent system BAW (1-butanol–acetic acid–water, 4:1:5) proved to be β-sitosterol upon HPLC analysis. Frac2 showed a compound at R_F 0.69 with solvent system BAW (4:1:5). It was stearic acid ester as detected in GC–MS. Frac3 contained rutin as identified by LC–MS and HPLC. The sub-fraction Frac4 contained vanillin and 3-*tert*-butyl-4-hydroxyanisole as identified by GC–MS. Quercetin was obtained by TLC analysis of Frac5 (mobile phase ethyl acetate–methanol [4:1]) and proved to be quercetin by HPLC and LC–MS.

HPLC analysis. The results of HPLC analysis of isolated compounds are shown in Table 3. The comparison of the retention times (RTs) of standard vanillin (RT = 3.131 min) and isolated one (RT = 3.121 min) indicated their identity, which was further confirmed by GC–MS.

The HPLC scan of standard stearic acid (RT = 7.417 min) and isolated one (RT = 7.413 min) showed similarity in the retention times. This was reconfirmed by GC–MS data. The saw-like pattern in the UV scan (380 to 570 nm range) is a prominent characteristic of fatty acids [13].

The compound 3-*tert*-butyl-4-hydroxyanisole and its standard had similar retention times (3.529 and 3.489 min, respectively). The structure was verified by GC–MS. Comparing the HPLC data of standard quercetin (RT = 6.377 min) and the isolated one (RT = 6.352 min) suggested its presence, which was verified by LC–MS. Quercetin, a natural antioxidant, is a flavonoid in nature. It has been previously isolated from the mesocarp of *L. siceraria* [5, 14].

The HPLC comparison also indicated the presence of rutin, which is a quercetin glycoside. It was obtained at RT of 3.077 min, which matched with the standard [14, 15]. The compound was further established using the MS fragmentation pattern from LC–MS.

Similarly, the HPLC chromatograms of isolated and standard β-sitosterol matched. The λ_{max} of both the standard and the isolated compound was at 206 nm, which further confirmed their identity. This compound has been reported from the fruit in the past [14].

GC–MS analysis. Four compounds isolated by TLC and HPLC were clearly identified by GC–MS analysis. They were vanillin, 3,4-dimethoxybenzaldehyde methyl monoacetal, octadecanoic acid methyl ester, and 3-*tert*-butyl-4-hydroxyanisole (Table 3).

LC–MS analysis. The LC–MS has been found especially useful to identify phytochemicals present in small amounts not sufficient for spectroscopic techniques [16]. In the present work, structures of quercetin and rutin were elucidated by LC–MS. The isolated compounds were dissolved in methanol. The solvent system used for the LC–MS analysis was methanol–acetonitrile (90:10. The data are tabulated in Table 4.

Quercetin (LC–MS RT = 5.95 min) showed a peak at m/z = 303.24. This was M + 1 peak. The fragmentation also showed M + 2 peak and another one at m/z = 613.30, which may be due to a dimeric adduct having a sodium atom and water molecule (302 + 302 + 23 − 18).

Rutin was identified as follows. The LC–MS peak at RT = 5.93 min showed a fragmentation pattern with a peak at m/z 303.28, indicating a quercetin unit, the aglycone of rutin. The peak at m/z = 334.00 was due to a condensation product of two rhamnose moieties. The m/z = 465.07 was due to quercetin attached to one rhamnose moiety (302 + 164). The peak at m/z 633.29 was possibly an Na salt of rutin.

Table 4. LC–MS analysis of the compounds isolated from the ethyl acetate fraction of the peel of *L. siceraria*

Retention time (min)	m/z	Compound indicated
5.95	303.24, 304.26, 613.30	Quercetin
5.93	303.28, 304.32, 334.00, 465.07, 633.29, 634.25	Rutin

Table 5. Compounds detected in the GC–MS analysis of Frac4 of the peel of *L. siceraria.* Two layers appeared when 1 mL of Frac4 was diluted with the same solvent system

Retention time (min)	Percentage	Compound	Molecular formula	Molecular mass
Lower layer which was oily				
6.920	58.748	Benzene, 1-methyl-4-(1-methylethyl)	$C_{10}H_{14}$	134
13.645	19.791	Heptadecane, 2,6,10,15-tetramethyl-	$C_{21}H_{44}$	296
13.845	21.461	Phenol, 2,4-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	206
18.343	8.315	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270
18.849	11.954	Hexadecanoic acid	$C_{16}H_{32}O_2$	256
19.982	6.880	9,12-Octadecadienoic acid (*Z*,*Z*)-, methyl ester	$C_{19}H_{34}O_2$	294
20.057	7.769	9,12,15-Octadecatrienoic acid, methyl ester (*Z*,*Z*,*Z*)	$C_{19}H_{32}O_2$	292
20.432	5.126	9,12-Octadecadienoic acid (*Z*,*Z*)	$C_{18}H_{32}O_2$	280
22.853	2.382	2,2′-Methylenebis[6-(1,1-dimethylethyl)-4-methylphenol]	$C_{23}H_{32}O_2$	340
23.897	46.830	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	$C_{16}H_{22}O_4$	278
Upper layer				
18.612	20.878	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_4$	270
19.125	27.342	Hexadecanoic acid	$C_{16}H_{32}O_2$	256
20.244	18.526	9,12-Ocatdecadienoic acid (*Z*,*Z*)-, methyl ester	$C_{19}H_{34}O_2$	294
20.326	20.395	9,12,15-Ocatdecatrienoic acid, methyl ester (*Z*,*Z*,*Z*)	$C_{19}H_{32}O_2$	292
20.713	12.859	9,12-Octadecadienoic acid (*Z*,*Z*)	$C_{18}H_{32}O_2$	280

NMR Spectral Study. Only four isolated compounds were in sufficient quantity to obtain their proton nuclear magnetic resonance (^{1}H-NMR) (300 MHz; $CDCl_3$) spectra, which matched with the reported ones and, hence, confirm their structures. They were stearic acid, vanillin, 3-*tert*-butyl-4-hydroxyanisole, and β-sterol.

GC–MS analysis of column eluate Frac4. The sub-fraction Frac4 obtained form the column was chosen for GC–MS analysis, as it gave a very prominent fluorescent spot on the TLC. Upon GC–MS screening, a number of compounds were clearly identified (Table 5). Most detected compounds were non-polar in nature or have long non-polar chain. Mostly, they were fatty acids and their esters.

The compounds containing phenolic rings are important for explaining the anti-bacterial effect of the peel, and this lures scientific interest as the plant is believed to be an anti-bacterial agent against mild bacterial infections.

Conclusion

The present study reports isolation and identification of compounds from ethyl acetate fraction of the methanolic extract of the peel of *L. siceraria* fruit; they included two flavonoids, quercetin and rutin, β-sitosterol, vanillin, 3-*tert*-butyl-4-hydroxyanisole, and a fatty acid. They are medicinally important substances, which support the use of this vegetable as a folkloric medicine. Besides this, a number of other compounds have also been identified by GC–MS analysis.

Conflict of Interest

The authors have declared no conflict of interest.

Abbreviations

HPLC: high performance liquid chromatography, GC–MS: gas chromatography–mass spectrometry, LC–MS: liquid chromatography-mass spectrometry, Frac: fraction, TLC: thin-layer chromatography; BAW: *n*-butanol–acetic acid–water

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