



Triterpenoid, flavonoids and sterols from *Lagenaria siceraria* fruits

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Abstract

The bioactive ethyl acetate and n butanol soluble parts of successive methanol extract of fruits of *Lagenaria siceraria* were subjected to column chromatography over silica gel (60-120 mesh) for separation of different phytoconstituents. Two flavonoids, a triterpenoid and a mixture of sterols were isolated. The structures I-V, were obtained through spectral analyses as oleanolic acid, β sitosterol, campesterol, isoquercitrin (quercetin-3-O- β -D-glucose) and kaempferol respectively. Their structures were elucidated by means of UV, IR, mass, and NMR spectroscopic analyses. These compounds were identified by comparison of their $^1\text{H-NMR}$, $^{13}\text{C-NMR}$ and mass spectra with the literature. The isolation of oleanolic acid, kaempferol and isoquercitrin from *Lagenaria siceraria* fruits is being reported first time. These compounds may explain the medicinal value of fruits of *Lagenaria siceraria*.

Key Words: *Lagenaria siceraria*, flavonoids, saponins, isoquercitrin, oleanolic acid.

Introduction

Fossil records date human use of plants as medicines at least to the middle Paleolithic age, some 60,000 years ago [1]. Plant material or its extracts contain a complex mixture of different components and it is necessary to separate them before exploring them further. *Lagenaria siceraria* (Molina) Standley (family cucurbitaceae), commonly known as lauki (Hindi) and bottle gourd (English), is a medicinal plant and utilizable species [2]. The plant enjoys the reputation of being one of the earliest plants to be domesticated on the earth [3]. It is a climbing or trailing herb with bottle, oval or dumbbell shaped fruit [4]. *Lagenaria siceraria* fruit is traditionally used for its cardioprotective, cardiotonic, general tonic and aphrodisiac properties [5]. It is also used in treatment of various allergic and inflammatory disorders like bronchial asthma, rhinitis, bronchitis [6] and rheumatism [7].

Few studies validating the folk medicinal uses of *Lagenaria siceraria* fruits have been reported. Various extracts of fruits of *Lagenaria siceraria* were found to have anti-inflammatory, analgesic [8], hepatoprotective [9], antihyperlipidemic [4], diuretic [10] and antibacterial activities [11]. Earlier researchers have reported different classes of chemicals in the fruits of *Lagenaria siceraria*. Methanol extract of fruits was found to contain flavonoids, saponins, tannins, carbohydrates and terpenoids [12]. More detailed investigations have revealed the presence of fucosterol and campesterol in fruits [10]. A novel ribosome inactivating protein, lagenin has been isolated from seeds of *Lagenaria siceraria* [10]. Fruits are reported to contain more soluble dietary fibers than insoluble cellulose fibers⁴. The fruits are considered as good source of vitamin C, β -carotene, vitamin B-complex, pectin and also contain highest choline level- a lipoprotic factor [13]. Few of these primary and secondary metabolites have been implicated in the reported uses of *Lagenaria siceraria* fruits. Previously we have reported the immunomodulatory activity of ethyl acetate and n butanol soluble parts of the successive methanol extract of *Lagenaria siceraria* fruits [12] and immunomodulatory activity of purified saponin mixture extracted from fruit of *Lagenaria siceraria* [14]. In spite of the numerous medicinal uses attributed to this plant, HPTLC study of different fractions of methanol extract of fruit of *Lagenaria siceraria* has not been published. To our knowledge there had been no documented evidence for the isolation of triterpenoid and flavonoids from these bioactive fractions. Hence, the present investigation is an attempt in this direction and includes phytochemical screening and HPTLC study of bioactive fractions of methanol extract of *Lagenaria siceraria* fruit and the isolation of two flavonoids, a triterpenoid and a mixture of sterols from bioactive ethyl acetate and n butanol soluble parts of successive methanol extract.

Materials and Methods

General experimental procedures

UV spectra of the isolated compounds were recorded in methanol over a scanning range of 200-400 nm and λ_{\max} of compounds were determined. Spectra were recorded with a Shimadzu double beam (UV -1700 E Pharma spec) UV-VIS spectrophotometer. IR spectra were obtained using JASCO FT IR 5300 (Japan) in KBr disc and absorption peaks in terms of wave numbers (cm⁻¹) were noted for each constituent in methanol. EIMS (electron impact mass spectrum) in positive mode, were recorded on Shimadzu LCMS 2010A (Japan) instrument. NMR spectra were acquired on Bruker supercon multi nuclei probe spectrophotometer (West Germany) at 300 MHz (¹H) and 75 MHz (¹³C). Chemical shifts were recorded as δ value (ppm) using TMS (tetra methyl silane) as internal standard. The spectra were observed on CDCl₃ and /or DMSO.D6.

Plant materials

The fresh fruits of *Lagenaria siceraria* were collected from outfield of Junagadh city, Gujarat state, India in March 2007. Plant was authenticated by the authority of department of botany, Bahauddin college, Junagadh, where a voucher specimen (BS/Bot./I-5/06-07) has been deposited for future reference.

Extraction

The air dried powdered fruits (1000g) were extracted exhaustively with petroleum ether (60-80⁰C) and then with methanol in a soxhlet apparatus. The methanol extract was concentrated by distilling off the solvent and evaporated to dryness. The residue (138 gm) was suspended in

water, extracted successively with ethyl acetate and n butanol (6×300 ml each) and then resulting solutions were concentrated to provide ethyl acetate (6.4gm), n butanol (29gm) and water soluble (101gm) parts.

Phytochemical screening

Methanol extract, its ethyl acetate and n butanol soluble fractions were tested for the presence of alkaloids, saponins, tannins, flavonoids, anthraquinones and sterols according to standard procedures. TLC (silica gel G 60 F254 TLC plates of E. Merck, layer thickness 0.2mm) fingerprint profile was established for the ethyl acetate and n butanol soluble fractions of methanol extract of *Lagenaria siceraria* fruit. Samples were spotted on TLC plates using Camag Linomat V automatic sample spotter. Solvent systems; n butanol: acetic acid: water (4:1:5) and chloroform: methanol (90:10) were used respectively for ethyl acetate and n butanol fractions. N butanol plate was then scanned (using TLC scanner 3, Camag) at 290 nm and then sprayed with vanillin sulphuric acid and heated at 100⁰C for 5 minutes and then again scanned at 580 nm. Photos of the plate were taken at 254 nm and 366nm before derivatization with detecting agent and at 366 nm and at visible range after derivatization (Fig 1). Number of spots, R_f values and % relative areas were recorded (Table 1).

Fig 1 HPTLC of n butanol fraction

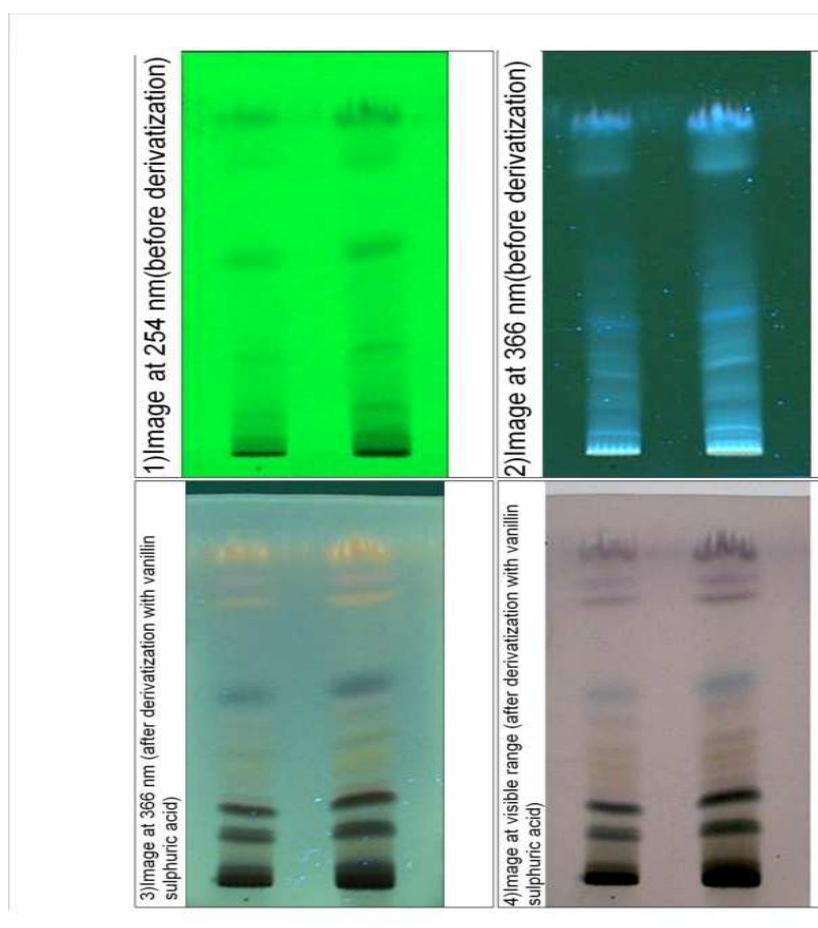


Table 1. TLC finger printing for n butanol and ethyl acetate soluble fraction

Scanning wavelength and mode	n butanol R _f	% relative area	Scanning wavelength and mode	Ethyl acetate R _f	% relative area
254nm, absorption	0.10	66.90	290nm, absorption	0.07	17.02
	0.26	6.90		0.14	37.60
	0.47	1.80		0.46	21.46
	0.54	13.36		0.74	20.56
	0.82	3.41		0.91	2.55
	0.94	7.63		600nm, absorption	0.36
366nm, fluorescence	0.05	21.19	0.47	20.05	
	0.15	10.32	0.74	6.28	
	0.19	3.23	0.91	0.81	
	0.22	21.24			
	0.35	18.15			
	0.46	6.02			
	0.53	4.40			
	0.81	1.98			
580nm, absorption	0.95	12.70			
	0.13	53.37			
	0.22	16.30			
	0.33	1.07			
	0.38	1.34			
	0.47	2.06			
	0.55	4.74			
	0.82	4.22			
	0.88	4.72			
	0.96	12.17			

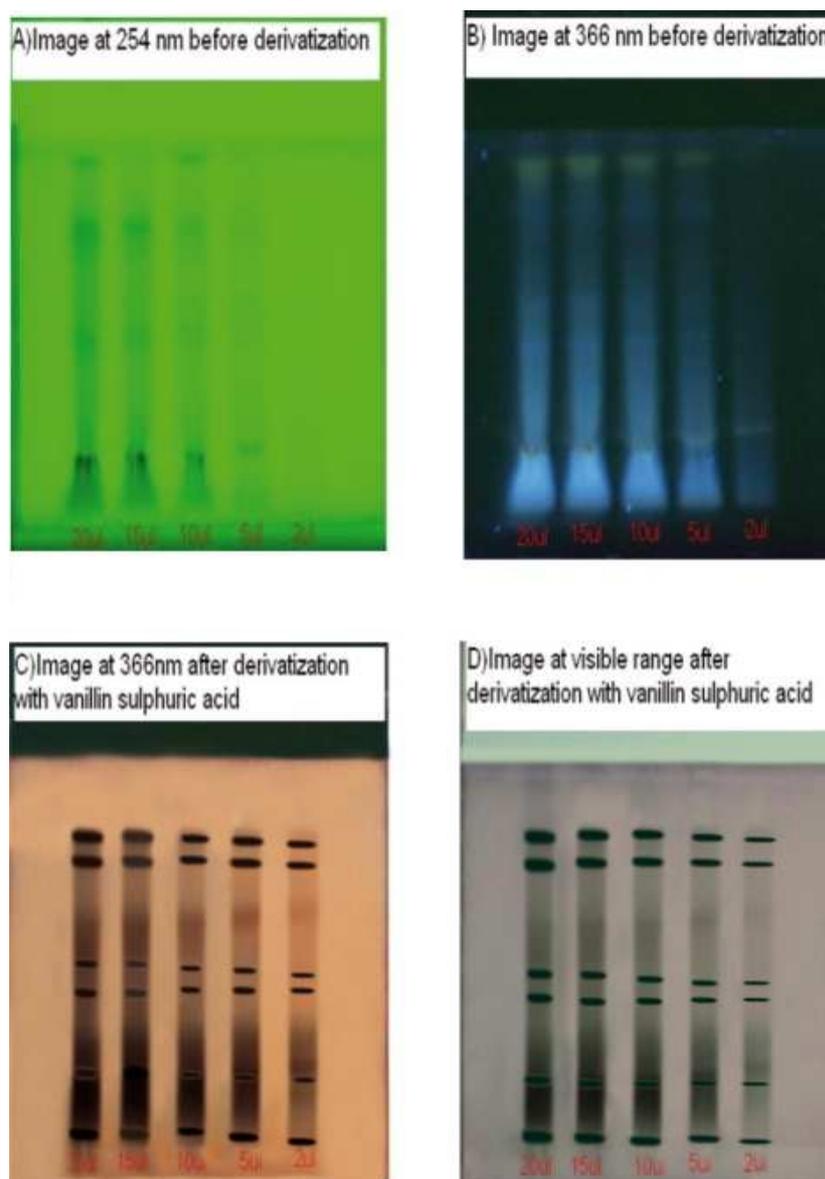
Ethyl acetate plate was also removed from chamber and dried. It was then scanned at 290 nm and then sprayed with anisaldehyde sulphuric acid and heated at 110°C for 5 minutes and then again scanned at 600 nm. Photos were taken at 254 nm and 366nm before derivatization with detecting agent and at 366 nm and at visible range after derivatization (Fig 2). Number of spots, R_f values and % relative areas were recorded (Table 1). Plate was also treated separately with 1 % AlCl₃ in methanol and observed under UV light.

Isolation

The n butanol soluble part (5gm) and ethyl acetate soluble part (5gm) were subjected separately to silica gel (60-120 mesh) column (60cm x 4.5 cm) chromatography for the isolation of phytoconstituents. n butanol soluble part was eluted gradiently with chloroform, chloroform-methanol mixtures 95:5, 90:10, 80:20, 50:50 and methanol. At uniform interval, the eluents (each of five ml) were collected and the progress of separation was monitored by thin layer chromatography (TLC) (silica gel G 60 F₂₅₄ TLC plates of E. Merck, layer thickness 0.2mm) using solvent system chloroform: methanol (90:10) and iodine vapor as detecting agent. Fractions eluted with chloroform: methanol (95:5) and 18-25 fraction of chloroform: methanol (90:10), which showed single spot on TLC, afforded the compound I (0.85 gm). Fraction 26-39

eluted with chloroform: methanol (90:10) afforded a mixture (1.1gm) of compound II and III. The same procedure was repeated for ethyl acetate soluble part using chloroform, chloroform: ethyl acetate (50:50), ethyl acetate, ethyl acetate: methanol (90:10) and methanol to give compound IV (0.6 gm) and V (0.275 gm). These compounds have shown single spot on TLC plate where the chromatograms were developed in n butanol: acetic acid: water (4:1:5) and the plates were sprayed with anisaldehyde sulphuric acid and heated for 5 minutes at 110°C. These compounds were purified by recrystallization from methanol.

Fig 2 HPTLC of ethyl acetate fraction



Results

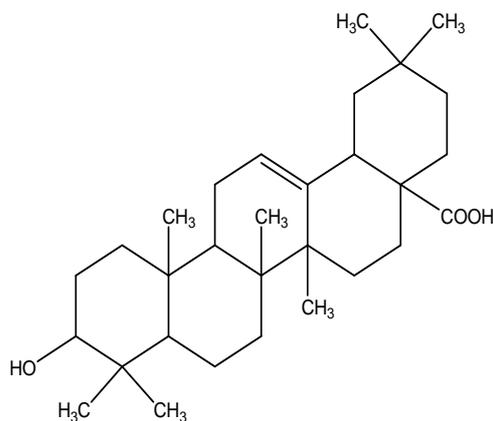
Phytochemical screening

Preliminary phytochemical screening of methanol extract showed the presence of flavonoids, saponins, sterols, tannins and carbohydrates. n butanol soluble fraction found to contain sterols, terpenoids and saponins while flavonoids and tannins were found in ethyl acetate soluble fraction of methanol extract. In HPTLC study, spots with chromatographic behavior identical to flavonoids (yellow color when exposed to uv light) and sterols (blue color) were observed for ethyl acetate and n butanol fractions respectively. HPTLC finger print profiles of ethyl acetate and n butanol fractions were recorded. Two yellow colored spots were seen in plates treated with 1% AlCl_3 .

Isolation

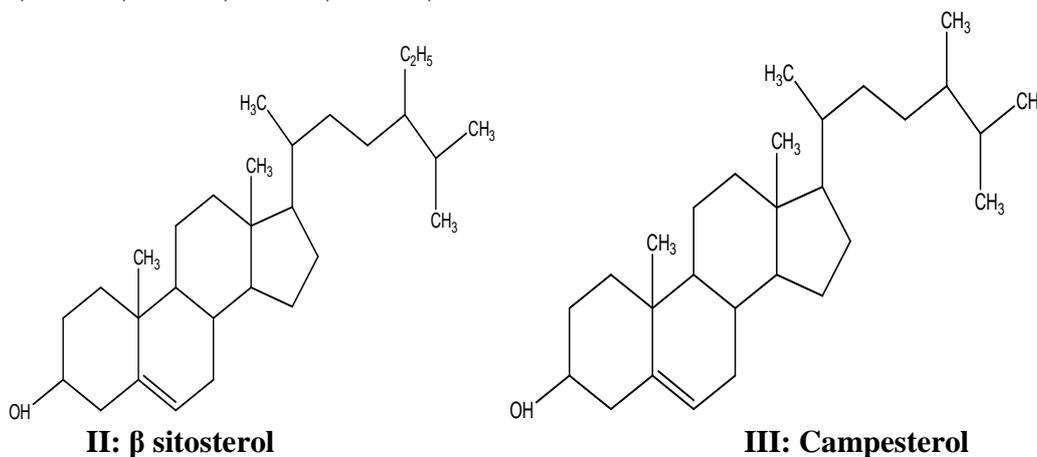
Flavonoid rich-ethyl acetate and saponin rich-n butanol soluble parts of successive methanol extract of *Lagenaria siceraria* fruits were submitted to silica gel column chromatography to yield compounds I–V. All the compounds were identified by IR, UV, $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ techniques. Compound I, II, III, IV and V were identified as oleanolic acid, β sitosterol, campesterol, isoquercitrin and kaempferol respectively by direct comparison of the spectra with those reported in literature [15-22].

Compound I: Isolated from n butanol soluble part of successive methanol extract of *Lagenaria siceraria* fruits as a white amorphous powder. UV_{max} : 202, $^1\text{H-NMR}$: δ 0.63-1.07(7 CH_3), 2.73(1H,d, $J=13.6$ Hz,H-18), 3.13(1H,dd, $J=9.6$ Hz,H-3), 5.22(1H, $J=3.6$ Hz,H-12). $^{13}\text{C-NMR}$: δ 15.13(C-25), 15.52(C-24), 16.88(C-26), 18.15(C-6), 22.84(C-11), 23.17(C-30), 23.43(C-16), 25.71(C-27), 26.97(C-2), 27.52(C-15), 27.98(C-23), 30.50(C-20), 32.29(C-22), 32.53(C-7), 32.94(C-29), 33.73(C-21), 36.84(C-10), 38.27(C-1), 38.56(C-4), 39.13(C-8), 40.94(C-18), 41.52(C-14), 45.87(C-17), 46.01(C-19), 47.42(C-9), 55.05(C-5), 78.42(C-3), 121.93(C-12), 144.5(C-13), 180.29(C-28). EI-MS m/z (relative intensity): 457, 439, 411, 247, 203, 191, 177. IR: 3468.32, 2945.57, 2862.62, 2652.36, 1697.51, 1464.10, 1386.94, 1363.80, 1323.29, 1304.00, 1273.13, 1238.41, 1209.48, 1184.40, 1091.81, 1032.01, 997.29, 949.06, 918.20, 885.41, 825.61, 754.23, 679.00, 655.86, 640.42, 603.77, 565.19.

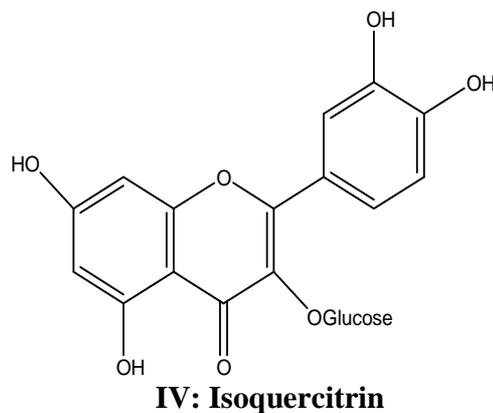


I: Oleanolic acid

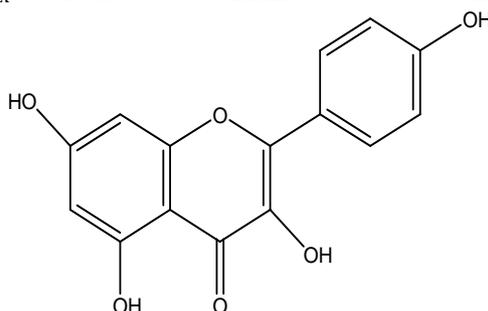
Mixture of compound II and III: Isolated from n butanol soluble part of successive methanol extract of *Lagenaria siceraria* fruits as a white crystalline powder. UV_{max} : 203, 1H -NMR: δ 5.33(1H, H-6), 0.68 (3H, s, Me-18), 1.14(3H, s, Me -19), 1.01(3H, d, Me-21), 0.82(3H, d, $J=6.8$ Hz, Me-26), 0.80(3H, d, $J=6.4$ Hz, Me-27), 0.84(3H, t, $J=6.6$ Hz, Me-29). ^{13}C -NMR: δ 11.92 (C-18), 11.97(C-29), 18.77(C-21), 18.85(C-27), 19.22(C-19), 19.51, (C-28), 21.16(C-11), 23.15(C-28), 24.37 (C-15), 26.2(C-23), 28.31(C-16), 29.26(C-25), 31.69(C-2), 31.98(C-7), 32.05(C-8), 32.47(C-22), 34.03(C-20), 36.21(C-10), 36.57(C1), 37.35(C-14), 39.86(C-12), 42.38(C-13), 45.91(C-24), 50.22(C-9), 56.15(C-17), 56.84(C-14), 77.42(C-3), 121.71(C-6), 140.84(C-5). EI-MS m/z (relative intensity): 414, 399, 397, 395, 383, 369, 353, 303, 301, 275, 263, 247, 219, 205, 192, 181, 153, 123, 101, 93, 80, 69, 54. I R: 425.89, 2935.92, 2868.41, 1639.64, 1466.03, 1379.23, 1240.34, 1192.12, 1134.25, 1107.24, 1060.94, 1022.36, 958.71, 839.11, 800.53, 738.80, 626.92.



Compound IV: Isolated from ethyl acetate soluble part of successive methanol extract of *Lagenaria siceraria* fruits as green amorphous powder. UV_{max} : 202, 255, 1H -NMR: δ 3.39-5.33(7H, m, 7H of sugar), 5.11(1H, d, $J=7.3$ Hz, H-1''), 6.30(1H, s, H-6), 6.30(1H, s, H-8), 6.83 (1H, d, $J=9.0$ Hz, H-5'), 7.73(1H, s, $J=2.1$ Hz, H-2''), 7.77(1H, dd, $J=2.1$ Hz, 9.0 Hz, H-6''), 12.29 (1H, s, OH-5'). ^{13}C -NMR: δ 60.14(C-6''), 68.56(C-4''), 72.91(C-2''), 75.48(C-3''), 76.77(C-5''), 92.57(C-8), 97.82(C-6), 102.07(C-1''), 103.05(C-10), 114.05(C-5'), 115.65(C-2'), 120.08(C-1'), 120.29(C-6'), 132.93(C-3), 143.29(C-3'), 147.28(C-4'), 155.45(C-9), 156.10(C-2), 160.23(C-5), 163.22(C-7), 176.51(C-4). EI-MS m/z (relative intensity): 465, 303. IR: 3260.00, 1660.86, 1606.85, 1504.61, 1454.46, 1359.94, 1304.00, 1197.90, 1060.94, 1010.79, 933.63, 798.60, 709.87, 644.28, 592.20.



Compound V: Isolated from ethyl acetate soluble part of successive methanol extract of *Lagenaria siceraria* fruits as light yellow amorphous powder. $^1\text{H-NMR}$: δ 6.12 (1H, *d*, $J=2.3$ Hz, H-6), 6.32 (1H, *d*, $J=2.3$ Hz, H-8), 6.86 (2H, *d*, $J=8.6$ Hz, H-3' and H-5'), 7.97 (2H, *d*, $J=8.6$ Hz, H-2' and H-6'). $^{13}\text{C-NMR}$: δ 93.20 (C-8), 98.07 (C-6), 102.95 (C-10), 115.11 (C-3' and C-5'), 121.67 (C-1'), 129.13 (C-2' and C-6'), 135.49 (C-3), 146.27 (C-2), 156.12 (C-9), 158.89 (C-4'), 160.65 (C-5), 163.67 (C-7), 175.54 (C-4). EI-MS m/z (relative intensity): 287, 288, 319, IR: 3317.86, 1662.79, 1612.64, 1568.27, 1506.54, 1440.96, 1383.09, 1302.07, 1251.91, 1224.91, 1176.68, 1087.95, 1008.86, 974.14, 883.48, 846.83, 817.89, 794.74, 723.37, 680.93, 621.13, 584.48, 495.75, 408.95. UV_{max} could not be determined because of insufficient quantity.



V: Kaempferol

Discussion

In the present investigation, methanol extract of *Lagenaria siceraria* fruits and its different fractions were subjected to phytochemical screening. Phytochemical screening showed the presence of variety of primary and secondary metabolites. TLC findings were in agreement with the data of qualitative chemical tests and the spots characteristic for flavonoids and saponins were observed. TLC fingerprint profile was established for the varied fractions of bioactive methanol extract. Column chromatography was performed to separate phytoconstituents from bioactive *n* butanol and ethyl acetate fractions by applying variety of mobile phases. Total five compounds were isolated in significant amount and their characterization was done using different spectral techniques ($^1\text{H-NMR}$, $^{13}\text{C-NMR}$, IR and UV analyses) based on the following deliberations, to reveal their identity as oleanolic acid (I), β sitosterol (II), campesterol (III), isoquercitrin (VI) and kaempferol (VII):

Compound I

Compound I was obtained as a white amorphous solid which gave positive Liebermann-Burchard test for triterpenes. The presence of Δ^{12} -oleane skeleton was confirmed in the $^{13}\text{C-NMR}$ spectrum with the signals in the region δ 15.13-34 ppm, at δ 121.93 and at δ 144.56 attributed respectively to seven methyl groups, to C-12, C-13 and Δ^{12} -oleane skeleton. The assignments of various carbon atoms in $^{13}\text{C-NMR}$ spectrum were made through comparison with published spectrum of oleanolic acid. The presence of this skeleton was also supported by the mass spectrum of the compound, which showed prominent fragments ion peak characteristic of cleavage of B and C rings at m/z 247 and 203. The ion fragment at m/z 203 indicated the presence of a carboxylic group in the ring D.

The base peak appeared at m/z 439 characteristic for pentacyclic triterpene of β amyrin series with a 12-13 double bond. It showed peak at m/z 457, in agreement with the molecular formula

$C_{30}H_{48}O_3$. IR spectrum of the compound showed characteristic bands at 3482.32(OH), 1697.51(olefinic C=C). The UV spectrum showed absorption band at λ_{max} 202 nm. 1H -NMR spectrum showed the presence of an olefinic proton resonating at δ 5.22(1H) (typical of Δ^{12} -oleane skeleton and a carbinyl proton at δ 3.13 (1H) which were assigned to H-12 and H-3 α respectively. The same spectrum also showed singlet for seven methyls in the region δ 0.0630-1.0740. Based on above data and by comparing its spectroscopic data with those reported in literature, compound was deduced as (C₃₀H₄₈O₃, 456.71).

Compound II and III

Mass spectrum of the mixture was characterized by protonated molecular ions (m/z 397) of the compound and an abundant signal corresponding to fragment ion due to the loss of a water molecule. A signal at m/z 383 was attributed to the $[M+Z-H_2O]^+$ ion of campesterol. Intense fragment ion m/z 397 was observed for β sitosterol indicating the loss of water $[M+Z-H_2O]^+$. It seems to be characteristic for sterols as dehydration was observed in earlier reports with phytosterols. Peaks at m/z 400, 383, 367, 275 and 263 were found to be identical to the corresponding mass spectrum of campesterol. The IR spectrum showed the presence of a hydroxyl group (3425.89 cm^{-1}) and c=c (1639 cm^{-1}) indicating unsaturation in the molecule. A band at 2935.92 cm^{-1} shows the presence of angular methyl group. The UV maximum was observed at 203nm. Mixture gave positive Liebermann-Burchard test for sterols. In the 1H -NMR spectrum of the mixture, a one-proton signal appeared at δ 5.33 which indicated the presence of an olefinic proton at C-6 owing to the double bond between C-5 and C-6. Singlets at δ 0.68 and 1.14 for the angular methyl groups C-18 and C-19 and doublets at δ 1.01, 0.82, 0.80 and a triplet at 0.84 confirm the presence of C-21, C-26, C-27, and C-29 methyl groups' respectively. ^{13}C -NMR showed signals corresponding to sterol nucleus. On the above deliberations, isolate was deduced as mixture of β sitosterol and campesterol.

Compound IV

^{13}C -NMR spectrum of the compound showed signals which suggested the presence of a flavonoids moiety and a sugar moiety. The sugar moiety was determined to be β glucose from analyses of 1H and ^{13}C -NMR spectroscopic data. The β -anomeric configuration of compound IV was derived from coupling constant ($J=6.9$) for glucoside. In mass spectrum, the signal at m/z 465 corresponded to the $[M+H]^+$ pseudomolecular ion, while $[A+H]^+$ at m/z 303 (which is a protonated molecule of quercetin), the most important peak was attributed to the quercetin aglycone [464-162+H]. Compound was unambiguously identified as isoquercitrin (C₂₁H₂₀O₁₂) based on its intense molecular ion $[M+H]^+$ peak at 465. IR spectrum showed a strong absorption band at 1660.86 cm^{-1} for a carbonyl and an intense broad band at 3260.00 cm^{-1} . UV spectrum showed absorption bands at 202 and 255 nm typical of 3-substituted flavonols. The aromatic region of the 1H -NMR spectrum of the compound has the characteristic set of signals of quercetin moiety. 1H -NMR signals at δ 6.14 (1H, s), 6.30(1H, s) are due to metacoupled protons of A ring (H-6 and H-8) of a flavonoids nucleus (5,7 dihydroxy flavonol). Signals at δ 6.83, d, $J=9Hz$; 7.73, d, $J=2.1Hz$ and 7.77, d $J=9Hz$ are assigned to H-5', H-2' and H-6' of the ring B respectively. This indicates 3', 4' dihydroxy structure corresponding to aromatic ring B of quercetin nucleus. A doublet at 5.11 ppm due to proton of anomeric carbon (C-1'') of the sugar moiety indicated unequivocally the configuration for the sugar (glucose) of isoquercitrin. 1H and ^{13}C -NMR spectra of compound IV revealed the archetypal signals of flavonol glycosides. The

$^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ data of this compound were consistent with reported data of isoquercitrin. On the above deliberations, compound IV was deduced as isoquercitrin.

Compound V

The $^1\text{H-NMR}$ spectrum of compound V displayed the characteristic signals of the kaempferol nucleus: two doublets at δ 6.12 and 6.32 ppm ($J = 2.3\text{Hz}$), assigned to the H-6 and H-8 protons respectively and a pair of A_2B_2 aromatic system protons at δ 6.86 and 7.97 ppm ($J = 8.6\text{ Hz}$), assigned to H-3' and 5' and H-2',6' respectively. A 100% base peak $[\text{M}]^+$ for compound was observed at m/z 287 in the mass spectrum indicating the compound as kaempferol. IR spectrum showed a strong absorption band at 1662.79 cm^{-1} and an intense broad band at 3317.86 cm^{-1} . $^{13}\text{C-NMR}$ signals were found to be in agreement with reported values. The molecular formula was deduced from ^1H , $^{13}\text{C-NMR}$ and mass spectrometry. On the basis of spectral evidence, the structure of compound IV was decided to be kaempferol ($\text{C}_{15}\text{H}_{10}\text{O}_6$, 286.2).

The flavonoids isolated, are extensively distributed in plant kingdom and all have been reported to have biological activities. Isoquercitrin has antibacterial and antiviral (against HSV-virus) activities and is also known as an inhibitor of ACE. It also suppresses release of inflammatory mediators. Kaempferol was also reported to have antibacterial, hepatoprotective, antioxidant activities²³. β sitosterol has been tested for its immunomodulatory and anti-inflammatory activities besides many other biological activities²⁴. Oleanolic acid is also reported to have immunomodulatory and anti-inflammatory activities²⁵. Our studies show that *Lagenaria siceraria* fruits contain flavonoids (isoquercitrin, kaempferol), β sitosterol, campesterol and oleanolic acid; which may account at least in part for their application in folk medicine. Since secondary metabolite content may vary as a function of multiple factors, such as environmental conditions and harvest period, reproduction of this analysis over a long period of time is needed before the effectiveness of our method is totally demonstrated.

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