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ACIDS CONTENT OF *SENNIELLA SPONGIOSA* (F.V. MUELL.) ALLEN

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Senniella spongiosa (F.v. Muell.) Allen variety *holocarpa* (F.v. Muell.) Allen belongs to family Chenopodiaceae, was collected from the selected habitat (Alexandria – Mersa Mattruh road) at 30 km during the vegetative growth seasons of 2003-2004.

The chromatographic investigation of the phenolic acids content of *S. spongiosa* stems and leaves revealed that the leaves contain phenolic acids, ferulic, chlorogenic, caffeic and gallic acids. While the plant stems contain the phenolic acids 2, 3-dihydroxy benzoic acid (*O*-protocatechuic acid), ferulic, chlorogenic and *p*- hydroxy benzoic acid.

These phenolic compounds were isolated using polyamide column chromatography, purified on a sephadex LH-20 column and identified through Rf-values and colour reactions. UV spectral data, ^{1}H-NMR, ^{13}C-NMR spectra and mass spectrum.

Using HPLC technique, it was observed that both plant stems and leaves contain the same organic acids (oxalic, malic, acetic, citric and fumaric) with different ranges of concentration. Acetic acid was the highest separated organic acid detected in the plant leaves and stems

Investigation of free amino acids using amino acid analyzer, showed that stems and leaves of *S. spongiosa* contained 13 and 14 free amino acids, respectively. Serine and proline were the highest separated free amino acid for the stems and leaves, respectively. Glutamic acid was the highest separated amino acid (after protein hydrolysis) for the stems and leaves, respectively.

Using gas liquid chromatography showed the presence of 13 and 10 hydrocarbons in the stems and leaves, respectively with different ranges of concentrations. n-eicosane was the highest detected hydrocarbon for the stems and leaves. Cholesterol and β-

sitosterol were detected in plant stems and leaves with different ranges of concentrations.

Using gas liquid chromatography revealed the presence of 7 saturated fatty acids beside 2 unsaturated fatty acids in plant stems and leaves, respectively with different ranges of concentrations. Myristic acid was the highest separated fatty acid for both plant stems and leaves.

Keywords: *Senniella spongios*, Chenopodiaceae, phenolic compounds, organic acids, amino acids, fatty acids.

Family Chenopodiaceae shows an interesting geographical distribution which is determined by the fact that they are almost halophytic. Vegetables of this family generally contain 1-*O*-ferulyl-β-D-glucose (Rizk, 1986).

The leaves of *Beta vulgaris* contain several phenolic compounds, including vitexin, a glucoside of quercetin and 3-hydroxytyramine, in addition it contains alkaloids, flavonoids and saponins (Rizk, 1986).

Mossa *et al.* (1987) reported that, *Chenopodium botrys* contains an essential oil and five flavonoids viz. hispidulin, salvigenin, 5-methyl salvigenin, 7-methyl eupatulin and sinensetin. While three alkaloids were isolated from *Kochia indica*, also they reported that the main constituents of *Salsola kali* were salsoline, salsolidine alkaloids, potassium salts, calcium, protein, fat, traces of oxalic acid and cellulose.

Senniella spongiosa (F.v. Muell.) Allen variety *holocarpa* (F.v. Muell.) Allen belongs to family Chenopodiaceae. It is an annual or short-lived perennial herb, 10-30cm, white-mealy, richly branched erect or ascending, monoecious herb; leaves 1.5 x 0.5-1.2 cm, scurfy on both surfaces, deltoid to rhombic, acute, the margins sinuate to serrate; flowers in axillary clusters; male flowers in small terminal headlets, female axillary, fruiting bracteoles 0.8-1.2cm, sessile, fused, globular to obovoid, shortly apiculate, inflated, scurfy to glabrous; seeds broadly elliptic. Introduced from Australia, nowadays naturalized in several places. It grows in roadsides and waste ground; in the Mediterranean coastal strip from the border with Libya near Sallum to Port Said (Boulos, 1999).

A unique feature of plant metabolism, when compared to that of animals and microorganisms, is the ability of plants to accumulate organic acids in the cell vacuole, sometimes in considerable amount. Organic acids are very important in intermediary metabolism and plant respiration. The simple organic acids that accumulate in plants fall conveniently into two groups: the tricarboxylic (krebs) cycle acids including citric, malic, isocitric, cis-aconitic, succinic, fumaric, oxalacetic and ketoglutaric acids and other acids including formic, acetic, monofluoracetic, oxalic, tartaric, malonic,

shikimic, quinic and ascorbic acids. Organic acids are water – soluble, colorless liquids or relatively low melting solids. The majority are non-volatile, they are generally chemically stable. Acids are easily recognized by their taste in solution and by the low pH of crude aqueous plant extracts, when they occur in quantity (Harborne, 1984).

This study aimed to investigate the phenolic acids and determine organic acids, free and combined amino acids and analysis of the lipid sample of *S. spongiosa* (leaves and stems).

MATERIALS AND METHODS

1. Plant Materials

Senniella spongiosa of family Chenopodiaceae was collected during the four seasons, winter, spring, autumn and spring of 2004. The leaves and stems of the plant were separately cleaned, dried in an oven at 40 °C, ground to fine powder and kept for further investigations.

2. Investigation of Phenolic Acids

2.1. Preparation of the extract

Leaves and stems powder of *S. spongiosa* collected from spring season (April) was separately extracted with 80% aqueous ethanol. The ethanolic extract was separately evaporated under reduced pressure and low temperature, then extracted with chloroform. The obtained residue was treated with excess of ethanol and filtered to remove inorganic salts and non-phenolic compounds.

2.2. Reagents

2.2.1. Reagents for phenolics

a) Ferric chloride, 1% ethanolic solution (Smith, 1960).

b) Gibb's reagent, a freshly prepared N-2 trichloro-p-benzoquinone-4-monoimin (0.5%) methanolic solution and saturated aqueous sodium bicarbonate solution (Neish, 1960).

2.2.2. Reagents for carboxylic acids (Aniline/glucose)

Two grams glucose was dissolved in 2ml water, and 2ml aniline were dissolved in 20ml ethanol. The two solutions were mixed and completed to 100ml by n-butanol. The chromatogram was sprayed with the reagent and heated for 5-10min. at 105°C, brown to red spots were detected (Smith, 1960).

2.2.3. Reagents for UV spectroscopic analysis

Sodium methoxide solution (2.5gm metallic sodium was added cautiously to 100ml dry methanol).

2.3. Solvent systems

n-Butanol: Acetic acid : Water (4:1:5 v/v/v) (BAW) = S_1.

Acetic acid: Water (15:85 v/v) (AcOH-15%) = S_2.

2.4. Chromatography

Each alcoholic extract of the leaves and stems of *S. spongiosa* was separately chromatographed on Whatman No. 1 paper chromatography (PC) using the solvent system BAW (S_1) for the first way and solvent system Ac-OH 15% (S_2) for the second way. The developed chromatograms were air dried, and examined under ultra violet (UV) light, then exposed to ammonia and re-examined under UV light.

The concentrated ethanolic extract of the leaves and stems of *S. spongiosa* were applied separately on the top of a polyamide column. Elution was started with distelled water followed by a mixture of water/ethanol and finally pure ethanol was used. The received fractions were evaporated and subjected to PC where similar fractions are collected together (Liu *et al.*, 1989).

Preparative paper chromatography (PPC) was applied on Whatman No.3 paper chromatography using the solvent system (S_1). The separated phenolic acids were purified on a sephadex LH-20 column using methanol / water system.

The pure isolated compounds were spotting on Whatman No.1 PC, using the solvent systems S_1 and S_2. The developed chromatograms were air-dried, examined under UV light. Phenolics were detected by spraying the dried chromatograms with the corresponding reagent.

2.5. Physical tests

2.5.1. Ultraviolet spectrophotometric analysis

Chromatographically pure materials were dissolved in pure methanol and subjected to ultraviolet spectrophotometer UV- 240.

2.5.2. ^{1}H and ^{13}C nuclear magnetic resonance analysis (NMR)

The NMR measurements were carried out on Buruker AMX-500ml, Varian Inova-500 and/or JEOL EX-270 NMR spectrometer apparatus as described by Mabry et al., (1970).

2.5.3. Mass spectrometric analysis (MS)

The isolated chromatographically pure compounds were subjected in most cases to fast atom bombardment (positive and negative) mass spectrometric analysis (FAB-MS). Some other compounds were subjected to electron impact and/or chemical ionization mass spectrometric analysis (EI, CI-MS). The spectra were conducted using Mass Spectrometer Varian Mat 711, Finnigan SSQ 7000 and MM 7070 E (Mabry *et al.*, 1970).

2.6. Chemical reactions

2.6.1. Alkali fusion

Fusion of the isolated chromatographically pure compounds with KOH pellets at 200-210 °C for about 2 minutes, followed by acidification led to the release of the corresponding phenol and phenolic carboxylic acids which were extracted with ether. Identification of the fusion products was

achieved through comparative paper chromatography (CoPC) against authentic phenolic materials.

3. Investigation of Organic Acids

3.1. Paper chromatography of organic acids (Qualitatively)

Two dimensional paper chromatography (TDPC) may be carried out with n-propanol : 1M NH_4OH (7:3 or 3:2) followed by n-butanol : formic acid : water (10:3:10); another good pair of solvents were 95% ethanol : 1M NH_4OH (19:1) and n-butanol: formic acid: water (4:1:5) after development, the paper was dried thoroughly to remove all traces of formic acid, then sprayed with bromothymol blue (0.04g in 100 ml 1.01M NaOH). The acids appear as blue spots on a yellow background; the contrast of the background can be adjusted by fuming paper with ammonia vapour.

3.2. Isolation and determination of organic acids using HPLC (Quantitively)

Organic acid extracts from stem and leaves of S.*spongiosa* were measured using a Waters HPLC (high performance liquid chromatography) by reverse phase ion-suppession on a C_{18} column using 0.05M KH_2PO_4 buffer (pH 2.5). At this time the components were unable to be identified with certainty by comparison of retention times to a range of acid standards where the extraction of organic acids from aqueous solution were done using extraction column Bakerbond Spe Quaternary amine, 3ml, 500mg using 100ml of sample (leaves and stems of the plant). 2ml 1M NaOH solution were added to the column and slowly aspirated through at a flow rate of 0.3ml/min and washed with 50ml of water at 3ml/min, followed by 5ml of diluted acetic acid (pH 2.05) at 0.5ml/min and finally with 50 ml of diluted acetic acid (pH 4.5) at 3ml/min. The sample was slowly aspirated through the column and allowed to percolate at a flow rate of 0.5ml/min. The column was washed with 30ml of water (3ml/min) and then the Spe column was vacuumed dry for 5 minutes with nitrogen or compressed air. The organic acids were eluted from the column with 2ml of 0.5M H_2SO_4 (0.3ml/min). This solution could be injected directly for HPLC analysis or diluted with mobile phase, depending on its total acidity. Final analytical of organic acids was done using HPLC-RP, column, C_{18} BDC column with guard 4.6x250mm, mobile phase was sulfuric acid (pH 2.45), flow rate of 0.7 ml/min, detector was UV at 210nm (Foy *et al.*, 1990).

4. Determination of Free and Protein Amino Acids (Quantitively)

Analysis of amino acids was carried out using amino acid analyzer according to the method described by Steven *et al.*, (1989). LKB alpha plus high performance amino acid analyzer LKB biochrom, LTD England, was used for this purpose. Retention times and areas were determined using Hewlett Packard 3390 recording integrator.

4.1. Preparation and identification of free amino acids using Amino Acid Analyzer

One gram of the defatted plant organs powder was extracted by boiling under reflux with 50ml of 50% ethanol for 3 times (each for 3 hours). The combined ethanolic solution were filtered and treated with trichloroacetic acid solution (10%) for clarification. The supernatant fluid was concentrated under reduced pressure to 5ml. The residue was washed with distilled water. The volume of the filtrate was adjusted to 100ml using distilled water. Five ml from diluted sample were dried at 70°C, then dissolved in 5ml loading buffer (0.2 N sodium citrate buffer pH 2). The sample was filtrated through 0.45 micropore filter and injected in amino acid analyzer.

4.2. System of amino acid analyzer

Beckman system 7300 high performance analyzer, column: Na high performance column 25 cm, injected volume: 50 µL and detector: visible light detectory. Retention time and separated area were obtained using Hewlett Packard 3390 recording integrator.

4.3. Preparation and identification of protein-amino acids

The hydrolyzed protein-amino acids were determined according to the method described by Steven *et al.* (1989) as follows:

Defatted plant powder (0.1g) was dissolved in 10ml of 6 N HCl in a sealing tube. The mixture was hydrolyzed at 110°C for 24 hours, then filtered and the hydrolyzed protein-amino acids were obtained by evaporation of the hydrolyzate to dryness. The residue was washed with distilled water. The volume of the filtrate was adjusted to 100ml using distilled water. The investigation of protein-amino acids was completed as in free amino acids.

5. Investigation of Fatty Acids (Saponifiable Fraction) Using Gas-Liquid Chromatography (GLC)

After the removal of unsaponifiable fraction (hydrocarbons and sterols) with ether, the soapy solution was converted into the corresponding free fatty acids by mean of 0.2% sulfuric acid. When the acids were completely liberated, they were removed by extraction with ether.

The extract was washed several times with distilled water until free from acids. The ether extract was dried over anhydrous Na_2SO_4 and filtered. The ether was removed by distillation and the last traces of ether were removed under vacuum at 60°C, and then cooled in a dessiccator.

The extracted fatty acids and the standard ones were converted to the corresponding methyl esters using ethereal solution of diazomethane (Farag *et al.*, 1986). The methyl esters of the fatty acids were analyzed with GCV Pye-Unicam gas chromatographic apparatus. The fraction of fatty acid methyl esters was conducted using a coiled glass column (150 mm x 4 mm), packed with diatomic C (100-120 mesh) and coated with 10%

polyethyleneglycol adipate (PEGA). The column oven temperature was programmed at 10°C/min. from 100°C to 200°C, then isothermally at 200°C for 15 minutes with nitrogen at 30ml/min. Peak identification was performed by comparing the relative retention time of each compound with those of standard materials. The relative proportions of each individual compound were estimated as the ratio of the partial areas to the total area as mentioned by Fryer *et al.*, (1960); Nelson *et al.*, (1969) and Farag *et al.*, (1986). The following conditions were used: column, coiled glass (150 mm x 4 mm), column, injector and detector temperature were: 300, 250 and 300°C, respectively. Rate of temp.: 8°C/minutes, initial and final temperature were 70 and 190°C, respectively. Initial and final time was 1 and 20 minutes. Chart speed: 2 minutes /cm.

RESULTS AND DISCUSSION

1. Investigation of Phenolic Acids

The developed chromatograms of the phenolic acids constituents of the leaves and stems of *S. spongiosa*, after drying, exposing to ammonia and subjected to ultraviolet light revealed that, the phenolic acids constituents of the leaves and stems are not identical (Table 1).

1.1. Isolation of the main phenolic acids constituents

When the concenterated extracts of the leaves and stems of *S. spongiosa* were applied on the top of a polyamide column using water/ methanol system, column of leaves gave 4 fractions, while column of stems gave another 4 fractions.

Preparative paper chromatography (PPC) of these fractions using the solvent system S_1 revealed the presence of four bands in both the stems and leaves. The separated phenolic acids were purified on a sephadex LH-20 column using methanol/water system. Then the purified compounds were subjected to two dimensional paper chromatography (TDPC) using S_1 for the first run and S_2 for the second run. The obtained chromatograms revealed that leaves of *S. spongiosa* contain compounds P_2, P_3, P_5 and P_6, while stems of the plant contain P_1, P_2, P_3 and P_4 as shown in table (1).

TABLE (1). Phenolic acids of *S. spongiosa*

Compound	Name	Stem	Leaf
P_1	2,3 dihydroxy benzoic acid	+ve	-ve
P_2	Ferulic acid	+ve	+ve
P_3	Chlorogenic acid	+ve	+ve
P_4	*p*-hydroxy benzoic acid	+ve	-ve
P_5	Caffeic acid	-ve	+ve
P_6	Gallic acid	-ve	+ve

1.2. Isolation and identification of compound P_1

TDPC of band (1), revealed the presence of one major fluorescent blue spot under UV light which gave purple colour with aniline / glucose spray reagent (Smith, 1960) specific for carboxylic acids.

An amorphous pure sample of P_1 was obtained by applying the PPC technique for fractionating the material of fraction (1), using S_1 as solvent for separation, led to the isolation of a chromatographically pure sample of P_1. Crystallization of the obtained amorphous material of P_1 from water gave colorless prisms of pure P_1, (m.p.202°C) which exhibited no optical activity when dissolved in methanol. Rf -values and UV spectral data of component (P_1) were illustrated in tables (2 and 3).

TABLE (2). R_f –values and colour reactions of the isolated phenolic acids

Compound	R_f -values		Colour reactions				
	BAW	ACOH15%	Visible	UV	UV+NH_3	$FeCl_3$	Aniline / glucose
P_1	0.87	0.69	-	Fluorescence blue	-	Blue	Dark purple
P_2	0.88	0.33	-	High blue	Fluorescence blue	Deep blue green	-
P_3	0.93	0.44	-	Fluorescence blue	Yellowish green	Blue	-
P_4	0.97	0.72	-	Brown	Brown	Bluish	Dark brown
P_5	0.78	0.49	-	Blue	Blue	Dark blue green	-
P_6	0.78	0.59	Fluorescence blue	Visible blue	-	Blue	-

BAW= n-Butanol : Acetic acid : Water (4:1:5 v/v/v)

TABLE (3). UV spectral data, λmax nm of the isolated phenolic acids

Compound	MeOH	MeONa
P_1	285, 308	-
P_2	285, 312	250, 290, 319
P_3	245, 300 (sh), 336	239, 265, 310 (sh), 382
P_4	250, 325 (sh)	275, 335 (sh)
P_5	240, 285 (sh), 325	252, 301, 345
P_6	218, 272, 335	220, 275, 345

TABLE (4). ^1H nuclear magnetic resonance (NMR) of some isolated phenolic acids

Compound	Symbol	signals at δ (ppm)
2, 3-dihydroxy benzoic acid	P_1	6.8 (t, j = 7.5 Hz, 5-H), 7.1 (dd, j = 7.5 Hz and j = 2.5 Hz, 6-H), 7.4 (dd, j = 7.5 Hz and j = 2.5 Hz, 4-H)
Ferulic acid	P_2	12.2 (broad, s, -COOH) 8.9 (s, -OH), 7.5 (1H, dd, j = 17 Hz, H-7) 7.15 (1H, d, j = 7.5 Hz, H-2), 7.09 (1H, d, j = 7.5 Hz and 2.5 Hz H-6), 6.95 (1H, dd, j = 7.5 Hz, H-5), 6.25 (1H, d, j = 17 Hz, H-8), 3.85 (3H, s, OCH3)
Chlorogenic acid	P_3	9.5 (s-OH), 9.0 (s-OH), 7.45 (1H, d, j = 17 Hz,- H-7), 7.1 (1H, d, j = 2.5 Hz, H-2), 6.95 (1H, dd, j =7 Hz and 2.5 Hz, H-6), 6.75(1H, d, j = 7.0 Hz, H-5), 6.15 (1H, d, j = 17.0 Hz, H-8), 5.55 (1H, broad, H-3),3.9 (1H, broad, H-5), 3.5 (1H, board, H-4), 1.9-2.1 (4H, multiblet, CH_2-2 and CH_2-6)
P-hydroxybenzoic acid	P_4	7.6 (2H, d, j =8 Hz, H-2 and H-6), 6.5 (2H, d, j =8 Hz, H-3 and H-5), 8.7(s, -OH)
Caffeic acid	P_5	12.1 (broad, s, COOH), 9.5 (s, OH), 9.15 (S, oh), 7.4 (1H, d j=17.0 Hz, H-7), 7.0 (1H, d, j=2.5 Hz, H-2), 6.95 (1H, dd, j=7.0 Hz and 2.5 Hz, H-6), 6.75 (1H, d, j=7.0 Hz, H-5), 6.15 (1H, d, j=17.0 Hz, H-8)
Gallic acid	P_6	6.98 (s, H-2 and H-6)

^{13}C-NMR spectral data

δ (ppm): 172.83 (C = O), 121.4 (C-1), 151.06 (C-2), 146.63 (C-3), 119.7 (C-4), 113.3 (C-5), 121.4 (C-6).

Alkali fusion

15 mg of component I were fused in an ignition tube together with 3 pellets of KOH for 2 minutes. The fusion product was left at room temperature, dissolved in 10 ml water and the aqueous solution obtained was rendered acidic by aqueous 1.5 N HCl. Extraction in a separating funnel of the acidified aqueous solution by ether followed by CoPC of the ether extract proved the presence of 3, 4- dihydroxy benzene, catechol (UV λmax, nm, in MeOH: 285, 308 nm).

The crystalline optically inactive material (P_1) has shown chromatographic (Table 2) and colour properties of a phenolic carboxylic acid (dark purple colour with aniline / xylose) which posses at least two ortho phenolic groups (blue colour with FeCl3). It exhibited UV spectral data (Table 3) identical with those reported for O- protocatechuic acid, a phenolic acid of rare natural occurrence. This view was supported by Electron Impact - Mass Spectrum (EI-MS) analysis of compound P_1.

Electron Impact - Mass Spectrum (EI-MS) analysis of P_1

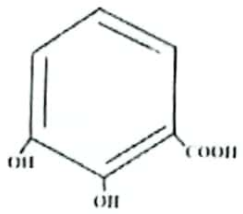
m / z (%): 154 (M^+, 9), 136 (37), 110 (100), 92 (14), 81 (15), 64 (42), 52 (17), 44 (61). The obtained spectrum showed a molecular ion peak at m/z 154 and a fragmentation pattern characteristics for phenolic carboxylic acids. The recovery of component (P_1) unchanged after alkaline hydrolysis in addition to its conversion to 2,3- dihydroxybenzene (catechol) on fusion at

245°C for 2 minutes, (CoPC) cofirmed the assumed structure of compound P_1.

Further confirmation of the identification was then received through ^{1}H-NMR analysis of P_1 as shown in table (4). The spectrum showed only a pattern of aromatic proton signals typical for 1, 2, 3- triunsymmetrically substituted benzene (t, j = 7.5 Hz, at δ (ppm) 6.8, H-5; dd, j = 7.5 Hz and 2.5 Hz at 7.1, H-6 and dd, j = 7.5 Hz and 2.5 Hz at 7.4, H-4), thus confirming the structure of component P_1 as 2, 3-dihydroxy benzoic acid, O-protocatechuic acid.

^{13}C-NMR spectrum of P_1 also, was recorded and assigned, the spectrum showed a carbonyl carbon signal at δ (ppm) 172.83.

This recognizable downfield shift (on comparison with about 168 δ ppm) for phenolic acids lacking ortho free hydroxyl group, e.g. 2-methoxybenzoic acid (Breitmaier and Voelter, 1974), gallic acid and 3,4-dihydroxy benzoic acid (Nawwar *et al.*, 1982) might be due to the hydrogen bonding with the adjacent free OH group. This hydrogen bonding also might affect the resonance of C-2 which was found resonating at δ (ppm) 151.06. The second oxygenated carbon (C-3) revealed its signal at δ (ppm) 146.63. The chemical shift values of the remaining four carbon signals are in consistence with the achieved structure of P_1 as 2,3- dihydroxy benzoic acid ($C_7H_6O_4$, molecular weight 154.122 and melting point 207 – 210°C).



2,3-dihydroxybenzoic acid
(O-protocatechuic acid)

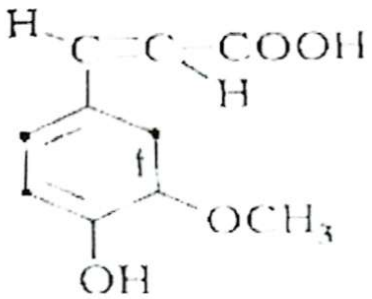
1.3.Isolation and identification of compound P_2

The band No. (2) on PC after eluted with methanol/water, drying under reduced pressure and purified on sephadex LH-20 column, using methanol/water (1:1) system and subjected to TDPC using the solvent system S_1 in the first way followed by the systems S_2 for the second way, revealed the presence of one major spot of phenolic nature (m.p.168-171°C). Its R_f -values and color reactions were outlined in table (2). Compound P_2 was obtained as long white needle crystals soluble in methanol and acetone

UV analysis of compound P_2 in methanol (Table 3) showed two bands at 285nm and 312nm which are characteristic for phenylpropanoids, also

with adding NaOMe, gives a bathochromic shift (250nm, 290, 319nm) proved the presence of free hydroxyl group.

^{1}H-NMR spectra of compound P_2 (Table 4) showed two doublets at (7.5 and 6.25ppm), j = 17.0Hz, characteristic of trans olefenic double bond H-7 and H-8, respectively. The two doublet at 7.15 and 6.95 (j = 7.5 Hz) and the doublet of doublet signal at dd, j=7.09 Hz, which is corresponding to *ortho* and *meta* coupling and it is a good evidence for the presence of trisubstituted benzene. The presence of singlet at 9.15ppm confirmed the UV analysis for the presence of free OH group. The ^{1}H-NMR showed signals at 12.2 (broad and singlet) for –COOH proton and 3.85 for 3 protons of OCH3 group. These data suggest that compound P_2 is 3-methoxy-4-hydroxy cinnamic acid (Ferulic acid). It is an acid which contribute to the effectiveness of pycnogenol. It is also a natural for UV protection.



4-hydroxy-3-methoxy cinnamic acid "Ferulic acid"

1.4. Isolation and identification of compound P_3

Elution of band No. (3) from PPC was performed with methanol/water, dried under reduced pressure, purified on sephadex LH-20 column, as described by Johnston *et al.*, (1968), using methanol and subjected to TDPC using the solvent systems (S_1 and S_2), where one major spot of phenyl propanoid nature was detected (compound P_3). Its Rf -values and color reactions were outlined in table (2). The change of its colour from fluorescence blue to yellowish green when exposed to ammonia vapour under UV light suggested that P_3 is chlorogenic acid. The UV spectral data of compound P_3 were outlined in table (3) were confirmed the presence of the absorption bands characteristic for phenylpropanoids. The bathochromic shift upon addition of NaOMe proved the presence of free hydroxyl groups.

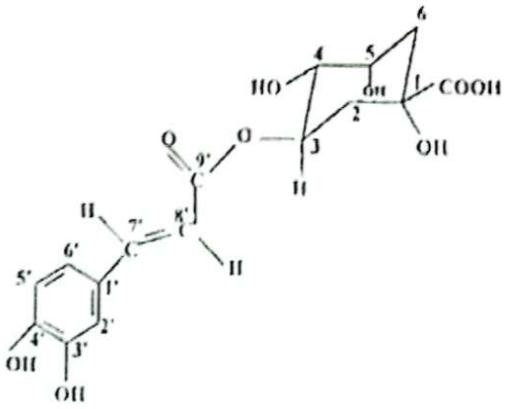
^{1}H-NMR spectral data shown in table (4) also showed signals at δ (ppm) 1.8, 2.2, 3.7, 4.5, 7.0 (different protons of quinic acid; CI-MS m/z rel. int. %) 36: 355 (M^{+1}, 1.52 %), 175 (quinic acid moiety, C7O5H11, 66%), 217 (M+, C8H17O2, 100 %), 157 (175-H2O 70 %). Its ^{1}H-NMR spectrum

showed all protons characteristic for 3, 4- dihydroxy-cinnamic acid beside the proton for quinic acid moiety.

The purified compound P_3 gave blue florescence spot under UV light changing to yellowish green, when exposed to ammonia, and re-examined under UV light compound P_3 gave blue colour with $FeCl_3$ reagent, proving the phenolic nature. Rf -values and color reactions indicate the phenolic nature compound P_3 as indicated in table (2).

UV spectral data of compound P_3 showed two major bands at 245, 330nm, in addition of sodium methoxide gave a bathochromic shift indicating the presence of free hydroxyl groups (Table 3).

The structure of the compound P_3 is further confirmed as chlorogenic acid by ^{1}H-NMR and mass spectrum. Chlorogenic acid ($C_{16}H_{18}O_9$) of molecular weight (354.31) and melting point (207- 209°C).



Chlorogenic acid

1.5. Isolation and identification of compound P_4

The band No. (4) on PC when eluted with methanol/water, dried under reduced pressure, purified on sephadex LH-20 column, and subjected to TDPC using the solvent systems S_1 followed by the S_2, revealed the presence of single spot of phenolic nature (compound P_4). Its Rf -values and color reactions were outlined in table (2).

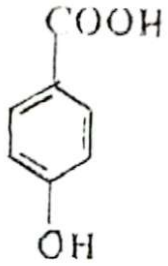
It is white amorphous powder, m.p.190-192°C, soluble in methanol and acetone, brown colour under UV, bluish colour with $FeCl_3$ dark brown colour with aniline/glucose reagent indicating its phenolic carboxylic acid nature.

UV analysis of compound P_4 in methanol showed band at 250nm and shoulder at 325nm which is similar to benzoic acid with – OH group (Silverstein *et al.*, 1981). The bathochromic shift after addition of sodium

methoxide, a shift at 275nm (Table 3), proved the presence of free hydroxyl group.

IR ν max (kBr) Cm-¹; 3388 (OH), 1677 (O=C); while ^{1}H-NMR (DMSO-d6) showed two doublets at 7.6 ppm (2H, d, j = 8Hz), 6.6 ppm (2H, d, j = 8 Hz), 8.3 ppm (s-COOH), 4.5 ppm (bs-OH).

EI-MS (Electron Impact – Mass Spectrum) m/z (rel. int. %) 139 (M^{+1}, 75.1 %), 138 (M^+, 49.1 %), 120 (M^+ - H2O, 100%), 93 (M^+ -COOH, 86.06%) and 65 (C6H5, 90.2%), which proved that compound P_4 could be *P*-hydroxybenzoic acid.



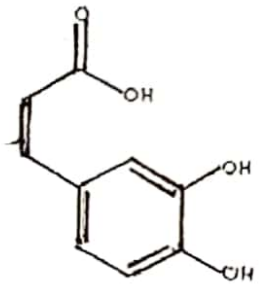
***P*- hydroxybenzoic acid**

1.6. Isolation and identification of compound P_5

Compound P_5 was obtained as white powder, soluble in methanol and acetone. (m.p.223 - 225 °C). Its Rf -values and color reactions were outlined in table (2), indicated that it has a phenolic nature especially its blue colour with $FeCl_3$

The UV spectra (Table 3) of P_5 exhibiting two absorption bands at 240 and 325 nm which are characteristic for phenyl propanoids (Harborne, 1984). The bathochromic shift on the addition of NaOMe proved the presence of free hydroxyl group.

^{1}H-NMR spectrum (Table 4) showed two doublets at δ 7.4 and 6.15 (j=17.0 Hz) characteristic for trans olefenic protons H-7 and H-8, respectively. The doublets at δ 7.0 and the doublet of doublet at δ 6.95 for H_2 and H_6, respectively are corresponding to *ortho* and *meta* coupling of trisubstituted benzene. The presence of two singlets at δ 9.15 and 9.5 confirmed the UV analysis for the presence of two free -OH groups. The presence of a signal at δ 12.1 (broad and singlet) which is attributed to –COOH proton. The above data suggested that compound P_5 is 3, 4-dihydroxycinnamic acid [caffeic acid $(HO)_2C_6H_3CH=CHCOOH$, molecular weight = 180.16], this suggestion was confirmed by EI-Mass Spectrum, which showed a molecular ion peak (M^+) at m/z 180, M^+ - 45) at m/z 135, (C_6H_6) at m/z 77 and the base peak (M^+ -3OH) at m/z 130.



Caffiec acid

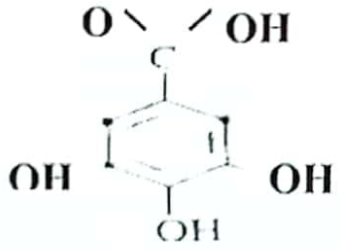
1.7. Isolation and identification of compound P_6

Compound P_6 was obtained as fine colourless needles from water of pure component (m.p. 250-251°C).

The isolated chromatographically pure compound P_6 was identified through R_f – values, UV spectral data, ^{1}H-NMR and ^{13}C-NMR analysis as gallic acid $\{C_6H_2(OH)_3COOH\}$.^{1}H-NMR spectral data (Table 4) showed signals at δ (ppm) 6.98 (s, 2-H and 6-H). ^{13}C -NMR spectral data at δ (ppm) 120.6 (C-1), 108.8 (C-2 and C-6), 145.3 (C-3 and C-5), 138.1 (C-4) and 167.7 (C-7).

Gallic acid called 3,4, 5-trihydroxybenzoic acid, $C_6H_2(OH)_3COOH$ (170.12), which are widely used in the manufacturing azo dyes and photographic developers and to treat certain skin diseases. Gallic acid and its derivatives are used in making dyes and inks, photographic developers and used as astringents in medically.

Synonyms of gallic acid; 3,4,5-trihydroxybenzoic acid; pyrogallol-5-carboxylic acid. Its molecular weight, 170.12; melting point 222 - 240 (dec); density, 1.69 and sparingly soluble in water.



Gallic acid (3, 4, 5-trihydroxy benzoic acid)

2. Organic Acids

2.1. Paper chromatography of the organic acids

The developed chromatograms of the organic acids of leaves and stems of *S. spongiosa* plants using the solvent system n-propanol: 1M NH_4OH (7:3) followed by n-butanol: formic acid: water (10:3:10); after drying, exposing to ammonia vapour and subjecting to ultraviolet light revealed that, the organic acid constituents of both leaves and stems are identical, they contained oxalic, malic, acetic, citric and fumaric acids.

2.2.Determination of organic acids using HPLC (Quantitively)

Both stems and leaves of the plant samples contain the same organic acids with different ranges of concentration. It is obvious from table (5) that, acetic acid was the highest separated organic acid detected in the leaves and stems of *S. spongiosa* (9.233 and 8.540 mg/100g), respectively using HPLC technique.

TABLE (5) Organic acids of *S. spongiosa* using HPLC.

No.	RT	Organic acids	% (mg/100g)	
			Leaves	Stem
1	4.553	Oxalic	5.139	4.154
2	5.596	Malic	3.098	3.012
3	6.720	Acetic	9.233	8.540
4	7.602	Citric	2.051	2.010
5	8.469	Fumaric	7.197	5.147

Acetic acid (CH_3CO_2H) may be considered the most important, since it serves as a universal precursor of fatty acids, lipids and many other organic plant products. It occurs in trace amounts both free and combined in the essential oils of many plants (Harborne, 1984).

Oxalic acid $(COOH)_2 2H_2O$ is used in the paint, cosmetics and ceramics industries and is found in many plants and vegetables. It can make antibiotic and other pharmaceuticals, also as kinds of raw materials for extraction rare metal solvents, precipitating agent of rare earth metal, dyestuff reducing agent, fabric bleaching agent, tanning hides agents, metal equipment purifying agent and organic synthesis industry. It is also used as an analytical reagent; automobile radiator cleanser; catalyst; and hemostatic agent (Sitting, 1985).

Malic acid, $HOCOCH_2CHOHCOOH$ (hydroxybutanedioic acid), is an odorless white powder with slightly more acid flavor than citric acid. It has excellent solubility in water (51% at 10°C). The function of malic acid is similar to those of citric acid (Sax, 1984).

Citric acid ($C_6H_8O_7$) helps to prevent the texture, color, aroma and vitamin content of canned products and is particularly useful as a chelant. Due to its excellent solubility, it is often used in beverages to impart an acid

flavor, as well as for its buffering capabilities. The acidity of particulary all edible fruits were due to citric acid accumulation (Verschueren, 1983).

Fumaric acid ($C_4H_4O_4$) is derived from malic acid, it is moderately hygroscopic, compared to the other organic acids. The acid flavor in solution is much stronger than citric and lasts for a longer duration. It dissolves in alcohol, a little dissolves in water, not dissolves in chloroform (Gosselin *et al.*, 1984).

3. Determination of Free and Protein Amino Acids (Quantitively)

The investigation of free amino acids using amino acid analyzer, showed that stems and leaves of *S. spongiosa* contained 13 and 14 free amino acids, respectively. Serine and proline were the highest separated free amino acid with concentration of (721.23 and 880.64µg/gm) for the plant stems and leaves, respectively (Table 6). It is obvious from the obtained data that glutamic acid was the highest separated amino acid (after protein hydrolysis) with concentration of (2465.26 and 2648.08 µg/gm) for the plant stems and leaves, respectively.

Proline is known to play an important role as an osmoprotectant in plants subjected to hyperosmotic stresses such as drought and soil salinity. Recent studies on Proline synthesis and catabolism genes have provided results that are consistent with diverse functions of Proline as a source of energy, nitrogen and carbon, and as an osmolyte in response to dehydration (Kohl *et al.*, 1988; Kavi Kishor *et al.*, 1995; Hua *et al.*, 1997; Zhang *et al.*, 1997).

4. Chromatographic Analysis of the Lipids Sample

4.1. Unsaponifiable matter (Hydrocarbons and sterols)

Data presented in table (7) revealed the presence of 13 and 10 hydrocarbons in plant stems and leaves, respectively with different ranges of concentrations. n-Eicosane was the highest detected hydrocarbon for plant stems (25.25 %) and leaves (29.02 %). Cholesterol and β-sitosterol were detected in plant stems and leaves with different ranges of concentrations. Cholesterol is a sterol occurring widely in animal tissues and also in some higher plants and algae (Martin, 1985). Also the wide occurrence of cholesterol in plants has been discovered recently (Trease and Evans, 1989).

4.2. Saponifiable matter (Fatty acids)

Data presented in table (8) revealed the presence of 7 saturated fatty acids beside 2 unsaturated fatty acids in plant stems and leaves, respectively with different ranges of concentrations. Myristic acid was the highest separated fatty acid (25.614 and 27.103%) for plant stems and leaves, respectively.

TABLE (6). Free and protein amino acids of *S. spongiosa* using Amino Acid Analyzer.

No.	RT	Amino acids	Free amino acids (µg/gm)		Protein amino acids (µg/gm)	
			Stem	Leaves	Stem	Leaves
1	11 74	Aspartic	267 16	588 53	1729 20	1444 21
2	14 89	Therionine	214 15	341 03	373 84	669 58
3	16 25	Serine	721 23	419 11	546 14	536 42
4	18 31	Glutamic	317 21	785 46	2465 26	2648.08
5	24 83	Glycine	20 48	107 38	546 42	651 78
6	26 07	Alanine	91 16	349 74	567 30	612 73
7	29 73	Valine	190.54	425 44	637 04	661 66
8	32 11	Proline	341 75	880 64	1535.56	1953 84
9	33 73	Isoleucine	72 09	261 63	239 90	369 17
10	34 97	Leucine	-	176 45	447 64	597 50
11	39 35	Tyrosine	-	-	145 08	224 11
12	41 88	Phenylalanine	137 39	415 88	239 66	329 18
13	51 09	Histidine	100 35	251 60	308 95	371 34
14	54 61	Lysine	40 51	160.34	376 78	419 92
15	61 14	Arginine	119 91	517 57	175 39	279 64

RT = Retention time

TABLE (7). Hydrocarbons and sterols of *S. spongiosa* using GLC.

RT	No. of carbon atom	Name	Stem	Leaf
Hydrocarbons				
5 653	-	Unknown	1.18	0 0
7.250	C 12	n-Dodecane	14.02	3 20
8 517	C 15	n-Pentadecane	2.49	0.0
10 833	C 18	n-Octadecane	11.66	3 90
12 150	-	Unknown	0.81	0 89
13 417	C 20	n-Eicosane	25 25	29 02
14 333	C 21	n-Heneicosane	14.47	25 20
15 200	C 22	n-Docosane	5 46	15 45
17 133	-	Unknown	0 29	0 0
18 483	C 27	n-Heptacosane	5 89	2 87
20 000	C 28	n-Octacosane	1 85	0 99
21 250	C 30	n-Triacontane	1 75	1 86
23 200	C 32	n-Dotriacontane	8 45	21 78
Sterols				
25 983	C 27	Cholesterol	4 70	0 78
30 750	C 27	β-sitosterol	3 26	5 90

RT = Retention time

TABLE (8). Fatty acids of *S. spongiosa* using GLC.

RT	No. of carbon atom	Name	Stem	Leaf
1.567	-	Unknown	2.701	-
3.283	-	Unknown	0.600	0.300
5.100	C 8	Caprylic acid (Octanoic acid)	10.104	6.617
6.000	-	Unknown	-	0.301
8.250	C 10	Capric acid (Decanoic acid)	4.350	7.532
10.150	-	Unknown	-	0.279
11.083	C 12	Lauric acid (Dodecanoic acid)	3.215	5.300
14.083	C 14	Myristic acid (Tetradecanoic acid)	25.614	27.103
15.017	-	Unknown	-	4.659
16.133	C 16	Palmitic acid (Hexadecanoic acid)	17.810	19.310
18.183	C 17	Margeric acid (Heptadecanoic acid)	19.203	11.173
20.217	C 18-1	Oleic acid (*cis*-9-Octadecanoic acid)	5.193	3.120
21.467	C 18-2	Linoleic acid (*cis, cis*-9, 12-Octadecadienoic acid)	6.280	6.223
23.350	20:0	Arachidic (Eicosanoic acid)	4.930	7.780

RT = Retention time

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محتوى الاحماض لنبات سينيلا سبونجيوزا

شلبية شحات امام وفاطمة على أحمد
قسم النباتات الطبية والعطرية – مركز بحوث الصحراء– المطرية – القاهرة– مصر.

ينتمى نبات سينيلا سبونجيوزا الى العائلة الرمرامية وقد تم جمع عينات النبات من بيئته الطبيعية بطريق الاسكندرية– مرسى مطروح الكيلو ٣٠ خلال موسم النمو (٢٠٠٣–٢٠٠٤).

تم التعرف على محتوى كلا من السيقان والاوراق من الاحماض الفينولية باستخدام طرق التفريد الكروماتوجرافى وتم فصل اربعة أحماض فينولية من أوراق النبات هى: فيريوليك وكلوروجينك وكافيك وجاليك بينما احتوت سيقان النبات على الاحماض الفينولية التالية:٢–٣ ثنائى هيدروكسى بنزويك و فيريوليك وكلوروجينك وبارا هيدروكسى بنزويك. كما تم تعريف كل مركب باستخدام التفاعلات اللونية و قياس طيف الكتلة و تحليل اطياف الاشعة فوق البنفسجية والرنين المغناطيسى لانوية الهيدروجين ١ والكربون ١٣ ومطياف الكتلة.

تم تقدير الاحماض العضوية كميا باستخدام جهاز HPLC واحتوت كلا من سيقان واوراق النبات على احماض أوكساليك، ماليك، أسيتك، سيتريك وفيوماريك بنسب منوية مختلفة. وكان اعلى نسبة هو حامض الاسيتك.

وباستخدام جهاز تحليل الاحماض الامينية تم دراسة الاحماض الامينية الحرة والداخلة في تركيب البروتين واحتوت سيقان واوراق النبات على ١٣،١٤حامض امينى حر على التوالى بنسب مختلفة ووجد ان اعلى نسبة كانت لحامض السيرين والبرولين. وقد أوضحت النتائج إرتفاع نسبة البرولين التى تدل على مقاومة النبات للملوحة والجفاف. كما احتوت كلا من سيقان واوراق النبات على ١٥ حامضا أمينيا يدخل فى تركيب البروتين بنسب مختلفة من التركيزات وكان حامض الجلوتاميك هوالأعلى نسبة.

كما تم تعيين المواد غير المتصبنة (الهيدروكربونات والاستيرولات) باستعمال طرق التحليل الكروماتوجرافى فى الحالة الغازية وقد تم التعرف على ١٣ و١٠هيدروكربونات بسيقان واوراق النبات على التوالى بنسب منوية مختلفة.وكان ايكوسين هو الأعلى نسبة بكلا من سيقان واوراق النبات كما تم التعرف على مركبين من الاستيرولات هما الكوليستيرول وبيتا سيتوستيرول بكلا من سيقان واوراق بنسب مختلفة.

كذلك تم فصل الأحماض الدهنية المتصبنه وتقدير نسبها باستعمال طرق التحليل الكروماتوجرافى فى الحالة الغازية وقد اتضح من الدراسه وجود سبعة احماض دهنية مشبعة واثنين غير مشبعين واتضح ان حمض الميريستيك هو الأعلى نسبة.