Extraction and Identification of Natural Antioxidant from the Seeds of the *Moringa oleifera* Tree Variety of Malawi

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ABSTRACT: The oil from the dried seeds of the *Moringa oleifera* tree (variety of Malawi) was extracted with a mixture of chloroform/methanol (50:50). The induction period measurements demonstrated a great resistance to oxidative rancidity. After degumming, there was a reduction of 74% in induction periods. The gums produced were extracted with diethylether, *n*-butanol, and water, yielding four fractions: Fraction 1 (81.8% w/w), Fraction 2 (0.04% w/w), Fraction 3 (0.05% w/w), and Fraction 4 (17.0% w/w). These fractions were tested for their protection of fresh sunflower oil against rancidity, at 50°C, using a UV accelerated method. The oxidation of the sunflower oil was measured using PV; absorbance $E^{1\%}_{1cm}$ and malondialdehyde concentration were measured by HPLC. The fraction that showed the highest antioxidant activity was further fractionated by HPLC, yielding seven fractions. Fraction HPLC 3 (present in a quantity of 330.8 and 29.11 ppm in gums and oil, respectively) showed the highest antioxidant activity. Its activity was also compared with that of the commonly used antioxidants BHT and α-tocopherol on sunflower oil using the same methods. At the same level of addition (200 ppm), HPLC 3 showed higher antioxidant activity than BHT and α-tocopherol. The identification of HPLC3 was done using ^{1}H NMR, ^{13}C NMR, MS, melting point, and UV absorption spectroscopy and proved to be 3,5,7,3′,4′,5′-hexahydroxyflavone (myricetin).

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The growing consumer preference for "natural" products forces the fats and oils industry increasingly to seek natural sources for antioxidants rather than investing in synthetic ones. Doubts about the safety and use of synthetic antioxidants first arose in the early 1960s. Toxicological evaluations of BHT and other synthetic antioxidants have been considered in several World Health Organization/Food and Agriculture Organization publications (1). BHA and BHT, the most widely used antioxidants, have unsurpassed efficiency in various food systems in addition to their high stability, low cost, and other practical advantages. However, their use in food has been declining due to suspected action as a promoter of carcinogenesis (2) as well as to a general rejection of synthetic food additives. Although these antioxidants are approved for food use, because of the above concerns the level of use is strictly regulated.

Extracts of many plants have been shown to have various degrees of antioxidant activity in different fats and oils. The antioxidant activity of these plants and their extracts can be attributed to the presence of flavonoid compounds, phospholipids, tocopherols, and ascorbic acid (1). Native phosphatides and gums in unrefined peanut oil were suspected to be factors responsible for the comparatively higher stability to oxidation (3).

In their experiments, Ibrahim *et al.* (4) proved that the formation of peroxides, in *Moringa* oil oxidized under different conditions, was not significant. Additionally, the negative results given by the Kreis test for all the samples indicated the absence of specific products from oxidative rancidity. Eckey (5) reported that the *Moringa* oil is unusually resistant to the development of rancidity and recommended it as suitable for enfleurage and as a lubricant. Morton (6) reported that the oil has excellent keeping quality, but the often-repeated statement that it never becomes rancid was untrue.

The present work sought to explain the oxidative stability of *Moringa oleifera* tree seed oil, to isolate natural components with antioxidant activity, and to evaluate their protection on vegetable oil.

EXPERIMENTAL PROCEDURES

Sample preparation. The *M. oleifera* seeds, variety of Malawi, were supplied by Kenya Forestry Research Institute (KE.F.R.I., Nairobi, Kenya). The oil was extracted with a mixture of 50:50 chloroform/methanol with the method described by Tsaknis *et al.* (7). Degumming followed the extraction of oil using the following procedure: The oil was put in a beaker and heated at 75°C. Then 20% of boiling water was added and mixed for 10 min. After cooling to 40°C, the mixture of oil and water was centrifuged for 10 min with a Sorvall General-Purpose RC-3 Automatic Refrigerated Centrifuge (Ivan Sorvall Inc., Newtown, CT) at 3,500 × *g* in 200-mL tubes. The gums were collected and freeze-dried at −10°C

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and 53 mTorr vacuum with a Virtis Sentry 5L (Virtis, Gardiner, NY), until a fine powder was produced. This powder was then collected and stored below –4°C (with N_2 introduced) for a maximum of 3 d in sealed bottles.

Determination of the antioxidant efficiency of freeze-dried gums. The method used to test the antioxidant efficiency of gums was a modification of the Quinn and Tang (8) method. A quantity of freeze-dried gums, approximating the percentage amounts removed during degumming, was mixed thoroughly for 10 min with a sample of the (degummed) oil from which they were produced. These samples were placed in the reaction vessels of the Rancimat 679 (Metrohm Ltd., Herisau, Switzerland) along with the sample of the original degummed *Moringa* oil (as control). The conditions were set at 120°C and 15 L/h.

Fractionation of compounds contained in gums. Gums were divided into 10 batches of 10 g. Each batch of fresh dried gums was homogenized for 10 min in 100 mL of water in a separation funnel. Then the solution was made alkaline to pH 8 with Na_2CO_3 and extracted with diethylether (10 × 50 mL). The organic (diethylether) phases were combined, evaporated in a rotary evaporator at a temperature of 35°C under vacuum, and weighed (this residue was named Fraction 1). The water phase that remained after the removal of the diethylether phase was acidified to pH 4 with HCl and extracted again with diethylether (10 × 50 mL). The new organic (diethylether) phases were combined and evaporated under the same conditions (the residue was named Fraction 2). The acidic water phase (remaining after the removal of the diethylether phase) was then extracted with *n*-butanol (10 × 50 mL). The organic (*n*-butanol) phases were combined, evaporated with the aid of the rotary evaporator at a temperature of 40°C under vacuum, and weighed (Fraction 3). The remaining water layer was collected, evaporated under vacuum at a temperature of 40°C, and weighed (Fraction 4). All fractions were stored below –4°C in brown gas-tight bottles.

Screening procedure of components contained in Fractions 1 to 4. The nature of the compounds in Fractions 1–4, separated as described in the previous section, was determined using the following method. The fractions were compared with two reference compounds, lecithin from soya (LFS) (Serva Fein Biochemica GmbH & Co., Heidelberg, Germany) and 3,5,7,4′-tetrahydroxy kaempherol (Fluka AG, Buchs, Switzerland), on two different chromatographic plates using different methods.

The first method used was reported by Ganshirt (9). A chromatographic tank was filled with 150 mL of chloroform/methanol/water (65:25:4) (10). Then 50 mg of LFS was dissolved in 1 mL of chloroform/methanol (1:2) and applied on a TLC plate (precoated with Silica gel G, 20 × 20 cm, layer thickness 0.25 mm; Merck Ltd., Darmstadt, Germany) on a line as thin as possible and with a length about 6 cm. Fractions 1–4 were also applied on the same plate using the same procedure. The run of the plate was completed within 18 min. Next, the spots were colored yellow in a chamber with iodine vapor and identified according to their R_f values by comparison with those reported by Mangold (10).

A TLC plate (precoated with cellulose, 20 × 20 cm, layer thickness 0.1 mm; Merck) with Fractions 1–4 and 3,5,7,4′-tetrahydroxy kaempherol was treated using the method reported by Markham (11). The mobile phase was *t*-butanol/acetic acid/water (60:20:20). After developing and drying, the plate was sprayed with 1% methanolic solution of diphenylboric acid 2-amino ethyl ester ($C_4H_{16}BNO$-Naturstoffreagenz A or Neu's reagent, Sigma Chemical Co., St. Louis, MO), which is adequate for identification of flavonoids by the color produced (11). The developed spots were observed under UV light (366 nm) to identify any flavonoids present.

Determination of the antioxidant activity of the isolated Fractions 1–4 from the gums. The Fractions 1, 2, 3, and 4 extracted from the gums were tested for their antioxidant activity on several samples of fresh sunflower oil (Elais S.A., Athens, Greece) using the UV accelerated method suggested by Morelle (12). Ten grams of sunflower oil and added fractions (the weight of the fractions was designed to give a final concentration in the sunflower oil of either 200 or 400 ppm) were accurately weighed into a glass petri dish (87 mm i.d. and 15 mm high) and mixed well. The dishes were placed (half immersed) in a water bath (50°C) directly exposed to UV light produced by a lamp situated 50 cm above (General Electric 260 nm UV Germicidal lamp G25T8, 25 watt, 45 cm in length, and 3 cm in diameter) for 12 h. Then the oils were stored in dark glass bottles with nitrogen at −20°C until required for analysis.

The extent of oxidation of the oils after 12 h of oxidation was assessed using PV, UV absorption at 232 nm (conjugated dienes), and determination of malondialdehyde (MDA) by HPLC. The method used for the determination of PV was adapted from that developed by Lea (13). The method used for the determination of specific extinction ($E^{1\%}_{1cm}$) at 232 nm (conjugated dienes) was adapted from IUPAC (14). The method used for determination of malondialdehyde (MDA) by HPLC was adapted from Tsaknis *et al.* (15). HPLC was performed using a Waters System consisting of a Waters 600E HPLC pump (Millipore, Waters Chromatography Division, Milford, MA) (detection limit: 6.3×10^{-8} mol/kg of oil).

Preparative-scale HPLC fractionation of Fraction 3. The method described below is a modification based on the method of Casteele *et al.* (16). Fraction 3 was diluted to a 0.25% concentration with water/methanol (1:3) in a dark glass screw-capped vial.

For the fractionation of Fraction 3, the HPLC system (Waters 600E HPLC pump and Waters 486 Tunable Absorbance Detector) was used. A mobile phase was made up of solvent A (water/formic acid, 5:95 vol/vol) and solvent B (methanol) with a flow rate of 2.5 mL/min. A Waters Zorbax RX-C18 semi-preparative column (9.4 × 250 mm, Zorbax porous silica microsphere, particle size 5 μm) was used. The elution profile was as follows: 0–2 min, 7% B in A (isocratic); 2–10 min, 7–15% B in A (linear gradient); 10–40 min, 15–75% B in A (linear gradient); 40–60 min, 75–80% B in A (linear gradient). The column temperature was kept constant at 35°C

with a Waters CHM oven for HPLC columns. The detector was set at 255 nm. Twenty 1-mL samples were separately injected onto the column.

Seven fractions were collected, from each injection, with the aid of a Waters Fraction Collector in 100-mL dark glass bottles, and those with the same retention times were combined and named according to their order of exit as HPLC 1–7. The solvents of the above fractions were removed with a vacuum rotary evaporator at 35°C. The residues of the seven fractions were tested for their antioxidant activity (using the same UV accelerated method), and the purity of the compound with the highest antioxidant activity (HPLC 3) was checked with a Waters Zorbax RX-C18 analytical column (4.6 × 250 mm, Zorbax porous silica microsphere, particle size 5 μm), using the same conditions as described above for the semipreparative column but with a flow rate of 1 mL/min (0.25 mg of this compound was dissolved in 500 μL with water/methanol in a ratio of 1:3). This solution was injected onto the column through a 20-μL loop of the Reodyne valve and only one peak was detected.

Identification of compound HPLC 3. The identification of fraction HPLC 3 was done using the following methods: ^{1}H NMR, ^{13}C NMR, MS, m.p., and UV absorption spectroscopy.

For the ^{1}H NMR and ^{13}C NMR determinations, the sample was prepared according to El-Ansari *et al.* (17), and 25 mg of sample was dissolved in 0.5 mL of hexadeuteriodimethylsulfoxide (DMSO). The solution was then analyzed using a Varian model Gemini 2000 spectrometer (Varian, Palo Alto, CA) at 300 MHz.

The positive electron impact (EI) method used for mass spectroscopy was adapted from Hedin and Phillips (18). Spectra were taken at 70 eV in the EI mode with a VG MassLab 20-250 Automated Mass Spectrometer (VG MassLab, Manchester, United Kingdom). The sample was introduced into the source of the instrument *via* a direct insertion probe. The source was maintained at a temperature of 200°C, and the probe was ballistically heated from ambient temperature to 300°C.

The method used for the determination of the m.p. was adapted from Markham (11) and carried out on a 535 Büchi apparatus (Büchi Laboratorius-Technik AG, Flawil, Switzerland).

The method used for UV absorption spectroscopy was adapted from Markham (11) and carried out on a Hitachi U-3210 spectrophotometer (Hitachi Co., Tokyo, Japan).

RESULTS AND DISCUSSION

Antioxidant activity of freeze-dried gums. The addition of gums back to the oil from which they were produced increased the induction period of the latter (Table 1). This is in agreement with Gopalakrishna and Prabhakar (3), who reported that by adding water-washed freeze-dried gums to the same batch of peanut oil from which they were produced, the resistance of the oil was increased to almost the same level that it had been before degumming.

The recovery of the original induction period was not 100% because during the degumming process (where high temperature was applied) tocopherols and possibly other constituents with antioxidant activity were partly destroyed. Tocopherols were reduced from 226.9 to 93.0 ppm (α-tocopherol), from 71.5 to 26.4 ppm (γ-tocopherol), and from 216.6 to 71.5 ppm (δ-tocopherol). Phospholipids contained in gums, which act synergistically with tocopherols, had a much lower content of tocopherols to interact with and, therefore, antioxidant activity was expected to be lower. This agrees with Kashima *et al.* (19), who reported that during their experiments none of the phospholipids (PC, PE, and PS) showed an antioxidant effect on perila oil free of tocopherols. The additions of PE or PC delayed the oxidation of the oil by acting synergistically with the endogenous tocopherols in the oil.

The same gums were added to sunflower oil at a level of 200 ppm. The results (Table 1) showed an improved resistance to oxidation of the sunflower oil after the addition of gums. The differences between the activities were statistically significant ($P < 0.05$).

Antioxidant activity of fractions isolated from gums. The four fractions (1, 2, 3, and 4) isolated from the gums were separately added to sunflower oil and the mixtures were oxidized using the accelerated-UV method. The methods used to measure the extent of lipid oxidation of the sunflower oil were PV, absorbance $E^{1\%}_{1cm}$ at 232 nm, and determination of MDA by HPLC. None of these methods by itself can be used to evaluate

TABLE 1
Antioxidant Activity of Freeze-Dried Gums Produced from the Oil Extracted with Chloroform/Methanol (50:50) from Seeds of the *Moringa oleifera* Tree Variety of Malawi[a]

Induction period of *Moringa* oil before degumming at 120°C (h)	Induction period of *Moringa oil* after degumming at 120°C (h)	Induction period of *Moringa* oil after degumming with added gums at 120°C (h)	Recovery of induction period (%)	Induction period of sunflower oil at 90°C (h)	Induction period of sunflower oil with added gums (200 ppm) at 90°C (h)
123.0 (7.2)	31.8 (0.6)	110.5 (5.9)	89.8[b]	8.65 (0.39)	12.99 (0.3)

[a]Values are means of triplicate determinations. SD is given in parentheses.
[b]The recovery was calculated as induction period of *Moringa* oil before degumming/induction period of *Moringa* oil after degumming with added gums × 100.

TABLE 2
Antioxidant Activity of Fractions 1, 2, 3, and 4 Isolated from the Gums of *Moringa oleifera* Malawi Seed Oil and HPLC Fractions (HPLC 1 to 7) Isolated from Fraction 3[a]

Fraction used	PV (meq O_2/kg oil)	$E^{1\%}_{1cm}$ at 232 nm	MDA by HPLC (mol MDA·10^{-6}/kg oil)
Sunflower oil (SO) (control)	298.7	27.04	6.23
	(2.4)	(0.03)	(0.04)
SO + 200 ppm α-tocopherol	167.8	14.36	3.18
	(1.9)	(0.06)	(0.02)
SO + 200 ppm BHT	156.7	13.46	2.97
	(2.0)	(0.03)	(0.02)
SO + 200 ppm Fraction 1	283.0	25.12	5.62
	(0.6)	(0.04)	(0.03)
SO + 400 ppm Fraction 1	268.0	23.06	5.46
	(1.2)	(0.07)	(0.04)
SO + 200 ppm Fraction 2	239.3	20.61	4.57
	(1.0)	(0.02)	(0.02)
SO + 400 ppm Fraction 2	218.6	18.74	4.07
	(0.6)	(0.04)	(0.08)
SO + 200 ppm Fraction 3	136.6	11.44	2.52
	(1.8)	(0.02)	(0.07)
SO + 400 ppm Fraction 3	116	9.87	1.57
	(2.8)	(0.04)	(0.04)
SO + 200 ppm Fraction 4	192	16.07	3.52
	(0.8)	(0.03)	(0.03)
SO + 400 ppm Fraction 4	208	17.89	3.91
	(0.8)	(0.01)	(0.03)
SO + 200 ppm HPLC 1	277.4	22.06	5.43
	(2.4)	(0.02)	(0.04)
SO + 400 ppm HPLC 1	286.1	22.64	5.59
	(2.0)	(0.04)	(0.02)
SO + 200 ppm HPLC 2	251.4	20.46	4.72
	(0.9)	(0.08)	(0.08)
SO + 400 ppm HPLC 2	234.3	18.83	4.26
	(1.8)	(0.01)	(0.05)
SO + 200 ppm HPLC 3	147.3	12.17	2.08
	(1.6)	(0.06)	(0.03)
SO + 400 ppm HPLC 3	131.2	10.95	1.66
	(0.7)	(0.06)	(0.08)
SO + 200 ppm HPLC 4	196.2	15.98	3.31
	(0.6)	(0.09)	(0.07)
SO + 400 ppm HPLC 4	201.2	16.15	3.44
	(0.8)	(0.03)	(0.04)
SO + 200 ppm HPLC 5	223.6	18.11	3.95
	(0.6)	(0.08)	(0.06)
SO + 400 ppm HPLC 5	209.5	16.99	3.63
	(1.6)	(0.06)	(0.03)
SO + 200 ppm HPLC 6	178.9	14.54	2.84
	(1.4)	(0.08)	(0.02)
SO + 400 ppm HPLC 6	170.7	13.86	2.71
	(1.4)	(0.07)	(0.04)
SO + 200 ppm HPLC 7	193.6	15.74	3.27
	(0.7)	(0.03)	(0.02)
SO + 400 ppm HPLC 7	184.2	14.89	3.02
	(1.0)	(0.09)	(0.01)

[a]Values are means of triplicate determinations. SD is given in parentheses.

oil deterioration adequately in all situations. However, in combination they can assess the extent of oxidation in both the beginning (primary products: PV, the specific extinction at 232 nm) and the end (secondary products: MDA). The antioxidant activity of the various fractions was compared with commercial antioxidants including α-tocopherol and BHT (Sigma Chemical Co.). The results (Table 2) indicate that the samples of sunflower oil containing 200 and 400 ppm of Fraction 3 (consisting 0.05% w/w of gums) showed the least oxidation. Therefore, according to the experimental results, this fraction seems to be a better antioxidant than BHT and α-tocopherol at a level of 200 ppm. Fraction 3 was found (using the screening

procedure) to contain mostly flavonoids and very few phospholipids (lysophosphatidylethanolamine). By using the TLC cellulose plate, R_f values of the spots developed were measured and their colors were determined in order to identify the flavonoid structure (11). The colors observed were orange fluorescence and dull yellow (flavonols with a free 3-OH and with or without a free 5-OH); fluorescent light blue (flavones, flavanones lacking a free 5-OH with or without 3-OH substitution, and isoflavones lacking a free 5-OH); orange red (anthocyanidin 3-glycosides), and yellow-green (aurones lacking a free 4′-OH, flavanones lacking a free 5-OH, and flavonols with a free 3-OH and with or without a free 5-OH) (20).

The other Fractions (1, 2, and 4) also showed some antioxidant activity. Fraction 1 (consisting 81.8% w/w of gums) contained (as identified by the screening procedure) the neutral lipids of the gums. These were the TG of the oil retained in gums, which were not separated by the physical procedures (hydration and centrifuging). Fraction 1 was expected to have a slight antioxidant effect because part of the other fractions may have been retained in it and were inseparable.

Fraction 2 (consisting 0.04% w/w of gums) was expected to contain the less polar flavonoids. Less polar aglycones, such as isoflavones, flavanones, and highly methoxylated flavones and flavonols, tend to be more soluble in solvents such as ether and chloroform (11). These components should possess some antioxidant activity. Thus, the increased resistance to rancidity of sunflower oil was expected after the addition of Fraction 2.

Fraction 4 (consisting 17.0% w/w of gums) showed some antioxidant activity but it was lower than that of Fraction 3. Fraction 4 contained the phospholipid fraction of gums and possibly a fraction of glycosides. According to Markham (11), the presence of an attached sugar tends to render the flavonoid more water-soluble, and thus combinations of solvents with water make better solvents for glycosides. The antioxidant activity of Fraction 4 could be attributed to the slight antioxidant activity of phospholipids or to any phenolic compounds mixed with the phospholipid or dissolved in water (such as glycosides). Pokorny et al. (20) reported that the apparent antioxidative activity of isolated plant phospholipids might be due to contaminating phenolic compounds.

HPLC. Fraction 3 was fractionated by HPLC. Seven fractions (named HPLC 1–7) were separated and collected.

The antioxidant activity of these fractions was tested on sunflower oil using the accelerated-UV method. PV, absorbance $E^{1\%}_{1cm}$ at 232 nm, and determination of MDA were used to measure the extent of lipid oxidation of the sunflower oil. Results are shown in Table 2. The antioxidant activity of the various fractions was compared with that of standard antioxidants including α-tocopherol and BHT.

The oxidation of the oil sample containing compound HPLC 3 (retention time 28.78 min) appeared significantly lower ($P < 0.05$) than that of the other samples. It was even lower than that of α-tocopherol and BHT at the same concentration (200 ppm). At a level of 400 ppm, fraction HPLC 3 showed an even greater antioxidant activity (significant at $P < 0.05$). According to the results obtained by TLC on cellulose plates, fraction HPLC 3 (color developed: dull yellow–light orange) was a flavonol with a free 3-OH and possibly a free 5-OH. The purity of the compound HPLC 3 was checked and confirmed using a Zorbax RX C-18 analytical column.

^{1}H NMR and ^{13}C NMR spectroscopy. The ^{1}H NMR spectrum of fraction HPLC 3 showed two doublet protons in the region of 6.15 ppm (H-6, coupling constant, *J*: 2.2 Hz) and 6.34 ppm (H-8, *J*: 1.8 Hz). The protons at C-6 and C-8 of flavonols that contain the common 5,7-dihydroxy substitution pattern give rise to two doublets in the range 6.0–6.5 ppm. The H-6 doublet occurs consistently at a higher field than the signal for the H-8 (21).

A doublet peak (relative intensity 2) was present in the NMR spectrum at 7.21 ppm (2′,6′-H). Mabry et al. (21), also reported that the C-2′ and C-6′ proton signals usually overlap in the region 6.5–7.5 in flavonoids having the 3′,4′,5′-oxygenation pattern.

The use of the integration ratios was very useful to assign the number of hydrogens because the 3, 7, 3′, 4′, and 5′ signals were very weak or joined to form a single broad peak. Four single protons were recorded at 9.23 ppm, which represent four hydroxyl groups bonded with four different carbon atoms at positions 7, 3′, 4′, and 5′. Two more signals were observed. The signal at 12.48 ppm was a typical one for a C-5 hydrogen-bonded hydroxyl group (21). The very weak signal at 10.8 represented another hydroxyl group bonded at position 3. Its position could possibly be explained by a hydrogen bond formed with the carbonyl at position 4.

The extracted sample HPLC 3 spectrum showed an unexpected signal at 3.32 ppm. The explanation is that the solvent used (DMSO) rapidly absorbs atmospheric moisture, and the signal obtained from the absorbed H_2O often obscures NMR signals resulting from some of the flavonoid protons (11).

When D_2O was added to the DMSO solvent of the sample, the six proton signals at 9.23, 10.8, and 12.48 ppm were not present, indicating that these protons are hydroxyl protons. Markham (22) reported that this method is helpful in the location of a hydroxyl.

The signals of HPLC 3 in DMSO were observed in the ^{13}C NMR spectrum. ^{13}C-^{1}H coupling data have been used to good effect in the distinction of C-6 from C-8 signals and C-5 from C-9 signals. The degree of coupling identifies each carbon and demonstrates that C-5 resonates downfield from C-9 and that C-6 resonates downfield from C-8 (23). The reverse of the order for H-6 and H-8 resonance takes place in the ^{1}H NMR (24). ^{13}C-^{1}H coupling constants for aromatic ring carbons range from 155–170 Hz for a directly attached proton down to 1–3 Hz for *ortho*- and *para*-related protons. A *meta*-related proton causes a 6–8 Hz splitting of the carbon signal (11).

The carbonyl carbon, C-4, resonates at around 172–177 ppm, and shifts are affected by the presence or absence of hydrogen-bonded hydroxyls (23). In contrast to proton resonance signals, the intensity of a ^{13}C resonance signal does not

necessarily reflect the number of carbons it represents, and so integration of ^{13}C NMR spectra is rarely of value (11).

According to the results of ^{1}H NMR and ^{13}C NMR spectroscopy, a 3,5,7,3′,4′,5′-hexahydroxyflavone structure was suggested for fraction HPLC 3.

MS. The mass spectrum of the extracted HPLC 3 fraction showed a value of 318 for the base peak (M^+) and 69, 108, 128, 136, 153, 246, 273, 289, 317, 319, and 358 for 11 fragments.

As reported by Hedin and Phillips (18) the fragments with values of 289 ($[M - 29]^+$) (which corresponds to the loss of CHO from the 4-keto group) and 153 ($[A_1 + 1]^+$) represented very informative ions that help to confirm that HPLC 3 has a 3,5,7,3′,4′,5′-hexahydroxyflavone structure.

Melting point. The m.p. of the isolated HPLC 3 fraction was shown to be 354–357°C.

UV spectrum. The UV spectrum of HPLC 3 dissolved in methanol, particularly the position of band I, provides information about the type of flavonoid as well as its oxidation pattern. Band I of flavones occurs in the range 304–350 nm, whereas band I of 3-hydroxyflavones (flavonols) appears at a longer wavelength (352–385 nm) (22). The UV spectrum of the compound HPLC 3 in methanol exhibited two major absorption peaks in the region 240–400 nm [Mabry *et al.* (21) reported that although the UV spectra are reproduced for the range of 220–500 nm, only the maxima for those peaks/shoulders occurring at wavelengths longer than 240 nm are indicated]. The λ_{max} of band I (300–380 nm) was 374.6 nm, and the λ_{max} of band II (240–280 nm) was 254.4 nm. These are in agreement with those reported by Markham (11) and Mabry *et al.* (21), for myricetin, which shows λ_{max} of 374 and 254 nm for bands I and II, respectively.

The results of ^{1}H NMR, ^{13}C NMR, m.p., and UV absorption spectroscopy showed that HPLC 3 fraction is 3,5,7,3′,4′,5′-hexahydroxyflavone (myricetin). To confirm the authenticity of HPLC 3 as myricetin, a standard 3,5,7,3′,4′,5′-hexahydroxyflavone (myricetin) was obtained from Fluka AG. The ^{1}H NMR, ^{13}C NMR, MS, m.p., and UV absorption spectroscopy of standard myricetin were determined (using exactly the same conditions), and the results were compared with those of compound HPLC 3. Results of compound HPLC 3 were also compared with those presented in the literature.

Both ^{1}H NMR spectra (HPLC 3 and standard myricetin) showed the same signals, which correspond to those expected for 3,5,7,3′,4′,5′-hexahydroxyflavone (myricetin). The protons at positions 6, 8, 2′, and 6′ had δ values of 6.10, 6.35, 7.18, and 7.18, respectively. The protons of the hydroxyl groups at positions 3′, 4′, 5′, and 7 had a δ value of 9.19. The protons of the hydroxyl groups at positions 3 and 5 had δ values of 10.73 and 12.4, respectively. As observed with compound HPLC 3, the standard myricetin sample spectrum also showed the unexpected signal at 3.32 ppm due to the atmospheric moisture rapidly absorbed by solvent DMSO.

Because of the lack of previously reported data in the literature on the ^{1}H NMR spectrum of myricetin in DMSO, Fraction HPLC 3 was compared with that reported by Mabry *et al.* (21) for myricetin in CCl_4. The structure of both spectra appears to be the same, with protons following the order H-6 > H-8 > H-2′,6′ (from higher to lower energy level). However, chemical shifts could not be compared because in Mabry *et al.* (21) myricetin was dissolved in a different solvent (CCl_4).

The myricetin ^{13}C NMR spectrum also could not be found in the literature. Therefore, the results were compared with those reported by Markham (11) and Markham and Chari (23) for myricetin (3-*O*-galactose) in DMSO and Markham and Chari (23) for myricetin (3-*O*-rhamnoside) in DMSO also. These are similar to those of the present work. However, there is a disagreement in δ of C-2. It was reported as 156.2 (C-2) for myricetin (3-*O*-galactose) and 156.4 (C-2) for myricetin (3-*O*-rhamnoside) by the above authors. In the present work, C-2 was reported at a lower δ value of 147.07. By comparing other spectra of polyhydroxyflavonols aglycones (quercetin and kaempherol) with their 3-*O*-glycosides (quercetin 3-*O*-glucoside, quercetin 3-*O*-rutinoside, quercetin 3-*O*-galactoside, quercetin 3-*O*-rhamnoside, quercetin 3-*O*-arabinopyranoside, quercetin 3-*O*-arabinofuranoside, kaempherol 3-*O*-rutinoside, and kaempherol 3-*O*-sophoroside) from Markham and Chari (23) and Markham (11), it could be concluded that the similar δ-values of C-2 and C-9 in glycosides were a consequence of 3-*O*-glycosylation. Aglycones produced two different signals for C-2 and C-9 at 146.9 and 156.2 ppm, respectively (quercetin), and at 146.8 and 156.2 ppm, respectively (kaempherol). These δ are in line with those reported in the present work for C-2 and C-9 of fraction HPLC 3.

During MS, both samples (HPLC 3 and standard myricetin) showed the same fragments but with higher intensities in the case of standard myricetin. One likely reason for this is that the spectrum may be affected by impurities in the sample acquired during the isolation procedure.

If the flavonoids are crystalline, one of the best methods of comparison is by m.p. and mixed m.p. determination. Pure flavonoids possessing the same or closely similar m.p. may or may not be the same compounds, but the m.p. of the mixture will be markedly depressed, often 20–30°C below the m.p. of either compound, if the compounds are different (11). Melting point determination of standard myricetin was in the range of 356–358°C. When the two samples were mixed, the m.p. was 355–357°C. This further proved that the samples are identical.

The UV spectrum of the fraction HPLC 3 and standard myricetin proved to be identical, with two major absorption peaks in the region 240–400 nm.

The above results confirmed the authenticity of HPLC 3 as myricetin. Its structure (multiple phenolic hydroxyl groups, especially the 3′,4′-dihydroxy configuration, a 4-CO group, and a free 3-OH group with a 5-OH group simultaneously present) is responsible for the important antioxidant activity

shown (24–26). The presence of a flavonoid with the above characteristics in the gums of the oil from the seeds of the *M. oleifera* tree can explain its extreme resistance to rancidity.

The use of myricetin (consisting of fraction HPLC 3 and present in a quantity of 330.8 and 29.11 ppm in gums and oil, respectively) as a potential natural antioxidant and a possible substitute for artificial ones should be considered. *M. oleifera* is a tree growing rapidly even in poor soil, is little affected by drought (6,27), and can be easily grown in Third World countries. The production and exploitation of a potential natural antioxidant from *M. oleifera* could be of economic benefit to the native population of the areas where the tree is cultivated.

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