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Antioxidant Activity of Methoxylated Flavonoids in Oils in Deep Frying Processes

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Abstract

Methoxylated flavonoids isolated from cold acetone leaf wash of *Polygonum senegalense*; 5-hydroxy-7-methoxyflavanone 1 and 5-hydroxy-6,7-dimethoxyflavanone 3, were tested for their ability to enhance thermal stability of vegetable oils. Determination of the peroxide value (P.V.) and the *p*-Anisidine value (*p*-A.V.) was done according to the standard methods of analysis. The compounds were tested for *in vitro* cytotoxicity against a mammalian cell-line, Chinese Hamster Ovarian (CHO) using the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazoliumbromide (MTT)-assay. Studies on changes in peroxide and *p*-Anisidine values for the oils heated to temperatures between 180˚C and 200˚C recorded better stability enhancement at 100 ppm concentration with these flavonoids than the commercial antioxidant, butylated hydroxytoluene (BHT). The plant-based flavonoids had no significant cytotoxic effect against the CHO cell-line and may serve as alternative antioxidants to synthetic ones which have previously raised great concern over the health of consumers.

Keywords

Polygonum senegalense, Antioxidant Activity, Methoxylated Flavonoids, Vegetable Oils, Cytotoxic Effect

1. Introduction

Stability enhancement of fish oil is necessary to improve its shelf-life because fish oil contains highly unsaturated oils which are more susceptible to oxidation [1]. The rate and extent of formation of oxidation products in oils depends on the nature of the fatty acid composition (monounsaturated or polyunsaturated), the temperature at which the oil is heated [2] and the presence of metal ions [3]

[4]). Fish oil largely comprises of arachidonic acid, C20:4ω-6, docosapentaenoic acid, C22:5ω-3, and docosahexaenoic acid, C22:6ω-3 [5] and is more easily oxidized than vegetable oil [6]. Lipid oxidation is a highly deteriorative process and health disorders such as atherosclerosis and cancerogenesis among others correlate highly to the consumption of highly oxidized oils [7].

Edible fats/oils may contain up to 200 ppm of synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) [8], purposely added to improve the shelf life of the edible fats/oils. The use of synthetic antioxidants is, however limited, because of their physical characteristics and the unfolding toxicological concerns on some of the synthetic antioxidants [9].

Flavonoid aglycones are reported to be deposited on the surface of leaves, twigs and seeds of *Polygonum senegalense* and *Psiadia punctulata,* with a higher deposition on younger plant parts than on the older parts [10] [11]). The existence of an exudate on the leaf surface, rich in methoxylated flavonoids [12] [13]), is fascinating. The constituent compounds of leaf surface exudates [14] supposedly serve some protective role on the plant [15]). Epicuticular layer and external flavonoids not only reflect radiation, but are also known to be good quenchers of singlet oxygen [16]. Some of the constituent compounds of the leaf surface exudates are relatively non-polar and could perhaps be endowed with invaluable bioactivity in the relatively non-polar fish oil and edible oils. The choice for an oil stability enhancer must critically address the solubility and thermal stability of the oil additive and evaluate their cytotoxicity levels.

The presence of some synthetic antioxidants in fats and oils may not guarantee stability at deep frying temperatures. Antioxidants of low boiling point may gradually vaporize and thus expose the oil to oxidation [17]. The oil may degenerate into various oxidation products, some of which may have injurious effects to the body. Antioxidant principles, stable at high temperatures, must be identified for use as oil additives to fortify the heat stability of the oils. The concentration levels of antioxidants may continue to diminish through vaporization at the high temperatures, leaving the oil exposed to further oxidation [18].

2. Materials and Methods

2.1. Plant Materials

The resinous leafy branches of *P. senegalense* were harvested, along the river banks of Njoro river within Egerton University, Nakuru County, Kenya, at an altitude of 2300 m, twenty five kilometers, west of Nakuru town. A voucher specimen is deposited in the National Museum of Kenya Herbarium.

2.2. Extraction

One kilogram of leaves detached from the branches of *P. senegalense* plant were stuffed into a 5 L erlmeyer flask for extraction. A 1.5 liter portion of acetone was introduced into the flask and shaken for two minutes and decanted as an orange solution [19] two more fresh portions of acetone were used to wash the leaves

clear of the orange exudate. The acetone extract was decanted and filtered through a filter paper into a 2.5 L brown glass bottle. The acetone was recovered using a rotary evaporator at a rotate speed of 100, heated at a water bath regulated at 60˚C. The concentrate was preserved in an open brown glass bottle (100 ml) and stored in a vacuum desiccator until it gave a constant mass of 47.5 g (*i.e.* 20.2% of dry leaves) on weighing.

2.3. Chromatographic Methods

20 g of the extract was introduced into a glass column (4 cm diameter), packed with 129 g of 230 - 400 mesh silica gel. The column was sequentially eluted with 500 ml portions of hexane, 40% CH_2Cl_2 in hexane and finally with 60% CH_2Cl_2 in hexane, effectively eluting four coloured bands ranging from yellow to red. The eluant was collected as 40 ml fractions from which pure compounds were isolated through fractional crystallization from dichloromethane-methanol mixtures. Melting points (uncorrected), were determined using a Gallen camp melting point apparatus. ^{1}H and ^{13}C NMR spectra were determined at 500 and 125 MHZ, respectively.

Identification of the compounds was achieved through correlation of spectral data, melting point values and comparison with literature data [20].

2.4. Determination of Peroxide and *p*-Anisidine Values

Sunflower and rapeseed oils and fractionated palm shortening (Rina oil and Chipsy fat and Canola oil) were procured locally from local market in Nakuru. Two aluminium based cooking pots (15 cm in diameter each) were used for electrically heating the oil in pot A and B (control) respectively.

250 g of each oil sample was electrically heated in the cooking pots up to a temperature of 180˚C - 200˚C for 7 hrs each day. 50 mg of the plant isolates were dissolved in 1 ml of acetone in a vial bottle and introduced into the oil in pot A. The vial bottle was rinsed twice with 1 ml portions of acetone and the washings transferred into the oil. The second pot served as a control into which 3 ml of acetone was added into the oil. Determination of the peroxide value (P.V.) and the *p*-Anisidine value (*p*-A.V.) was done according to the standard methods of analysis [21]. The experiment was repeated three times for each case.

2.5. Cytotoxicity Screening Tests

Compounds were tested for *in vitro* cytotoxicity against a mammalian cell-line, Chinese Hamster Ovarian (CHO) using the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazoliumbromide (MTT)-assay [22]. All samples were tested in triplicate on a single occasion. The MTT-assay is used as a calorimetric assay for cellular growth and survival, and compares well with other available assays [23] [24]). The tetrazolium salt MTT was used to measure all growth and chemosensitivity. Flavanone 1 (Polsen 1) was dissolved in 10% methanol while flavanone 3 (polsen 3) was dissolved in 10% DMSO. Compounds were tested as a suspension

due to poor solubility. The initial concentration of the stock solutions was 2 mg/ml for all samples. All compounds were stored at −20˚C until testing. Ementine was used as the positive control in all experiments. The initial concentration of all samples were 100 mg/ml, which was serially diluted in complete medium with 10-fold dilutions to give 6 concentrations, the lowest being, 0.001 µg/ml. The 50% inhibitory concentration (IC_{50}) values for these samples were obtained from dose response curves, using a non-linear dose response curve fitting analyses via GraphPad Prism v, 4.0 software.

3. Results and Discussion

Column chromatography of the acetone leaf extract using dichlomethane-hexane solvent system mixtures yielded known methoxylated flavonoids 1 and 3 (Figure 1). ^{1}H NMR and ^{13}C NMR spectra are shown on Figures 2-5.

5-Hydroxy-7-methoxyflavanone **1** was obtained as colourless crystalline flakes, melting point of 101˚C. The ^{1}H NMR spectrum of **1** showed the presence of one methoxyl group at δ 3.79. It also exhibited three sets of double doublets of an AMX system at δ 5.408 (1H, J = 3.0, 10.1 Hz), δ 3.074 (1H, J = 13.0, 4.17 Hz) and δ 2.81(1H, J = 3.17, 14.08 Hz) which were characteristic of H-2, H-3_{ax} and H-3_{eq}, respectively, of the ring C of a flavanone moety (Rao *et al.*, 2004). Two meta-coupled doublets, at δ 6.05 and 6.06 (J = 2.38 Hz), each integrating for one proton, were assigned to H-6 and H-8, respectively. Noesy correlation of the methoxy group signal to these protons and HMBC correlations assigned the methoxy group to C-7 (δ_C 167.98). A D_2O exchangeable downfield signal at δ 12.01 (IH) was assigned to a hydrogen-bonded hydroxyl group at C-5 (δ_C 164) [25].

5-Hydroxy-6,7-dimethoxyflavanone **3** was obtained as light yellow crystals (61.9 mg), melting point 147˚C - 148˚C. The flavanone nucleus was confirmed by the presence in the NMR spectrum of an ABX system centered at δ 2.81, 3.08 and 5.40 for the C-2 and C-3 protons. Signals at δ 3.83 (3H) and 3.86 (3H) were assigned to a hydrogen bonded OH for C-5, δ_C 155.0. HMBC correlations of the signal at δ_H 11.85 with one of the methoxylated C-atoms (δ_C 130.6) required the signal at δ_H 6.11 to be attached to C-8. A multiplet centered at δ_H 7.41 (5H) was attributed to a mono-substituted ring C. Flavanones **1** and **3** have previously been isolated from *Onychium ciliculosum*, a medicinal herb [26].

Four brands of vegetable oils; Sunflower oil, Canola oil, Rina oil and Chipsy fat when heated to 180˚C - 200˚C displayed heat instability on assessment of

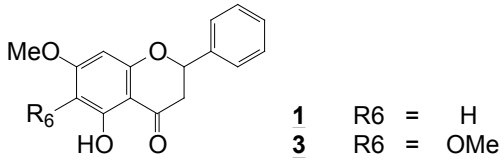
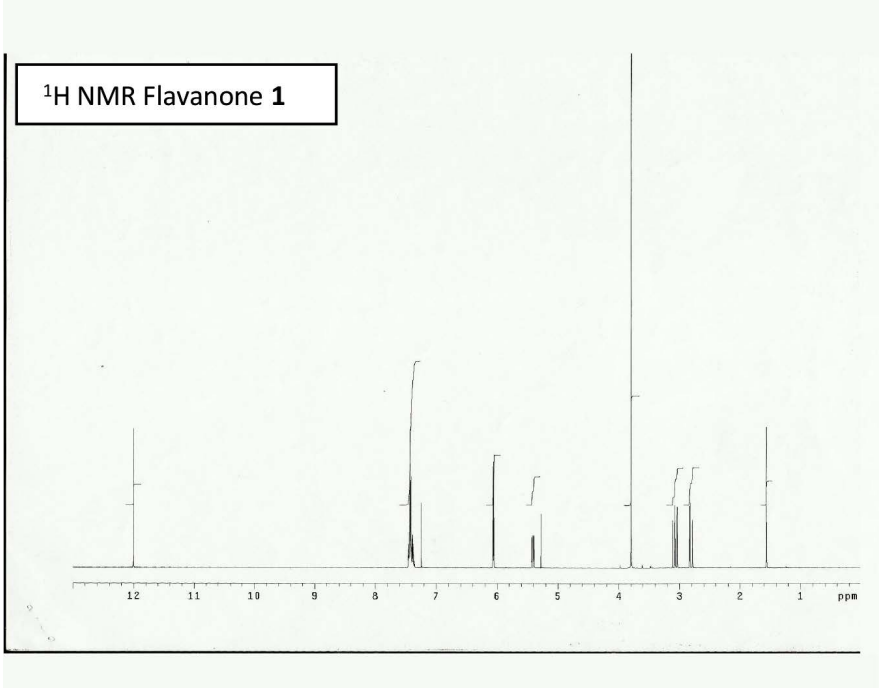
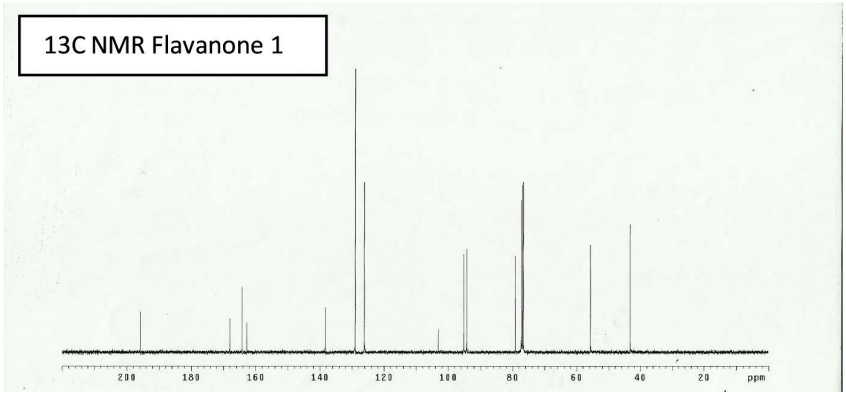


Figure 1. Flavanones 1 & 3.



INDEX	FREQUENCY	PPM	HEIGHT
1	4801.665	12.006	34.0
2	4801.268	12.005	43.8
3	2980.671	7.453	7.5
4	2974.125	7.436	43.8
5	2973.133	7.434	40.2
6	2966.785	7.418	28.9
7	2964.603	7.413	6.7
8	2961.032	7.404	6.2
9	2959.446	7.400	9.1
10	2959.049	7.399	9.1
11	2956.668	7.393	7.7
12	2954.486	7.387	10.4
13	2951.908	7.381	6.5
14	2948.734	7.373	6.2
15	2947.940	7.371	7.5
16	2895.969	7.241	16.8
17	2895.572	7.240	20.6
18	2426.239	6.066	15.4
19	2423.859	6.061	33.6
20	2420.288	6.052	35.8
21	2417.908	6.046	16.1
22	2170.745	5.428	9.6
23	2167.769	5.420	10.2
24	2157.653	5.395	10.3
25	2154.677	5.387	10.0
26	2111.434	5.279	12.1
27	2111.037	5.278	17.3
28	1517.329	3.794	200.0
29	1244.379	3.111	15.1
30	1231.287	3.079	14.4
31	1227.121	3.068	19.9
32	1214.227	3.036	19.5
33	1132.897	2.833	19.7
34	1129.724	2.825	20.8
35	1115.640	2.790	15.4
36	1112.466	2.782	14.5
37	625.280	1.563	26.6
38	624.487	1.561	44.5
39	623.495	1.559	14.4

Figure 2. ^{1}H NMR spectrum.



INDEX	FREQUENCY	PPM	HEIGHT
1	19688.072	195.774	14.4
2	16893.100	167.982	11.9
3	16505.825	164.131	23.0
4	16369.043	162.770	10.5
5	13912.730	138.345	15.8
6	12960.198	128.874	100.0
7	12683.338	126.121	59.8
8	10371.224	103.129	8.0
9	9566.186	95.124	34.5
10	9478.843	94.256	36.4
11	7966.822	79.220	34.1
12	7775.657	77.320	53.4
13	7743.521	77.000	58.9
14	7711.386	76.680	60.0
15	5600.325	55.688	37.7
16	4361.868	43.374	45.0

Figure 3. ^{13}C NMR spectrum.

their patterns in both the *p*-anisidine and the peroxide values as observed in Figure 6 and Figure 7.

Stability enhancement for these oils was implied based on the continued rise in peroxide and p-anisidine values for 14 hours on average when flavanones isolated from the *P. senegalense* were introduced into the oils at concentrations of 100 ppm as observed in Figures 8-11.

5-Hydroxy-7-methoxyflavanone suppressed the rise in p-Anisidine values and the peroxide values respectively and resulted in predictable patterns for all the oils as observed in Figures 8-11. It also prevented oil browning and registered a delay in rise in peroxide values, recording a maximum after 14 hours on average. 5-Hydroxy-6,7-dimethoxyflavanone **3** also displayed similar stability enhancement patterns when introduced into Sunflower oil at a concentration of 100 ppm.

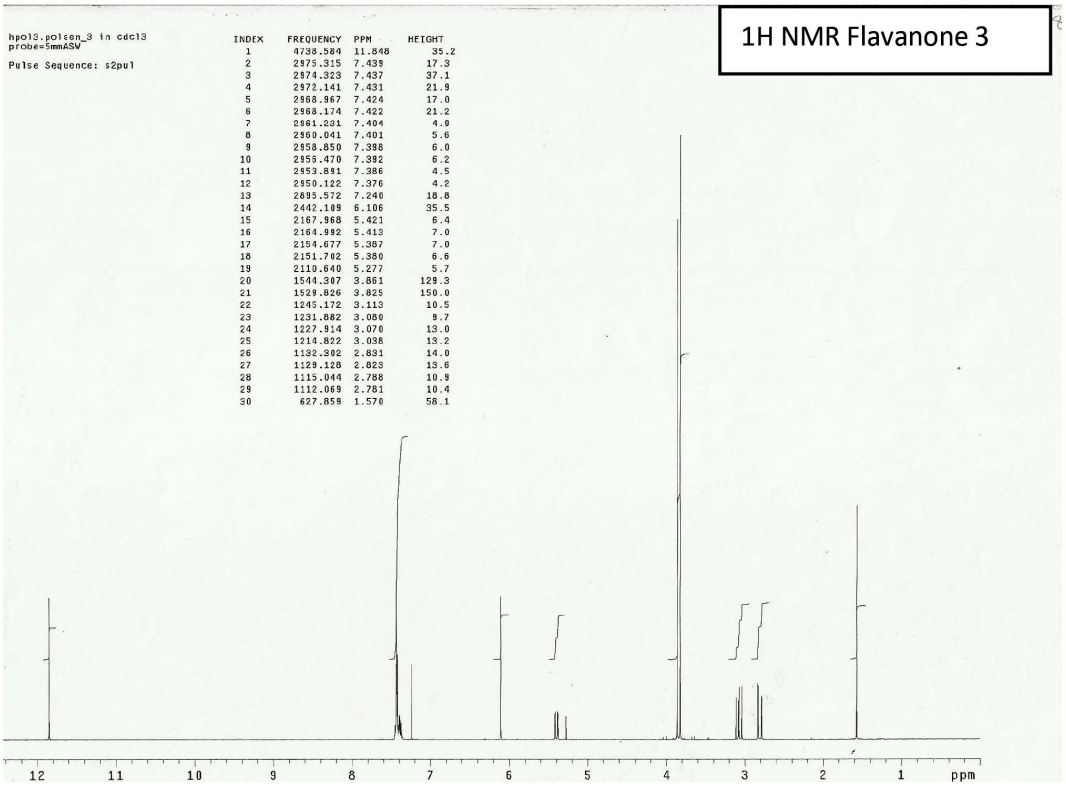


Figure 4. 1H NMR Spectrum.

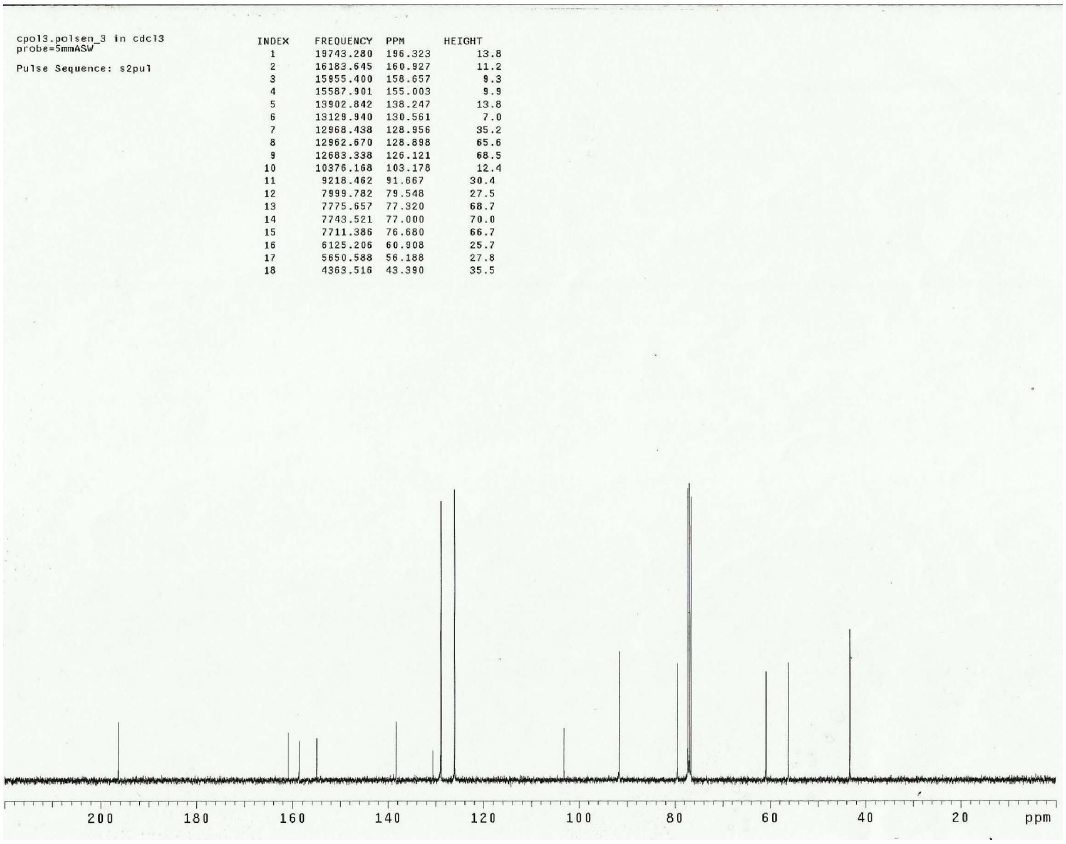


Figure 5. ^{13}C NMR Spectrum.

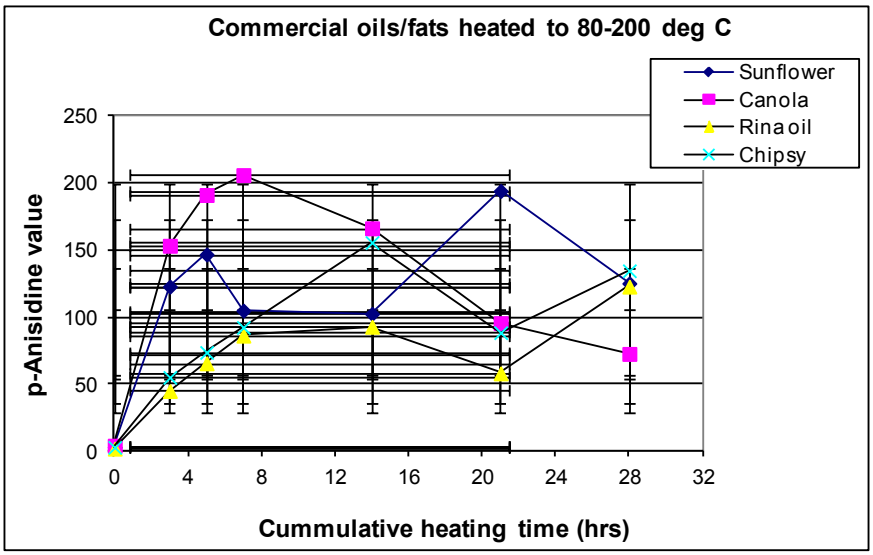


Figure 6. Changes in p-AV for plain oils heated to 180˚C - 200˚C.

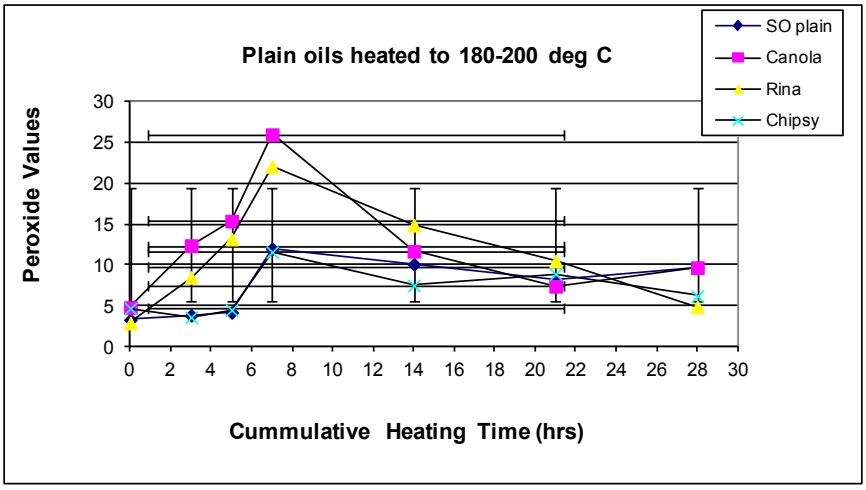


Figure 7. Changes in peroxide values for plain oils heated to 180˚C - 200˚C.

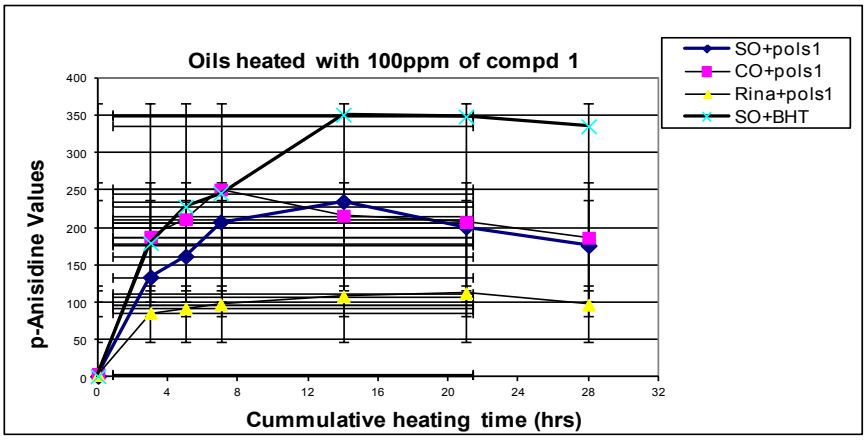


Figure 8. Changes in p-AV values for Oils heated with 100 ppm of 5-hydroxy-7-methoxyflavanone **1**.

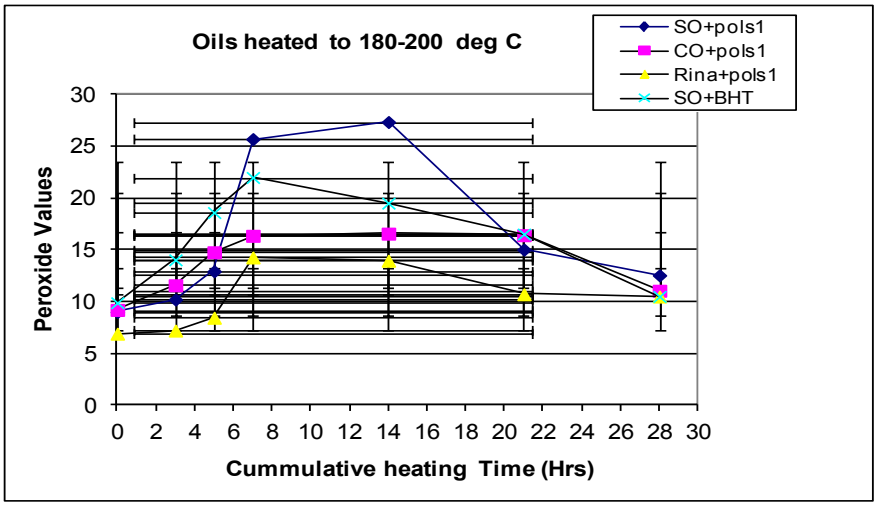


Figure 9. Changes in peroxide values for Oils heated with 100 ppm of 5-hydroxy-7-methoxyflavanone **1**.

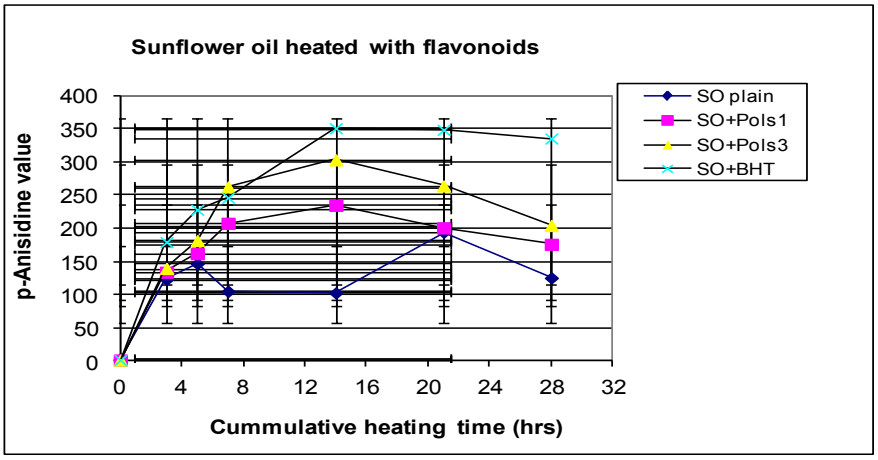


Figure 10. Changes in p-AV values for Sunflower oil heated with 100 ppm of compounds **1** and **3**.

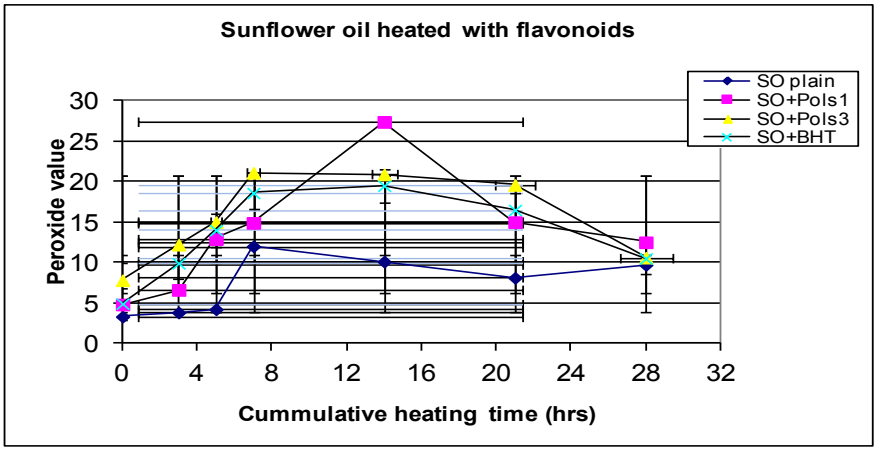


Figure 11. Changes in peroxide values for Sunflower oil heated with 100 ppm of compounds **1** and **3**.

Cytotoxicity Screening Tests

Compounds **1** and **3** had no significant cytotoxic effect against the CHO cell-line as observed in Table 1 and Figure 12 on dose-response curves.

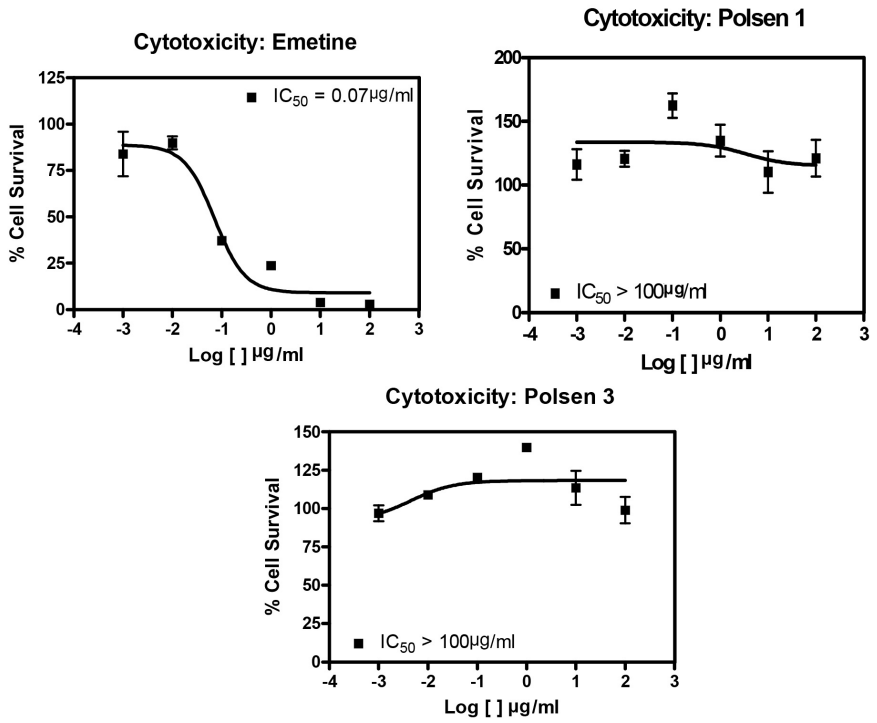


Figure 12. Dose-response curves of fractions using the CHO cell-line.

Table 1. IC_{50}-values of fractions using the CHO cell-line.

Compound	IC50 (µg/ml)
Polsen 1 (pols 1)	>100
Polsen 3 (pols 1)	>100
Emetine	0.07

The propensity of a flavonoid to inhibit free-radical mediated events is governed by its chemical structure. The number of substituent groups and their positions on the flavonoid structure can influence the radical-scavenging activity [27].

4. Conclusion

The present study indicate that 5-hydroxy-7-methoxyflavanone (pols **1**) and 5-hydroxy-6,7-dimethoxyflavanone (pols **3**) are effective anti-oxidants in retarding the formation of primary and secondary oxidation products during deep frying using vegetable oils. The two flavanones had no significant cytotoxic effect and are therefore potential food additives intended to serve as antioxidant principles in oil deep frying processes and in the formulation of animal feeds. The

melting points of these flavanones are well above the melting point of BHT (70˚C).

Acknowledgements

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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