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Full Length Research Paper

Antioxidant activities and inhibitory action of *Solanum macrocarpon* and *Hibiscus esculentus* phenolic containing leaf extracts against lipid oxidation

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There is a growing interest in preventive health care for the development and evaluation of natural antioxidants from plant food. Therefore, this study sought to investigate the phenolic composition of *Solanum macrocarpon* and *Hibiscus esculentus* leaves using HPLC/DAD/MS techniques, evaluate the radical scavenging activities, ferric reducing properties and assess the inhibitory action of the vegetable extracts against Low Density Lipoprotein (LDL) and egg yolk lipid- rich oxidation. The HPLC analysis revealed the presence of cynamoyl derivative, two chlorogenic acid derivatatives, rutin and Kaempferol-3-rutinoside in *S. macrocarpon* and five dicaffeoyl derivatives, unidentified flavonoid and isoquercitrin in *Hibiscus esculentus* leaf. The result indicated that the total phenolic content (mg tannic acid equivalent/g) and 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging (µmol trolox equivalent/g) activities were higher in *Hibiscus esculentus* phenolic extract (12.96± 0.04; 2.44E-05 ± 4.52E-06), respectively. Similarly, ferric reducing antioxidant power (mg ascorbic acid/g) and 1, 1-diphenyly-2-picrylhydrazyl radical scavenging (µmol trolox equivalent/g) activities were slightly higher in *Hibiscus esculentus* extract (10.60 ± 0.14; 2.68E-05 ± 1.56E-05), respectively. The result also showed that the studied vegetable extracts inhibited both human LDL and lipid rich egg homogenate oxidation. Overall, the antioxidant action and protection against lipid oxidation could be linked with the presence of the phenolic compounds in the vegetable extracts and the consumption will be of immense benefit in the prevention of a number of free radical mediated diseases.

Key words: Antioxidant Activities, *Hibiscus esculentus,* Lipid oxidation, Phenolic compounds, *Solanum macrocarpon.*

INTRODUCTION

Lipid peroxidation occurs in the body by the oxidation of unsaturated fatty acids. This process could be enzymatic or non-enzymatic events. It occurs in three steps initiation, propagation and termination stage (Gutteridge, 1995). During the initiation step, one atom of hydrogen is extracted from the unsaturated fatty acid (LH) by hydroxyl radical (*OH) resulting in the formation of lipid radical ($L^{*)}$ leading to the formation of conjugated diene. The propagation step is characterized by the reaction of conjugated diene with oxygen to form peroxyl radical ($LOO^{\cdot}$) which in turn attacks another unsaturated fatty acid to form unstable hydroperoxide (LOOH) and a new radical. In the termination step, a reaction between two of the formed radicals occurs to form non-radical products. In the body, endogenous antioxidant mechanisms exist to limit the formation and to scavenge free radicals. These include antioxidant enzymes; superoxide dismutase in the accumulations of O_2 radical by converting it to H_2O_2, which has lower toxicity. H_2O_2 is metabolized to H_2O by either glutathione peroxidase or catalase (Halliwell et al., 1992). In addition to antioxidant enzymes, some other molecules in the body act as antioxidants like glutathione. Other exogenous products act as antioxidants and free radical scavenging molecules like, vitamin E and C and

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plant derived natural antioxidants like carotenoids and polyphenols (Rice-Evans, 1995).

Phenolic compounds, important constituents in many plants, have received considerable attentions as potentially protective factors against cancer and heart diseases because of their antioxidant potency and their ubiquity in a wide range of commonly consumed foods of plants origin (Rice-Evans, 2001; Muselik et al., 2007; Kamei et al., 1995). Many studies have reported that phenolic compounds possess other biological activities such as anti-inflammatory, antiulcer, antispasmodic, anti-secretory, antiviral, anti-diarrhea, antitumor (Carlo et al., 1999). The presence of phenolic compounds in plants is an important factor for normal growth, development and defense against infection and injury. Also, phenolics may have an important effect on the oxidative stability.

Phytochemicals, including phenolics are suggested to be the major bioactive compounds contributing to the health benefits of vegetables and fruits (Yang et al., 2004; Sinelli et al., 2008). It was shown that the health properties of these natural products depend on the contents of bioactive compounds, mainly phenolics, and partly on dietary fibers (Chun et al., 2005). Green leafy vegetables provide a high amount of carotene, ascorbic acid and micro elements which play important roles in nutrient metabolism and slowing down of degenerative diseases (Yi-Fang et al., 2002).

Solanum macrocarpon otherwise known as the African Eggplant or Gboma is a tropical perennial plant of the *Solanaceae* family. The part of the plant that is consumed is the fruits and its young leaves. The roots, leaves, and fruit of *S. macrocarpon* have been reported to have some medicinal potential. In Nigeria, the fruit is used as a laxative, and as a means to treat cardiac diseases (Oboh et al., 2005). *Hibiscus esculentus* L. (Family Malvaceae), also known as *Abelmoschus esculentus*, is an annual vegetable that is more commonly known as okra. In some areas, it is called gambo or lady fingers. Originally from Africa, this member of the mallow family is now grown in warmer climates all over the world and is very popular in the southeastern United States. It produces showy pale yellow flowers, but is grown for its edible seed pods. The leaf of *Hibiscus esculentus* is an important medicinal plant and is usually used against stomach troubles, diarrhea, hypertension, skin diseases and in urinary troubles and dental-caries in Assam (Kalita et al., 2012).

The aim of the present investigation is to assess the antioxidant activities and the inhibitory action of the phenolic containing extracts against human Low Density Lipoprotein (LDL) and egg yolk homogenate lipid-rich media.

MATERIALS AND METHODS

Materials

The vegetables (*S. macrocarpon and Hibiscus esculentus*) were collected from local farms in Akure, south-western Nigeria, and voucher specimens were deposited at the Department of Biochemistry, Federal University of Technology, Akure, Nigeria and Department of Pharmaceutical Science, University of Florence, Italy (April, 2010). The samples were air-dried (5 to 7 days) and then oven-dried at 30^{o}C to a constant weight. The dried samples were then kept in sealed air-tight polythene bags until analysis. The dried samples were finely powdered immediately before extraction. All the standards used were purchased from Extrasynthese (Geney, France), with the exception of rutin from Sigma–Aldrich (St. Louis, MO, USA).

Extraction

A dried sample (1 g each) was extracted with 40 ml (20 ml x 2) of ethanol/water 7:3 (v/v) with water acidified by formic acid (pH 2.5). The samples were filtered and the clear solution directly analyzed by HPLC/DAD/MS (*Salawu et al.,* 2009).

HPLC/DAD/MS analysis of phenolic compounds

Analyses were performed using an HP 1100 liquid chromatograph equipped with HP DAD and 1100 MS detectors; the interface was an HP 1100 MSD API-electro spray. All the instruments were from Agilent Technology (Palo Alto, CA, USA). The MS analyses were carried out in negative mode with a fragmentor range between 80-150 V.

METHODS

A C12 column, 150 x 4mm (4µm) Synergi max (Phenomenex- Torrance CA) maintained at 30^{o}C and equipped with a 10 x 4 mm pre-column of the same phase was used with a flow rate of 0.4 ml min^{-1}. The eluents were H_2O acidified to pH 3.2 by formic acid (A) and acetonitrile (B). The following linear solvent gradient was applied: from 95% A to 85% A in 5 min, to 75% A in 8 min and a plateau of 10 min, to 55% A in 12 min and a plateau of 5 min, to 10% A in 3 min, and a final plateau of 2 min to wash the column. The total time of analysis was 45 min (Salawu *et al*., 2009).

Quantitative evaluation of phenolic compunds

The standards chlorogenic acid, rutin and luteolin 7-O-glucoside were used for the quantitative evaluation. Three five-point calibration curves were prepared as

follows: chlorogenic acid at 330 nm (range 0.038–0.3 mg/ml and r2 of 0.9996) was used to evaluate all the cynnamoyl compounds; rutin at 350 nm (range 0.13–1.02 mg/ml and r2 of 0.9999) was used to quantify all the derivatives of quercetin and kaempferol

Determination of antioxidant activities

Total phenolic content

The total phenolic contents of the ethanol/water extracts were determined by the Folin-Ciocalteu assay as described by Waterman and Mole (1994). The hydro-alcoholic extract (0.25 ml), was placed in a 25 ml volumetric flask and 5 ml distilled water was added. Folin-Ciocalteu's phenol reagent (1.25 ml) was added to it and mixed. After 2 min, 3.75 ml 20% (w/v) sodium carbonate solution was added. The contents were mixed and distilled water was added to make up 25ml and mixed. The mixture was left to stand for 2 h after addition of the sodium carbonate and the absorbance of the mixture was measured at 760 nm using a Lambda EZ150 spectrophotometer (Perkin Elmer, USA). The used standard was tannic acid and the results were expressed as mg tannic acid equivalents per gram of the sample.

Ferric reducing antioxidant power

The reducing power of the extracts was determined by assessing the ability of the extract to reduce $FeCl_3$ solution as described by Oyaizu (1986). Briefly, appropriate dilution of the extract (2.5 ml) was mixed with 2.5 ml 200 mM sodium phosphate buffer (pH 6.6) and 2.5 ml 1% potassium ferricyanide. The mixture was incubated at 50^{o}C for 20 min and then 2.5 ml 10% trichloroacetic acid was added. This mixture was centrifuged at 353 x g for 10 min. The supernatant (5ml) was mixed with an equal volume of water and 1 ml of 0.1% ferric chloride. The absorbance was measured at 700 nm. The ferric reducing antioxidant power was expressed as mg ascorbic acid equivalent/g of the sample..

ABTS antiradical assay

Antioxidant activity of the extracts was determined using the 2, 2'-azinobis-(3- ethylbenzothiazoline-6-sulfonic acid) ABTS antiradical assay (Awika et al., 2003). The $ABTS^{\bullet+}$ (mother solution) was prepared by mixing equal volumes of 8 mM ABTS and 3 mM potassium persulphate ($K_2S_2O_8$) (both prepared using distilled water) in a volumetric flask, which was wrapped in foil and allowed to react for a minimum of 12 h in a dark place. The working solution was prepared by mixing 5 ml of the mother solution with 145 ml phosphate buffer (pH 7.4). A range of trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-carboxylic acid) standard solutions (100–1000 µM) were prepared in acidified methanol. The working solution (2.9 ml) was added to the ethanol/water extracts (0.1 ml) or Trolox standard (0.1 ml) in a test tube and mixed with a vortex. The test tubes were allowed to stand for exactly 30 min. The absorbance of the standards and samples were measured at 734 nm with a Lambda EZ150 spectrophotometer. The results were expressed as µ mol Trolox equivalents/g sample, on dry weight basis.

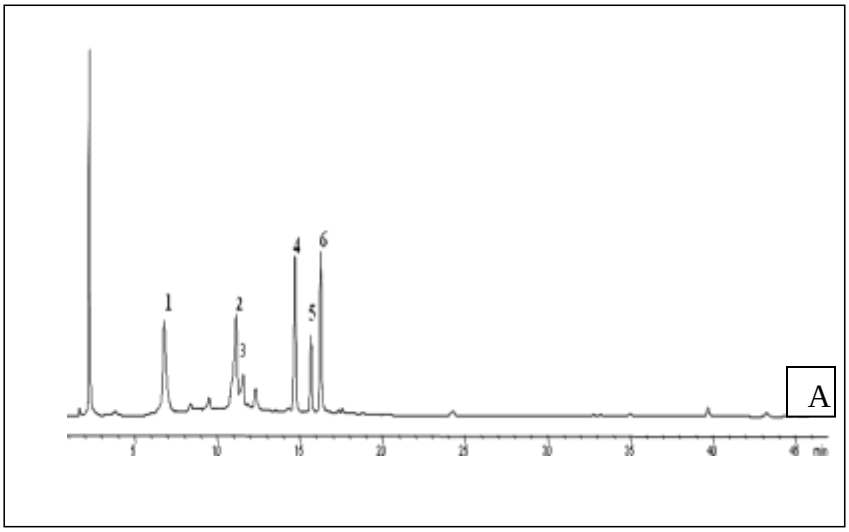
DPPH antiradical assay

The DPPH assay was done according to the method of Brand-Williams *et al.* (1995) with some modifications. The stock solution was prepared by dissolving 24 mg DPPH with 100mL ethanol and then stored at -20^{o}C until needed. The working solution was obtained by mixing 10mL stock solution with 45mL ethanol to obtain an absorbance of 1.1 units at 515 nm using the spectrophotometer. Vegetable extracts (150 mL) were allowed to react with 2850 mL of the DPPH solution for 6 h in the dark. Then the absorbance was taken at 515 nm. Results are expressed in µmol Trolox Equivalent/g sample. Additional dilution was needed if the DPPH value measured was over the linear range of the standard curve.

INHIBITION OF LIPID OXIDATION

Oxidation inhibition assay using human low density lipoprotein

The ability of the extracts to protect against LDL oxidation was determined spectrophotometrically by measuring the amount of thiobarbituric acid reactive substances (TBARS) produced after Cu^{2+}-induced oxidation of LDL in the presence of the extracts (Liu and Ng 2000). Briefly, 170 µl of an LDL solution (50µg/ml) in PBS was incubated with 100 µM final concentration Cu^{2+} in the presence or absence (control) of 20 µL of diluted hydroalcoholic extracts. The oxidation was performed in screw capped 2 ml eppendorf tubes at 37^{o}C in a shaking water bath for 3 h in the dark. Oxidation reaction was stopped by adding 10 mM EDTA (final concentration). Trichloroacetic acid (TCA) (200 µl, 20% w/v) and 200 µl of 0.67% (w/v) thiobarbituric acid (TBA) in 0.2 M NaOH were added to the post-incubation mixture. The mixture was heated at 80^{o}C for 30 min and cooled. After centrifugation at 1500 × g for 15 min to remove precipitated proteins, the absorbance of the supernatant was measured at 532 nm. Lipid peroxidation inhibitory



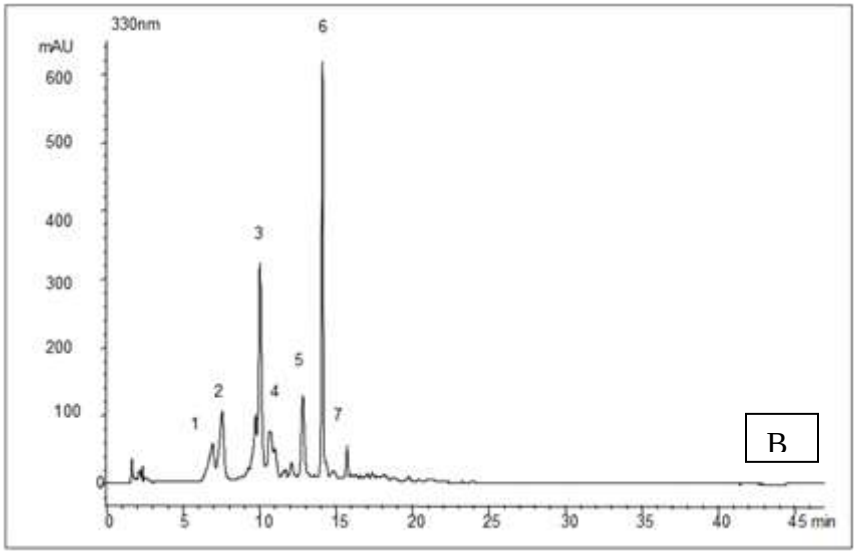


Figure 1. Chromatographic Profile at 330nm of The Hydro Alcoholic Extracts: (A) *Solanum macrocarpon* (B) *Hibiscus esculentus* At 330nm. The Profile Was Obtained With Synergy Max Column.

ratio was estimated as a function of the absorbance of the positive control.

Oxidation inhibition assay using egg yolk homogenate lipid rich media

Egg homogenate (0.5ml, 10% v/v) and 0.1ml of each extract were added to a test tube made up to 1ml with distilled water (Ruberto, et al., 2000). 0.05 ml $FeSO_4$ (0.07M) was added to induce lipid peroxidation and incubated for 30min. Then 1.5ml of 20% acetic acid (pH adjusted to 3.5 with NaOH) and 1.5ml of 0.8% (w/v) TBA in 1.1% sodium deodecyl sulphate and 20% TCA were added and the resulting mixtures were vortexed and then heated at 95^{O}C for 60min. After cooling, 5.0 ml of butan-1-ol was added to each tube and centrifuge at 300 rpm for 10min. The absorbance of the organic layer was measure at 532nm. The percentage inhibition of lipid peroxide formation by the extract was calculated.

Statistical analysis

All analysis for antioxidant activity determination, biological properties and HPLC phenolic composition were run in triplicate. The mean value and standard deviation were calculated using the Microsoft Excel software (Microsoft Corporation, Redmond, WA).

Table 1. List of the Identified Compounds in *Solanum macrocarpon* (SM) and *Hibiscus esculentus* (HE) Leaves by HPLC/DAD and HPLC/ESI/MS.

Peak no. /Sample	compounds	Rt (min)	χmax (nm)	$[M-H]^-$	Fragment ions
SM					
1.	Cynamoyl derivative	6.8	287/313	527	293, 135
2.	Chlorogenic acid derivative	11.2	240/328	693	353
3.	Chlorogenic acid derivative	11.5	238/326	693	353, 915
4.	Rutin	14.7	256/354	609	303
5.	Kaempferol -3-rutinoside	15.7	266/348	593	287
6.	Kaempferol -3-rutinioside	16.3	266/348	593	287
HE					
1.	Dicaffeoly derivative	7.0	242/326	743	191, 371
2.	Dicaffeoly derivative	7.4	244/326	743	191,371
3.	Dicaffeoyl derivative	10.1	244/328	743	191,371
4.	Dicaffeoyl derivative	10.7	243/326	417	
5.	Dicaffeoyl derivative	12.9	238/328	775	191,415
6.	Flavonoid	14.2	258/356	595	
7.	Isoquercitrin	15.7	256/358	453	301

RESULTS AND DISCUSSION

Phenolic compounds are very important plant constituents because they exhibit antioxidant activity by inactivating free radicals or preventing decomposition of hydroperoxides into free radicals. Many reports support the use of antioxidant supplements in reducing the level of oxidative stress and in slowing or preventing the development of complications associated with diseases (Rose et al, 1982). The process of lipid peroxidation has been suggested to proceed *via* a free radical chain reaction, which has been associated with cell damage in biomembranes (Halliwell, 1989).

The HPLC/DAD/MS phenolic profile of the studied green leafy vegetables is as shown in Figure 1 and Table 1. The phenolic compounds of *S. macrocarpon* and *Hibiscus esculentus* were characterized by using some standard compounds, the spectra data (Retention time, $[M-H]^-$, Fragment ions) and literature information. The chromatographic analyses revealed the presence of cynamoyl derivative, chlorogenic acid, rutin and kaempferol-3-rutinoside in *Solanum macrocarpon* while di-caffeoyl derivatives, unidentified flavonoid and isoquercitrin (*Quercetin*-3-β-glucopyranoside) were identified in *Hibiscus esculentus* leave. Results of Salawu et al. (2009) on some other popularly consumed green leafy vegetables (*Occimum gratissimum, Corchorous olitorius, Vernonia amygdalina and Manihot utilissima*) confirmed the presence of some of this important phytoconstituents (Salawu *et al*., 2009). The presence of quercitrin in *Hibiscus esculentus* leaf is in agreement with the report of Atawodi et al. (2009). Though, the compound quercitrin was identified in the fruit of *Hibiscus esculentus* in addition to an unidentified flavonoid and it is not unlikely that phenolic compound found in the fruit will not be found in the leaf since polyphenols are product of secondary metabolism in plant.

Though, very few data are available on the phenolic composition of Nigerian green leafy vegetables. However, the available information showed that the phenolics are mostly phenolic acids and flavonoids (Salawu et al., 2009). Table 2 showed the quantitative estimates of the phenolic compounds identified in the two vegetable extracts. The results showed the two identified Kaempferol-3-rutinoside (2.09± 0.01; 3.81 ± 0.03mg/g) as the most abundant phenolic compound in *S. macrocarpon* while the five dicaffeoly derivative were shown to be the predominant phenolic compounds in *Hibiscus esculentus*. Essentially, the phenolic compounds in the selected vegetables were identified to be cinnamics and flavonoids. On the basis of this classification, cinnamics will be considered to be the most

Table 2. Quantitative Estimates of Phenolic Compounds in *S. macrocarpon* and *Hibiscus esculentus* Leave.

Sample/Peak no	Compounds	mg/g (Mean ± SD)
Solanum macrocarpon		
1.	Cynamoyl derivative	2.79 ± 0.0069
2.	Chlorogenic acid derivative	2.95 ± 0.0200
3.	Chlorogenic acid derivative	1.12 ± 0.0077
4.	Rutin	4.37 ± 0.0231
5.	Kaempferol -3-rutinoside	2.09 ± 0.0145
6.	Kaempferol -3-rutinioside	3.81 ± 0.0347
Hibiscus esculentus		
1.	Dicaffeoyl derivative	0.89 ± 0.0135
2.	Dicaffeoyl derivative	1.30 ± 0.028
3.	Dicaffeoyl derivative	2.69 ± 0.028
4.	Dicaffeoyl derivative	0.43 ± 0.005
5.	Dicaffeoyl derivative	1.08 ± 0.012
6.	Flavonoid	5.00 ± 0.012
7.	Isoquercitrin	0.47 ± 0.024

Values are presented as Mean ±SD (n=4).

Table 3. Antioxidant Activities of *Solanum macrocarpon* and *Hibiscus esculentus* Leaves.

Sample	Total Phenol (mgTAE/g)	FRAP (mgVit.CEq/g)	ABTS (µmol Trolox Eq./g)	DPPH (µmol Trolox Eq./g)
Solanum macrocarpon	4.36± 0.06	8.56 ±0.11	6.59E-06± 7.94E-07	2.01E-05 ± 7.51E-06
Hibiscus esculentus	12.96± 0.04	10.60± 0.14	2.44E-05± 4.52E-06	2.68E-05 ±1.56E-05

Values are presented as Mean ±SD (n=4).

abundant phenolic in the two evaluated vegetables.

Hydroxycinnamic acids (e. g. ferulic and caffeic acids) are a well-known group of natural compounds, which are present in the human diet in representative amounts. Apart from being widely used as food additives (Serrano et al., 1998; Silvia et al., 2001), some phenolic acid derivatives have been found to act as inhibitors of deleterious oxidative processes – e. g. in the prevention of cardiovascular and inflammatory diseases and cancer (Esterbauer et al., 1992). Cinnamic acid esters, in particular, were shown to display remarkable growth-inhibition properties towards some human cancer cell lines (Nakayama, 1994). Chlorogenic acid occurs ubiquitously in food and posseses series of biological effects *in vitro* and *in vivo*, such as antioxidant capacity, radical scavenging activity, antimutagenic/ anticarcinogenic

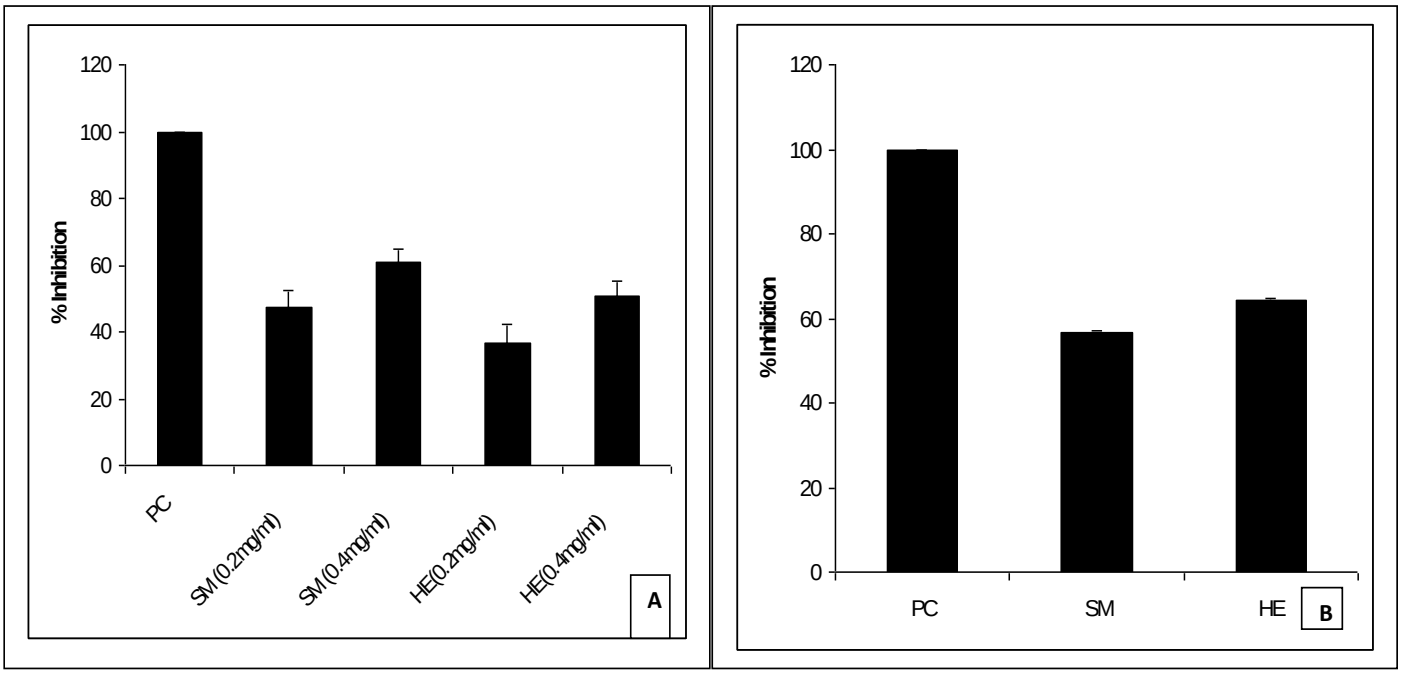


Figure 2. Inhibitory Action of Extracts of *Solanum macrocarpon* and *Hibiscus esculentus* against Lipid oxidation using (A) Human LDL (B) Egg Yolk Lipid-Rich Homogenate. SM-*Solanum macrocarpon*; HE-*Hibiscus esculentus*; PC- Positive Control. Error bars represent standard deviations. Graph bars with the same letters are not significantly different (P< 0.05).

effect, and inflammation inhibiting and endothelial protective properties (Morishita and Ohnishi, 2001).

Kaempferol glycosides found in *S. macrocarpon* have been previously reported to be a natural plant product with potentially useful pharmacological and nutraceutical activities common in vegetable, fruits, plant and herbal medicines. Kaempferol is known for its health promoting effect. Studies have shown that it reduces cancer, arteriosclerosis, cardiovascular disorder, and serve as antioxidant and anti-inflammatory (Yoshida et al., 2008; Kowalski *et al.,* 2005a, b). Rutin also is known for its anti-inflammatory and vasoactive properties, as well as for its capability to diminish capillary permeability and to reduce the risk of arteriosclerosis, thereby reducing coronary heart disease, possibly through the diminishing of platelet aggregation (La Casa et al., 2000; Jiang et al., 2007).

The result of the antioxidant properties and the radical scavenging activities of the phenolic containing vegetable extracts are as shown on Table 3. The results revealed that the two vegetable extract demonstrate some level of antioxidant action. However, *Hibiscus esculentus* leaf have the highest total phenolic content (12.96 ± 0.04 mg tannic acid equivalent/g of the sample) and ferric reducing power (10.60 ± 0.14 mg ascorbic acid equivalent/g of the sample). Similar trend was observed for ABTS and DPPH radical scavenging activities with *Hibiscus esculentus* leave having the highest values (2.44E-05 ± 0.00, 2.68E-05 ± 1.56E-05 µM TE/g) respectively. The *in vitro* antioxidant activity of methanolic extract of *Hibiscus esculentus leaf* has been reported by Patil et al. (2011). They observed that leaves and stem of *Hibiscus esculentus* have a good antioxidant activity. Also, the antioxidant activity of *S. macrocarpon* has been reported by Odukoya et al. (2007), Olajire and Azeez (2011). However, the result of the present investigation showed a direct relationship between the phenolic content and the evaluated antioxidant activities. This is in agreement with previous studies that reported a direct correlation between phenolic content and antioxidant capacity (Yang *et al*., 2002).

The inhibitory action of the phenolic extract against Low Density Lipoprotein oxidation and oxidation of egg yolk lipid rich homogenate is as shown in Figure 2. Both extracts demonstrate some level of inhibitory action against lipid oxidation. At a concentration of 0.2 and 0.4 mg/ml, the extracts showed inhibitory action against LDL oxidation in a concentration dependent manner. Also, at a concentration of 25mg/ml, the two vegetable extracts inhibited the oxidation of egg yolk lipid-rich homogenate. The inhibitory activities of both vegetables against human LDL and egg yolk lipid-rich homogenate oxidation will possibly be linked with their phenolic constituents. Flavonoids and phenolic acids have been reported to directly inhibit lipid peroxidation (Morton et al., 2000; Middleton et al., 2000). Polyphenolic compounds in the diet enhance the stability of low-density lipoprotein (LDL)

to oxidation, and evidence exists that LDL oxidation plays a significant role in atherosclerosis and coronary heart disease (Steinberg et al., 1989).

CONCLUSION

The extract of the studied leafy vegetables showed the presence of some phenolic compounds (phenolic acids and flavonoids) and demonstrated some level of antioxidant activities. It is well known that phenolic compounds containing plant food is of immense benefit to human in the prevention of a number of degenerative diseases. Also, the inhibitory action against lipid oxidation will make the vegetable rank high among the league of arsenals in ameliorating cardiovascular, coronary heart diseases and other diseases that are associated with lipid oxidation.

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