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Full Length Research Paper

Cellular antioxidant activities and cytotoxic properties of ethanolic extracts of four tropical green leafy vegetables

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The present investigation sought to evaluate cellular antioxidant activities of some green leafy vegetables: *Vernonia amygdalina,* Asteraceae *(Va,); Manihot utilissima* Pohl*.,* Euphoriaceae *(Mu);Corchorous olitorius* L.*,* Malvaceae *(Co) and Occimum gratissimum* L., Lamiaceae *(Og)* against peroxyl radical-induced oxidation in HepG2 cells and also evaluate their cytotoxic properties against drug sensitive and drug resistant Lovo and Lovo/Adr cancer cell lines respectively. HPLC/DAD/MS investigation of the ethanolic extract of the vegetal matter revealed the presence of 4, 5, 6, and 7 phenolic compounds in *Mu, Co, Og and Va* respectively. The cellular antioxidant activity was tested with different concentrations (0.1, 0.25, 0.5 and 1.00 mg/ml dried weight) of the extracts. At a concentration of 1.00 mg/ml, the quenching of peroxyl radical in HepG2 cells as shown by the fluorescence units revealed that *Og* has the best inhibition capacity, followed by *Co, Va* respectively and with the least inhibition capacity recorded for *Mu*. However, pro-oxidant activities were observed at lower concentrations of *Va* (0.1 mg/ml) and *Mu* (0.1, 0.25 and 0.5 mg/ml). Cytotoxic investigation revealed that the extracts were active against both cell lines with EC50 (µg/ml) ranging from 1.3 to 2.6 for Lovo and 1.5 to 2.7 for Lovo/Adr respectively, and that *Va* was the most active, while the least activity was recorded for *Mu* and *Co* for Lovo and Lovo Adr respectively. The observed cytotoxic properties and the elicited antioxidant activities would be due to the phytochemical constituents among which could be the rich content of flavonoids and cynammoyl derivatives as revealed by the chromatographic investigations.

Key words: Phenolic compounds, green leafy vegetables, cellular antioxidant activities, cytotoxicity.

INTRODUCTION

Plant foods have been an important source of medicine for thousands of years. Even today, the World Health Organization estimates that up to 80% of people still rely mainly on traditional remedies such as herbs for their medicines (Tripathi and Tripathi, 2003). Medicinal plants foods constitute one of the main sources of new pharmaceuticals and healthcare products. A whole range of plant-derived dietary supplements, phytochemicals and pro-vitamins that assist in maintaining good health and combating disease are now being described as functional ingredients and nutraceuticals. The role of medicinal plants food in disease prevention or control has been attributed to antioxidant properties of their constituents, usually associated to a wide range of amphipathic molecules, broadly termed polyphenolic compounds (Ivanova et al., 2005).

Overwhelming scientific data, from epidemiological

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studies, indicate that diets rich in fruit and vegetables are associated with a lower risk of several degenerative diseases, such as cancers (Steinmetz and Potter, 1996) and cardiovascular diseases (Rimm et al., 1996). This association is often attributed to different antioxidant components, such as vitamin C, vitamin E, carotenoids, lycopenes, polyphenols and other phytochemicals.

Phenolic compounds originating from edible and non-edible plant parts possess antioxidant activity. They display the capability to inhibit or delay the oxidation of lipids, proteins, and DNA by affecting the initiation or propagation of oxidizing chain reactions. Natural phenolic antioxidants can scavenge reactive oxygen and nitrogen species (RONS), thereby preventing the onset of oxidative diseases in the body (Halliwell and Gutteridge, 1992; Willet, 1994). A positive correlation between the consumption of phenolic-rich foods and several chronic diseases has been shown to exist from epidemiological studies (Kris-Etherton et al., 2002; Kushi et al., 1999).

The occurrence of various kinds of antioxidative phenolic compounds including flavonoids and cinnamic acid derivatives in plant foods was reviewed by several authors amongst whom was Herrmann (1989). Polyphenols in vegetables, fruits, and teas can prevent degenerative diseases including cancers through antioxidative action and/or the modulation of several protein functions and it has been estimated that over two-thirds of cancer related death could be prevented through lifestyle modification, particularly through dietary habits (Barnard, 2004).

The aim of the present work is to evaluate the cellular antioxidant activities and the cytotoxic properties of some leafy vegetables commonly consumed in Nigeria, namely; *Vernonia amygdalina,* Asteraceae *(Va); Manihot utilissima* Pohl., Euphoriaceae *(Mu); Corchorus olitorius* L., Malvaceae *(Co) and Occimum gratissimum* L., Lamiaceae *(Og).*

MATERIALS AND METHODS

Collection and preparation of materials

The vegetables (*O. gratissimum* L., *V. amygdalina, C. olitorius L* and *Manihot utilissima* Pohl.) were harvested from local farm in Akure, South-Western Nigeria. Fresh sample of about 4 kg was collected and dried in an oven at 30°C and powdered before storage. Voucher specimens were deposited at the Department of Biochemistry, Federal University of Technology, Akure, Nigeria and Department of Pharmaceutical Science, University of Florence, Italy.

Extraction method

The powdered vegetal materials (40 g) were extracted with 200 ml ethanol (96%) for 24 h and subsequently filtered. The alcoholic extracts were evaporated to dryness under reduced pressure using a rotary evaporator and the dried extract dissolved in 2% Dimethyl Sulphoxide (DMSO) and stored prior analysis.

Cellular antioxidant activities

Cellular antioxidant activities were evaluated according to the method of Wolfe and Liu (2007) in HepG2 cells. Human hepatocellular carcinoma HepG2 cells were seeded at a density of 6×10^4/well on a 96-well microplate in 100 µL of growth medium/well (William's Medium E; WME). Twenty-four hours after seeding, the growth medium was removed and the wells were washed with Phosphate Buffer Saline (PBS). Triplicate wells were treated for 1 h with 100 µL of vegetable extracts plus 25 µM of 2'7'-dichlorofluorescin-diacetate (DCFH-DA) dissolved in treatment medium. When a Phosphate Buffer Saline (PBS) wash was utilized, wells were then washed with 100 µL of PBS. Then 600 µM 2'2' azobis-dihydrochloride (ABAP) was applied to the cells in 100 µL of Hank's Buffered Salt Solution (HBSS), and the 96-well microplate was placed into a Fluoroskan Ascent FL plate-reader (ThermoLabsystems, Franklin, MA) at 37°C. Emission at 538 nm was measured with excitation at 485 nm every 5 min for 1 h. Each plate included triplicate control and blank wells: control wells contained cells treated with DCFH-DA and oxidant; blank wells contained cells treated with dye and HBSS buffer without oxidant.

In vitro assay for cytotoxic activity

Cytotoxicity was determined by means of the tetrazolium-dye assay (MTT) of Mosmann (1983). LoVo and LoVo/Adr cell lines were used. The cells were kindly provided by Dr A. Michelutti, Udine, Italy and were cultured in plastic flasks or multi-well plates with RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 µg/ml streptomycin and maintained at 37°C in a 5% CO_2 atmosphere and 95% humidity. The medium was changed every other day; exponentially growing cells were used in experiments.

3-*(4, 5-Dimethylythiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay*

Cells were seeded into 96 well plates at a cell density of 0.2×10^6 cells/ml (200 µl/well) in complete medium with increasing concentrations of the extracts (0.04-2 mg/ml) and incubated for 72 h. 20 µl of a 5 mg/ml MTT solution were added in each well, and the incubation continued for additional 4 h. The medium was then removed by vacuum aspiration and cellular formazan crystals were solubilized with 200 µl of dimethyl sulfoxide (DMSO). Absorbance was measured on an Automated Microplate Reader EL 311s (Bio-Tek Instruments, Inc., Winooski, VT) with a reference wavelength of 630 nm and a test wavelength of 540 nm. Each experiment was performed using 8 replicate wells for each extract concentration.

HPLC/DAD/MS analysis

The alcoholic extracts of the vegetal matter were filtered and the clear solution directly analysed by HPLC/DAD/MS. 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, the MS analyses were carried out in a negative mode with a fragmentor range between 80-150 V.

Method 1

A C12 column, 150 × 4 mm (4 µm) Synergi Max (Phenomenex-Torrance CA) maintained at 30°C and equipped with a 10 × 4 mm pre-column of the same phase was used; flow rate was 0.4 mL min^1. The eluents were H_2O acidified to pH 3.2 with formic acid (A)

Table 1. List of the identified phenolic compounds in ethanolic extract of the selected vegetables by HPLC/ESI/MS.

Sample	Compounds	Rt (min)	χmax (nm)	[M-H]	Fragment ions
Manihot utilissima Pohl					
1.	Rutin	14.6	356	609	300
2.	Kaempferol 4'-O-rutinoside	15.6	260/348	593	285
3.	Kaempferol 3-O-rutinoside	16.2	260/348	593	285
4.	Amentoflavone	38.1	268/336	537	
Corchorous olitorius L.					
1.	Caffeoyl quinic acid derivative	11.3	330	729	375; 353; 191; 179
2.	Hyperoside	15.4	256/358	463	301
3.	Isoquercitrin	15.6	256/358	463	301
4.	1, 5, dicaffeoyl quinic acid	19.1	328	515	353; 191; 179; 161
5.	Dicaffeoyl quinic acid	19.7	326	515	353; 161
Ocimum gratissimum					
1.	Vicenin-2	11.2	270/336	593	387
2.	Caffeic acid	12.3	330	179	135
3.	Rutin	14.6	256/356	609	301
4.	Caffeoyl derivative	33.8	330	-	-
5.	Cirsimaritin	38.8	276/334	313	297
6.	Nevadencin				
Vernonia amygdalina					
1.	Caffeoyl quinic acid	11.4	330	353	191
2.	Chlorogenic acid	12.0	330	353	191
3.	Luteolin-7-O-glucoside	15.6	260/348	447	285
4.	1, 5 dicaffeoyl quinic acid	19.1	328	515	353; 191
5.	Dicaffeoyl quinic acid	19.7	328	515	353;191;173
6.	Dicaffeoyl quinic acid	20.9	328	515	353;191;179
7.	Apigenin 6-O-glucuronide	22.1	268/334	445	269

and acetonitrile (B). The following linear solvent gradient was applied: from 95 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 90% B in 3 min, and finally a plateau of 2 min to wash the column. The total time of analysis was 45 min.

Method 2

A different column was used for the analysis of the *O. gratissimum* samples. The column was a Polaris-ether (Varian) 250x4.6 mm, 5 um; the eluents were H_2O acidified to pH 3.2 with formic acid (A) and acetonitrile (B); the flow rate was 0.8 mL min^{-1}, oven temperature 30°C. The following linear solvent elution method was applied: from 92 to 80% A in 10 min, to 75% A in 18 min, to 55% A in 12 min, to 95% B in 3 min, and finally a plateau of 6 min to wash the column. The total time of analysis was 44 min.

RESULTS AND DISCUSSION

The measurement of antioxidant activity is an important screening method to compare the oxidation/reduction potentials of fruits and vegetables and their phyto-chemicals in various systems. Many classical chemistry methods are currently in wide use to evaluate the antioxidant potentials of fruits and vegetables. Despite wide usage of these chemical antioxidant activity assays, their ability to predict in *vivo* activity is questioned for a number of reasons because such chemical analysis is performed at non-physiological pH and temperature, without taking into account the bioavailability, uptake, and metabolism of the antioxidant compounds (Liu and Finley, 2005). Cell culture models therefore provide an approach that is cost-effective, relatively fast, and address some issues of uptake, distribution, and metabolism.

Phenolic profile and cellular antioxidant activity (CAA) of the ethanolic extract was as shown in Table 1 and Figures 1 to 4 respectively. Previous report on the pheno-lic composition of methanolic extracts of the studied

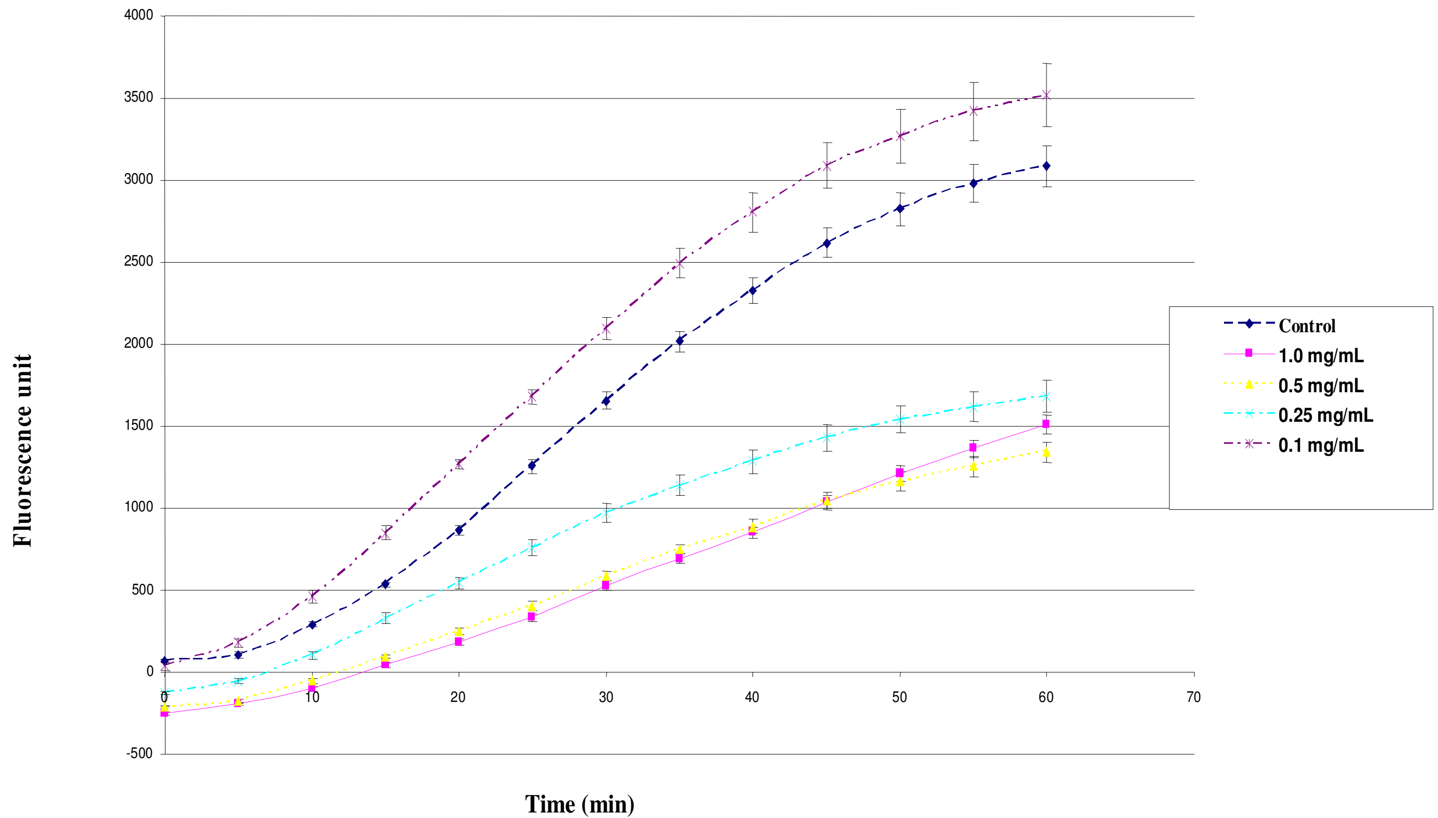

Figure 1. Cellular antioxidant activity of *Vernonia amygdalina* extract against peroxyl radical-induced oxidation in HepG2 cells.

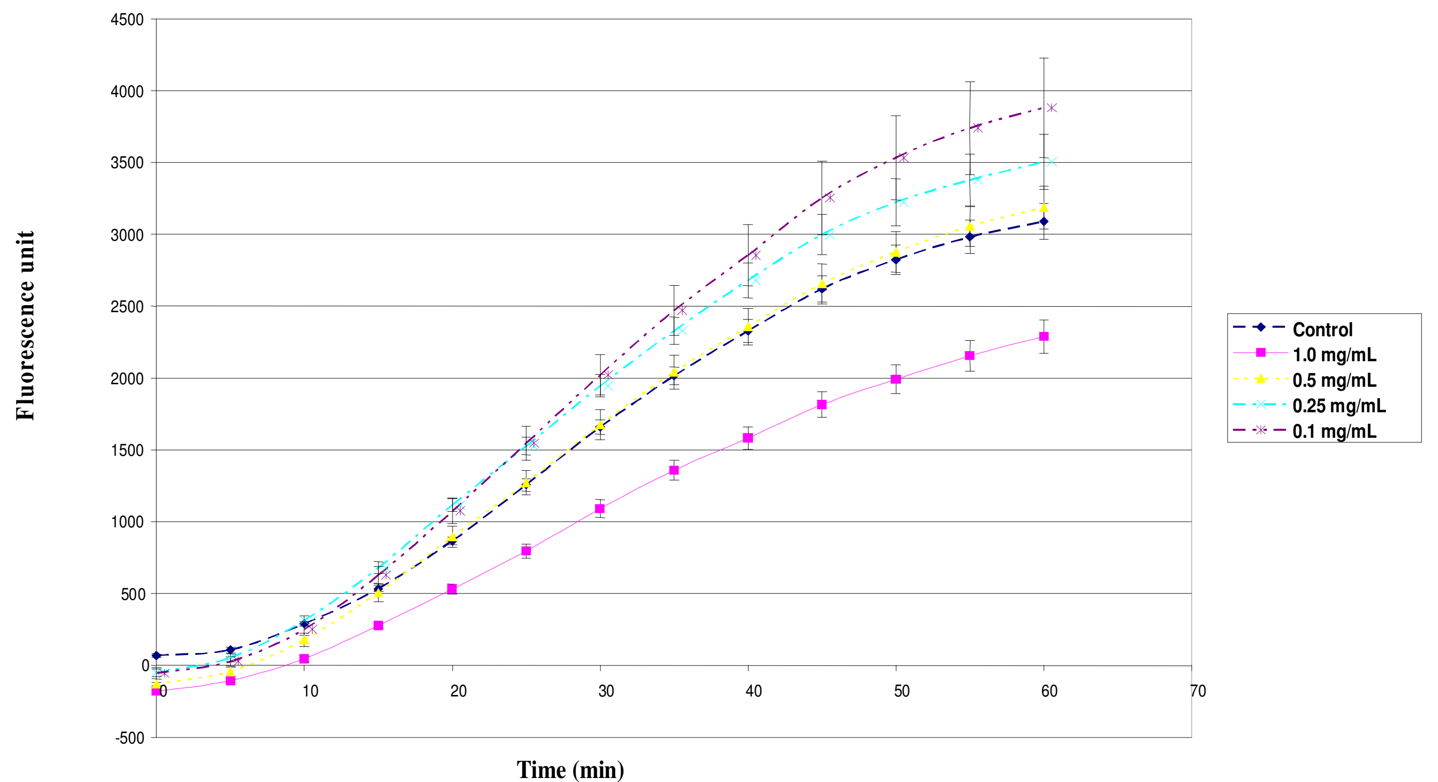

Figure 2. Cellular antioxidant activity of *Manihot utilissima* extract against peroxyl radical-induced oxidation in HepG2 cells.

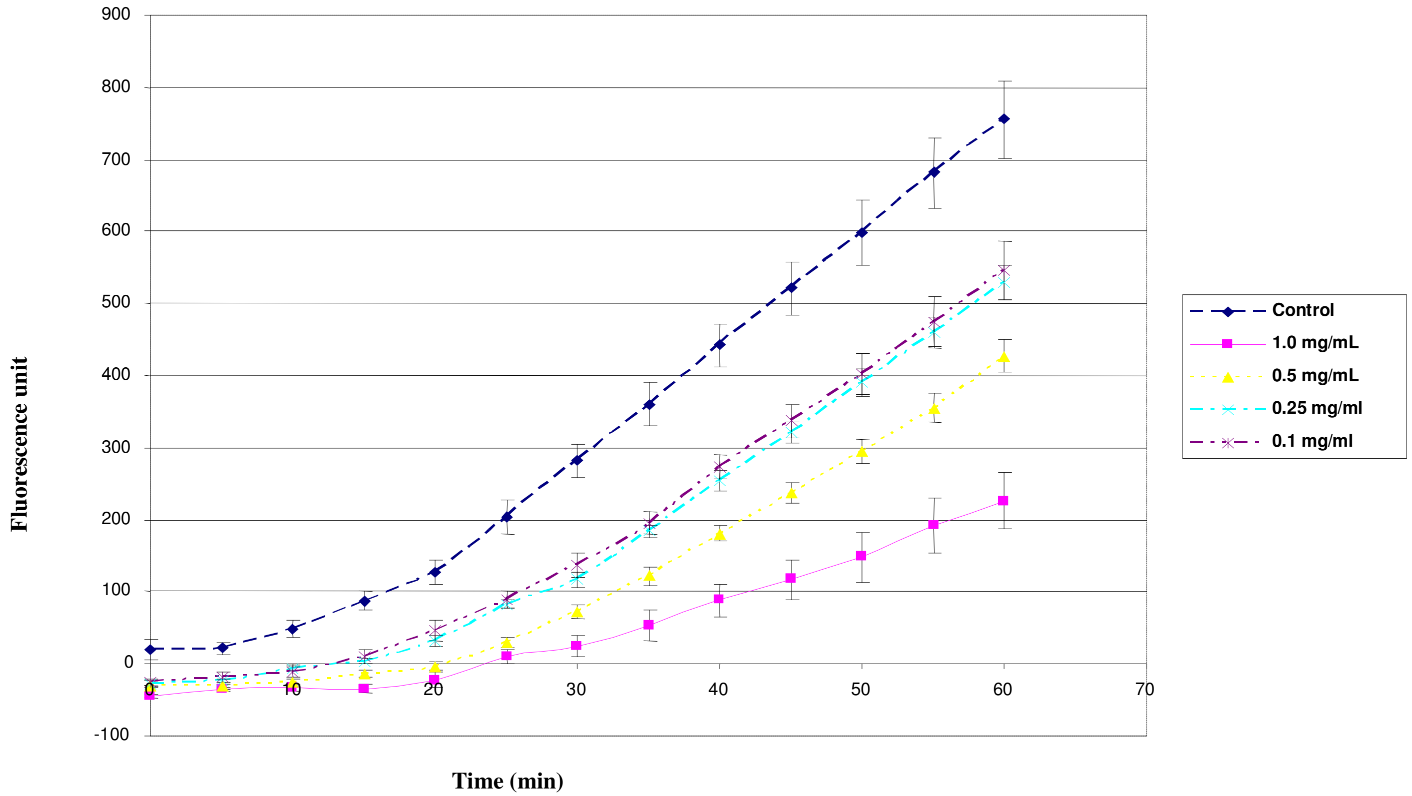

Figure 3. Cellular antioxidant activity of *Ocimum graticimum* extract against peroxyl radical-induced oxidation in HepG2 cells.

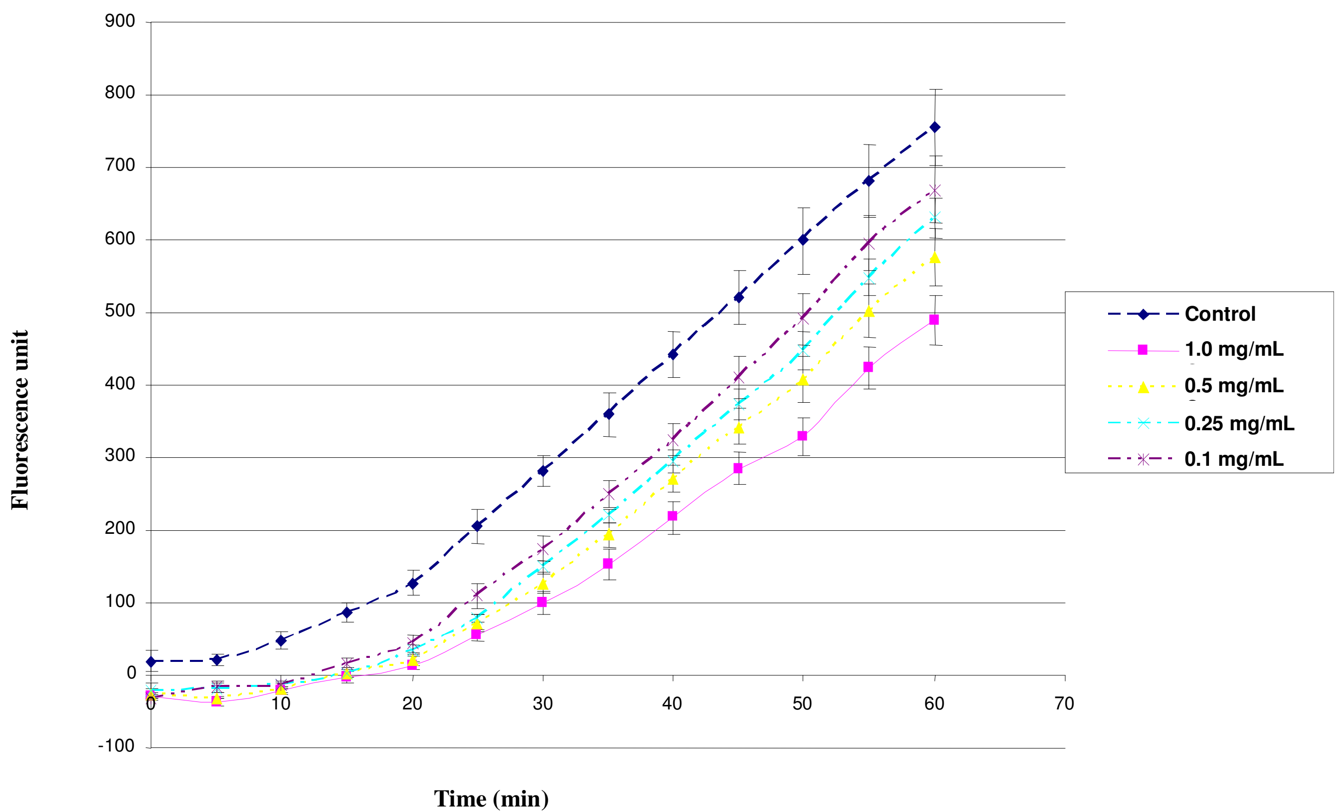

Figure 4. Cellular antioxidant activity of *Corchorous olitorius* against peroxyl radical-induced oxidation in HepG2 cells.

Table 2. EC_{50} values of the vegetable extract (on dry weight basis) against lovo and lovo Adr cancer cell lines.

Extract	EC_{50} of vegetable extract (µg/ml)		
	Lovo	Lovo Adr	Lovo Adr /Lovo
V. amygdalina	1.3 ± 0.07	1.5 ± 0.10	1.15
M. utilissima	2.6 ± 0.02	2.6 ± 0.04	1.00
O. gratissimum	2.5 ± 0.02	2.6 ± 0.12	1.04
C. olitorius	2.4 ± 0.05	2.7 ± 0.03	1.13

vegetable (Salawu et al., 2009) showed some qualitative difference in the identified polyphenol with respect to the present investigation, in which some compounds present in the methanolic phenolic extract were not found in the ethanolic extract. It is generally known that the yield of chemical extraction depends on the type of solvents with varying polarities and pH, extraction time, and temperature as well as on the chemical compositions and physical characteristics of the sample (Rubilar et al., 2003; Pinelo et al., 2004). The chemical characteristics of the solvent and the diverse structure and composition of the natural products ensure that each material-solvent system shows different behaviour.

The cellular antioxidant activity against peroxyl radical-induced oxidation in HepG2 cells (Figures 1-4) was evaluated at different concentration (0.1, 0.25, 0.5 and 1.00 mg/ml dried weight) of the extracts. The extent of inhibition of oxidation is measured by the ability of the phenolic extract to prevent the oxidation of 2'7'-dichlorofluorescin (DCFH) and membrane lipids and reduced the formation of fluorescent oxidized 2'7'-dichlorofluorescin (DCF). In all the extracts, cellular antioxidant activity were elicited with the use of 1.0 mg/ml dried weight, while pro-oxidant activity were observed for lower concentration in *Vernonia amygdalina* (0.1 mg/ml), *M. utilissima* (0.1, 0.25 and 0.5 mg/ml) with the exception of *C. olitorius* and *O. gratissimum* that were active against peroxyl radical-induced oxidation in all selected concentration. Compounds with antioxidant activity may exhibit pro-oxidant behaviour under certain conditions. Pro-oxidant activity can accelerate damage to molecules such as DNA, carbohydrates, or proteins (Aruoma et al., 1997). Previous report on the antioxidant and pro-oxidant activities of phenolic compounds showed that prooxidant activities are mostly elicited at lower concentration (Fakumoto and Maza, 2000). It is a well known fact that antioxidant play a pivotal role in cancer chemoprevention. However, it has also been documented that cancer therapeutics exploits the pro-oxidant activity of the chemical agent used in the management of cancer (Hadi et al., 2010).

At a concentration of 1.0 mg/ml, the quenching of peroxyl radical and subsequent inhibition of DCFH oxidation in HepG2 cells showed that *O. gratissimum* has the highest antioxidant activity with about 220 fluorescent unit followed by *C. olitorius, V. amugdalina* with about 500 and 1500 fluorescent unit respectively, while the least activity was recorded for *M. utilissima* with about 2250 fluorescent unit.

There has been an increasing popularity towards the use of natural compounds to improve human health (Ullah and Khan, 2008). There are epidemiological reports suggesting that the vegetarian food habit is associated with reduced risk of cancer, cardiovascular and neurodegenerative disorder. The association of the reduced risk of cancer in vegetarian diet is believed to be related to plant derived phenolic compounds, which are promising nutraceuticals for control of various disorders such as cardiovascular, neurological and neoplastic disease (Ullah and Khan, 2008). In addition to the potential of polyphenol as anticancer agents, an important role of plant polyphenol as natural modulators of cancer multidrug resistance has been realized (Ullah, 2008). Flavonoids, a major class of plant polyphenol has been found to inhibit breast cancer resistance protein, an ATP-Binding Cascade Transporter (ABC- transporter), which plays an important role in drug disposition leading to chemoresistance in breast cancer (Shuzhong et al., 2005). Cancer is believed to be the result of external factors combined with a hereditary disposition for cancer. It is a neoplasm characterized by the uncontrolled growth of the anaplastic cells that tends to invade surrounding tissue and to metastasize to distance body sites. Colorectal cancer is usually a slowly progressing disease that may be present without symptoms for at least several years. However, there is epidemiologic evidence supporting an inverse association between vegetables and fruit intake and the risk of colon cancer.

The cytotoxic investigation of the vegetal extract (*V. amygdalina*, *O. gratissimum*, *M. utlissima* and *C. olitorius*) was carried out using ethanol extract. The concentration of each vegetable extract (µg/ml) required to produce a 50% reduction in cell viability of Lovo and Lovo/Adr respectively is as shown in Table 2: *V. amygdalina* (1.3 ± 0.07, 1.5 ± 0.10); *M. utilissima* (2.6 ± 0.02, 2.6 ± 0.04; *Ocimum graticimum* (2.5 ± 0.02, 2.6 ± 0.12) and *C. olitorius* (2.4 ± 0.05, 2.7 ± 0.03). The result of the cytotoxic effect of the vegetable in a dose dependent manner expressed as percent survival on Lovo and lovo /Adr colon cancer cells by MTT assay is shown in Figure 5.

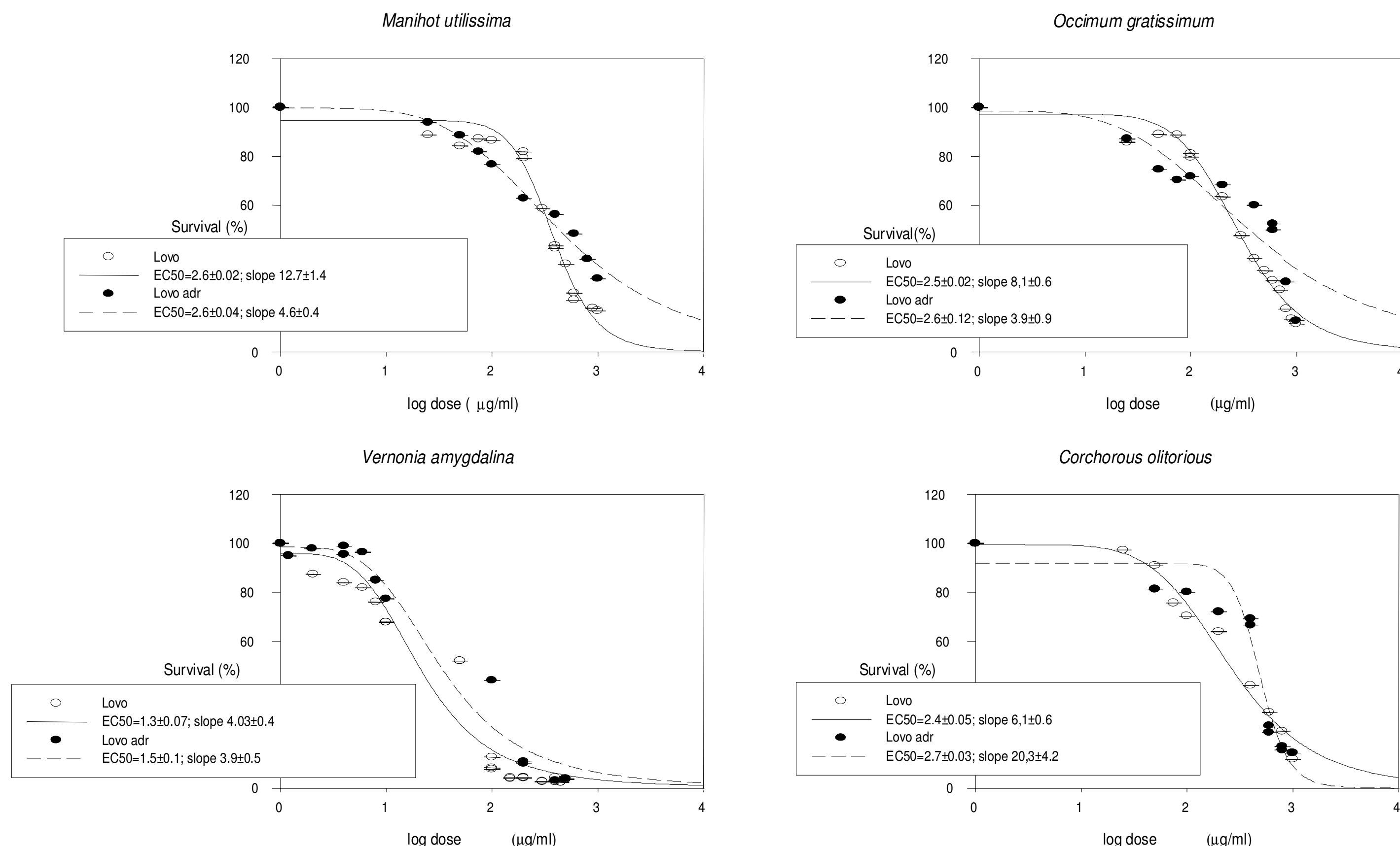

Figure 5. Dose response curve of the cytotoxic properties of vegetable extract.

The result of the present investigation showed *V. amygdalina* to be the most effective with EC_{50} of 1.3 ± 0.07, and 1.5 ± 0.10 µg/ml respectively for Lovo and Lovo/Adr. There are anecdotal reports of the biological activities of *V. amygdalina* against a wide range of human diseases. The anticancer activity of organic extracts of *V. amygdalina* was first reported for human carcinoma cells of the nasopharynx by Kupchan et al. (1969). Jisaka et al. (1993) showed that components of organic extracts of *V. amygdalina*; vernodaline and vernolide, retarded the growth of P-388 and L1210 leukemia cells with EC_{50} values of 0.11 and 0.17 µg/ml for vernodaline and 0.13 and 0.11 for vernolide, respectively. Obaseiki-Ebor et al. (1993) reported an antimutagenic activity of organic solvent extracts of *V. amygdalina*. Izevbigie (2003) reported anticancer activities of a water soluble extract of *V. amygdalina* with an EC_{50} value of 0.0056 ± 0.0002 mg/ml. All these reports are in support of the potential anticancer property of *V. amygdalina*.

On the other hand, the other three extracts *(M. utilissima, C. olitorius and O. graticimum)* are able to inhibit the growth of both the drug sensitive and the drug resistant forms of the investigated adenocarcinoma cells, though at a very high concentration with EC_{50} (2.6 ± 0.02, 2.6 ± 0.04; 2.5 ± 0.02, 2.6 ± 0.12; 2.4 ± 0.05, 2.7 ± 0.03) doubling that which is observed for the cytotoxic effect of *V. amygdalina*.

Cassava (*Manihot* sp.) has been used to control different forms of cancer or the other in the Chinese traditional medicine (Yeoh et al., 1998) without scientific validation. Similarly, research based on both aqueous and methanol crude cassava extracts showed potential of anticancer activity (Iyuke et al., 2004). Cassava contains linamarin, a potentially toxic glucoside, the cytotoxic property of purified linamarin on human breast cancer (MCF-7) cell line, the human colon adenocarcinoma (HT-29) and the acute myelogenous leukemic line (HL-60) was reported (Idibie et al., 2007). The present investigation was carried out on the succulent portion of the leaves which is usually eaten as vegetable, the result of which showed that the leaves could be a good candidate in the prevention of colon cancer.

O. graticimum is a traditionally used medicinal herb, which shows anti-oxidant, anti-carcinogenic, radio-protective and free radical scavenging properties. So far no detailed studies have been done on its effects on human cancers. The first report showing that *O. graticimum* leaf extract has some inhibitory effect on human cancer was reported by Nangia-Makker et al. (2007). The result of the investigation showed that

aqueous *O. graticimum* leaf extract inhibits proliferation, migration, anchorage independent growth, 3D growth and morphogenesis and induction of COX-2 protein in breast cancer cells. The present investigation equally showed that *O. graticimum* might be considered as additional additive in the arsenal of components aimed at combating and preventing colon cancer.

Although, there are dearth of information on the use of *C. olitorius* as an anticancer agent, our result showed that it could be useful in the prevention of cancer as shown in the result obtained for drug sensitive and drug resistant type of colon cancer. One interesting observation of the present investigation was that the vegetable extracts was able to suppress the growth of even the drug resistance Lovo/Adr which is characterized by an over-expression of P-glycoprotein, which is present at a low level in parental cells.

Conclusion

The exhibited antioxidant activity and cytotoxic properties could be partly due to the rich content of flavonoids and cynamoyl derivatives in addition to other phytochemical constituents of the vegetal materials. This by implication is that regular consumption of green leafy vegetables could be a promising nutraceuticals in cancer chemoprevention and related diseases.

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