Full Length Research Paper

In vitro antioxidant activity of *Guibourtia tessmannii* Harms, J. Leonard (Cesalpinoidae)

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The *in vitro* antioxidant activity of aqueous and hydroethanolic extracts of *Guibourtia tessmannii* was evaluated by determination of flavonoid, anthocyanidins and tannins content. Scavenging activity on NO° and OH° radicals, metal chelating activity and antilipoperoxidative activity (thermal and chemical peroxidation of olive oil and hepatic lipid peroxidation) were also carried out. Results have shown that hydroethanolic extracts of *G. tessmannii* had more polyphenols content that aqueous extracts. Antiradical activity has shown that aqueous extract had more effect on NO° radical (IC_{50}: 2309.272 µg/ml) than hydroethanolic extracts (IC_{50}: 10698.500 µg/ml). Inhibitory effect on hydroxyl radical revealed that hydroethanolic extract had the best inhibitory effect than aqueous extract (IC_{50}: 1923.939 and 19750.000 µg/ml, respectively). Metal chelating activity has revealed that extracts of *G. tessmannii* had high chelating activity. Aqueous and hydroethanolic extracts had IC_{50}: 185.515 and 162.786 µg/ml, respectively. Thermal and chemical peroxidation of olive oil showed that extracts of *G. tessmannii* inhibit peroxidation of olive oil but aqueous extracts (IC_{50}: 0.489 µg/ml) had best effect than hydroethanolic extracts. Anti-lipid peroxidation activity of *G. tessmannii* revealed that hydroethanolic extracts of *G. tessmannii* had the best inhibition profile on hepatic lipid peroxidation ((IC_{50}: 1.711 × 10^{-5} µg/ml) than aqueous extracts (IC_{50}: 0.667 µg/ml). The study indicated that *G. tessmannii* possessed the highest antioxidant activity and phenolic compounds concentrations and has potential applications in health against nutritional diseases.

Key words: *Guibourtia tessmannii*, peroxidation, antioxidant.

INTRODUCTION

Oxidative stress depicts the existence of products called free radicals and reactive oxygen species (ROS) which are formed under normal physiological conditions but become deleterious when not being eliminated by the endogenous systems. In fact, oxidative stress results from an imbalance between the generation of reactive oxygen species and endogenous antioxidant systems. ROS are major sources of primary catalysts that initiate oxidation *in vivo* and *in vitro* and create oxidative stress which results in numerous diseases and disorders

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(Rackova et al., 2007).

Plants have been an important source of medicine for thousands of years. Antioxidants are radical scavengers which protect the human body against free radicals that may cause pathological conditions such as ischaemia, asthma, arthritis, inflammation, neurodegeneration, Parkinson's disease, mongolism, ageing process and perhaps dementias (Polterait, 1997). Flavanoids and flavones are widely distributed secondary metabolites with antioxidant and antiradical properties. Antioxidant based drugs or formulations are used for the prevention and treatment of complex diseases like atherosclerosis and stroke. The main characteristic of an antioxidant is its ability to trap free radicals. These free radicals may oxidize nucleic acids, proteins, lipids and DNA and can initiate degenerative diseases. Antioxidant compounds like phenolic acids, polyphenols and flavanoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases (Pourmorad et al., 2006).

Plant and plant products are being used as a source of medicine since long. The medicinal properties of plants have been investigated in the recent scientific developments throughout the world due to their potent antioxidant activities, with no side effects and economic viability (Auudy et al., 2003). Flavonoids and phenolic compounds are widely distributed in plants which have been reported to exert multiple biological effect, including antioxidant, free radical scavenging abilities, anti-inflammatory, anti-carcinogenic (Miller, 1996). It was also suggested that plants and plant products have a potential iron chelator (Boyer et al., 1998). Novel natural anti-oxidants from some plants have been extensively studied in the past few years for their antioxidant and radical scavenging properties. Barks of *Guibourtia tessmannii* Harms, J. Leonard (family Cesalpinoidae) have hypolipidemic activity (Nyangono et al., 2012).

MATERIALS AND METHODS

Collection and identification of plant

Leaves and barks were collected at the National School of Water and Forests (Cameroon, Mbalmayo) and identified at the Cameroon National Herbarium under No. 1037/HNC. The barks were dried at 50°C indoor drying room for 48 h and ground into uniform powder using grinding machine.

Preparation of aqueous extract

The stem barks powder was boiled in distilled water (1:8 w/v) for 15 min. After boiling, mixture was cooled and filtered through Whatman #2 filter paper (Whatman International Limited, Kent, England) using a funnel and concentrated to about 10% of the original volume by a rotary evaporator before drying in the oven at 50°C for

72 h. The extracts obtained were stored at room temperature (20% yields).

Preparation of hydroethanolic extracts

The stem barks powder was dissolved in distilled water/alcohol (1:1) reagent. After homogenization, mixture was macerated for 48 h at room temperature and filtered through Whatman #2 filter paper (Whatman International Limited, Kent, England) using a funnel and concentrated to about 10% of the original volume by a rotary evaporator before drying in the oven at 50°C, before evaporation to oven (50°C) for 72 h (10% yields).

Evaluation of antioxidant activity

Determination of total flavonoids

Total flavonoids were determined using the method of Aiyegoro and Okoh (2010). The aluminium chloride colorimetric method was used whereby; 1 ml of sample (1 mg/ml) was mixed with 3 ml of metha-nol, 0.2 ml of 10% aluminum chloride, 0.2 ml of 1 M potassium acetate and 5.6 ml of distilled water. The mixture was allowed to stand at room temperature for 30 min. The absorbance of the reaction mixture was measured at 420 nm with ultra violet (UV) visible spectrophotometer (Spectronic 20 Genesys™). Quercetin solution (0 to 1000 µg/ml) was used as standard (Ordonez et al., 2006). The concentration of flavonoids was expressed in µg/ml quercetin equivalents.

Determination of total proanthocyanidins

Total proanthocyanidins were determined using the method of Aiyegoro and Okoh (2010). The mixture of 3 ml of vanillin-methanol (4% v/v), 1.5 ml of concentrated hydrochloric acid was added to 0.5 ml (1 mg/ml) of extract and vortexed. The resulting mixture was allowed to stand for 15 min at room temperature followed by the measurement of the absorbance at 500 nm. Cyanidin solution (0 to 1000 ug/ml) was used as standard. The concentration of flavonoid was expressed in µg/ml cyanidin equivalents (Aiyegoro and Okoh, 2010).

Determination of tannins

Tannin content was determined using the method described by Bainbridge et al. (1996). 1 ml of the extract was collected and mixed with 5 ml of reaction solution (50 g of vanillin + 4 ml of hydrochloric acid in 100 ml distilled water) and the mixture was incubated at 30°C for 20 min. The absorbance was read at 500 nm against a blank (without extract). Gallic acid (1 mg/ml) was used as standard. The results were expressed in µg/ml gallic acid equivalents.

Determination of antiradical activity

Scavenging activity against NO° radical

Nitric oxide was generated by sodium nitroprusside at physiological pH on interaction with oxygen to produce nitrite ions. The nitrite ion was measured using Griess reagent (Green et al., 1982); the more

the extract is reactive, the lesser the production of nitrite ion and the weaker the absorbance (Sreejayan and Rao, 1997). To 1 ml of extract was added to 2 ml of sodium nitroprusside 10 mM (in phosphate buffer 50 mM, pH 7.4) and homogenised and incubated at 25°C for 15 min. After incubation, 0.5 ml of the mixture was mixed with 1 ml of sulfanilic acid (0.33% in glacial acetic acid 20%). The mixture was allowed to stand for 15 min for diazotation then, 1 ml of naphtylethylenediamine dihydrochloride (NED: 0.1%) was added, homogenised and incubated at room temperature for 30 min. A pink chromophore was formed in diffused light whose absorbance was read at 540 nm against a blank (consisting of the reaction mixture without nitroprusside). The control was prepared in the same way except that the extract was replaced by the methanol. All measurements were done in triplicate and the % inhibition was calculated as follows:

%inhibition = [(Ac – As) / Ac] × 100

Where Ac = absorbance of control, As = absorbance of sample.

OH° radical scavenging activity

Hydroxyl radical is one of the potent reactive oxygen species in the biological system. It reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and causes damage to cell (Halliwell and Gutteridge, 1981). The model used is ascorbic acid-iron-ethylenediaminetetraacetic acid (EDTA) model of $HO^{.}$ generating system. This is a totally aqueous system in which ascorbic acid, iron and EDTA conspire with each other to generate hydroxyl radicals.The hydroxyl radical scavenging activity was determined as earlier described by Halliwell et al. (1987). The reaction mixture consisted of $FeCl_3$ (300 µM), EDTA (780 µM), 2-deoxyribose (2.8 mM), ascorbic acid (300 µM), H_2O_2 (4 mM) and aliquots of extracts in a final volume of 1 ml. All reagents were dissolved in potassium phosphate buffer (20 mM, pH 7.4). This was then incubated at 37°C for 1 h. After incubation, 1 ml of TCA (2.8%) and TBA (1%) were added to the reaction mixture and incubated at 100°C for 20 min. A control tube was prepared similarly effect except that the extract was replaced with methanol. The absorbance was read spectrophotometrically at 532 nm. All measurements were done in triplicate and the % inhibition was calculated as before.

Metal chelating activity

Ferrozine can quantitatively chelate with Fe^{2+} and form a complex with a red color. This reaction is limited in the presence of other chelating agents and results in a decrease of the red color of the ferrozine-Fe^{2+} complexes. Measurement of the color reduction estimates the chelating activity to compete with ferrozine for the ferrous ions (Soler-Rivas et al., 2000). Chelation of ferrous ions is estimated using the method of Dinis et al. (1994). This method is based on the competition between ferrozine and the bio reactive components of the plant extracts in trapping ferrous ions. This is translated by a reduction in the absorbance at 562 nm of the ferrozine (Fe^{2+}) complex. To 0.5 ml of plant extract was added 25 ul of 2 mM $FeCl_2$ and 100 µl of ferrozine (5 mM). The mixture was vigorously shaken and left to stand at room temperature for 10 min. A control tube was prepared similarly except that the extract was replaced with methanol. The absorbance was measured spectrophotometrically at 562 nm. All measurements were done in triplicate and the % inhibition was calculated as follows:

% Inhibition = [(Ac – As) / Ac] × 100

Determination of antilipo – peroxidative activity

Inhibiting effect of extracts of G. tessmannii on the thermal olive oil peroxidation

Quantifying thiobarbituric acid reactive substances (TBARs) was done following the method of Okhawa et al. (1979) as modified by the laboratory. 300 µl of plant extract (10 to 1000 µg/ml) were added to 01 ml of an emulsion of olive oil (10 g olive oil whose oxidation was initiated by heat, 10 mM phosphate buffer, pH 7 and tween 20%). After incubation at 37°C for 16 h in the dark, the reaction was stopped by cooling and addition of EDTA (100 µl, 20 mM) and 100 µl of vitamin C (4 mM). After incubation and stopping the reaction, 1 ml of 20% trichloroacetic acid (TCA) and 1 ml of thiobarbituric acid (TBA) (0.78%) were introduced simultaneously. The mixture was again incubated at 95°C for 45 min. The samples were then cooled to room temperature and then centrifuged and the absorbance read at 532 nm. In the positive control, the extract was replaced with methanol. The levels of TBARs are calculated using the molar extinction coefficient of MDA.

% Inhibition = [(Abs positive control - Abs sample) / Abs positive control] × 100

Inhibiting effect of extracts of G. tessmannii chemical olive oil peroxidation

Quantifying TBARs was done following the method of Okhawa et al. (1979). 300 µl of plant extract (10 to 1000 µg/ml) were added to 0.1 ml of an emulsion of olive oil (10 g of olive oil, 10 mM phosphate buffer, pH 7 and tween 20%). The oxidation is initiated by the $CuSO_4$ (100 µl) freshly prepared at a final concentration of 40 µM in phosphate buffer. After incubation at 37°C for 16 h in the dark, the reaction was stopped by cooling and addition of EDTA (100 µl, 20 mM) and 100 µl of vitamin C (4 mM). After incubation and stopping the reaction, 1 ml of 20% TCA and 1 ml of TBA (0.78%) were introduced simultaneously. The mixture was again incubated at 95°C for 45 min. The samples were then cooled to room temperature and then centrifuged and the absorbance read at 532 nm. In the positive control, the extract was replaced by methanol. Levels of TBARs are calculated using the molar extinction coefficient of MDA.

% Inhibition = [(Abs positive control - Abs sample) / Abs positive control] × 100

Anti-lipid peroxidation of extracts of G. tessmannii

Homogenate of liver was prepared as earlier described by Auddy et al. (2003). Male wistar rats were randomly selected, anaesthetized with diethyl ether and killed by decapitation after an overnight fasting. The liver were removed and carefully washed and rinsed with KCl (0.15 M) (Okhawa et al., 1979). Homogenates were prepared, filtered and the supernatant was separated. Supernatant was used as source of PUFAS. In vitro lipid peroxidation was done as follows: To 300 µl of extract was added 500 µl of liver homogenate, 100 µl of KCl (0.15 M), and lipid peroxidation was initiated by adding 100 µl of $FeSO_4$ 15 mM. Mixture was incubated at 37°C for 30 min. After incubation, 1 ml of equal volume of TBA:

Table 1. Polyphenols content of *G. tessmannii*.

Guibourtia tessmannii	Aqueous extract	Hydroethanolic extract
Flavonoids content (μg/ml) with Quercetin	2.892±0.389[a]	5.619±0.183[b]
Hydrolyzable tannins content (μg/ml) gallic acid	250.260±2.570[a]	293.413±2.540[b]
Condensed tannins content (μg/ml) with cyanidine	256.000±3.665[a]	281.000±2.088[b]

Values are shown as mean ± standard mean of three replicates. Means followed by the different letters in the same line are significantly different ($p < 0.05$).

TCA (1:1) and 1 ml of ascorbic acid (6 mM) were added. Final mixture was incubated at 80°C for 20 min, refridgerated and centrifuged. The absorbance was read spectrophotometrically at 532 nm (Prassanth et al., 2000). A control tube was prepared similarly except that the extract was replaced with methanol. All measurements were done in triplicate and the % inhibition was calculated as follows:

% Inhibition = [(Ac – As) / Ac] × 100

Where Ac = absorbance of control, As = absorbance of sample.

Statistical analysis

Results are expressed as mean ± standard error mean. Data analysis was performed using statistical package for social sciences (SPSS) 10.1 for windows. Statistical test used was one way analysis of variance (ANOVA) followed by least significant difference (LSD), and Tamhane post hoc were used to evaluate antioxidant activity. IC_{50} was evaluated using linear regression analysis. Changes were considered significant for *p* values less than 0.05.

RESULTS

The quantification of polyphenols showed that hydroethanolic extracts of *G. tessmannii* have more poly-phenols than aqueous extracts (Table 1). *G. tessmannii* have inhibitory activity on nitric oxide radical (Figure 1), aqueous extracts of *G. tessmannii* present the best activity on nitric oxide inhibition (IC_{50}: 2309.272 μg/ml) than hydroethanolic extracts (IC_{50}: 10698.500 μg/ml). Inhibitory effect on hydroxyl radical revealed that hydroethanolic extracts have the best inhibitory effect than aqueous extracts (IC_{50}: 1923.939 and 19750.000 μg/ml, respectively) (Figure 2). Metal chelating activity has revealed that extracts of *G. tessmannii* have high chelating activity. Aqueous and hydroethanolic extracts had IC_{50}: 185.515 and 162.786 μg/ml, respectively (Figure 3).

Thermal and chemical peroxidation of olive oil showed that extracts of *G. tessmannii* inhibit peroxidation of olive oil (Figures 4 and 5). Thermal olive oil peroxidation showed that all extracts of *G. tessmannii* have an effect on thermal peroxidation of olive oil, but aqueous extracts (IC_{50}: 0.489 μg/ml) have best effect than hydroethanolic

extracts (IC_{50}: 329.090 μg/ml). The same profile has been observed in chemical olive oil peroxidation, aqueous extracts (IC_{50}: 156.865 μg/ml) have the best effect than hydroethanolic effect (IC_{50}: 7237.114 μg/ml). Anti-lipid peroxidation activity of *G. tessmannii* revealed that hydroethanolic extracts of *G. tessmannii* have the best inhibition profile on hepatic lipid peroxidation ((IC_{50}: 1.711 to 10^{-5} μg/ml) than aqueous extracts (IC_{50}: 0.667 μg/ml) (Figure 6).

DISCUSSION

It is generally believed that plants which are having a more important phenolic content show good antioxidant activity, that is there is a direct correlation between total phenol content and antioxidant activity (Chanda and Dave, 2009). Qualitative phytochemical study showed that extracts of *G. tessmannii* contained alkaloids and flavonoids (Nyangono et al., 2012). It has been recognized that alkaloids and flavanoids show antioxidant property and their effects on human nutrition and health care are considerable (Kumpulainen and Salonen, 1999). Mechanism of action of alkaloids is through inhibition of peroxidation (Kessler et al., 2003). Compounds such as flavanoids are responsible for inhibition of lipid peroxidation (Das and Pereira, 1990).

Nitric oxide is a free radical product in mammalian cells, involved in the regulation of various physiological processes like regulation of vascular tone, inhibition of both platelet and leukocyte aggregation and adhesion, and inhibition of cell proliferation (Bredt and Snyder, 1990). However, excess production of nitric oxide (NO) is associated with several diseases. In the present study, the nitrite produced by the incubation of solutions of sodium nitroprusside in standard phosphate buffer at 25°C was reduced by the aqueous extract of *G. tessmannii*. This may be due to the antioxidant principles in the extract which compete with oxygen to react with nitric oxide, thereby inhibiting the generation of nitrites (Sangameswaran et al., 2009).

Hydroxyl radicals are reactive oxygen species that initiate peroxidation of lipid membranes (Halliwell, 1991). Hydroxyl radicals interact with purines and pyrimidine
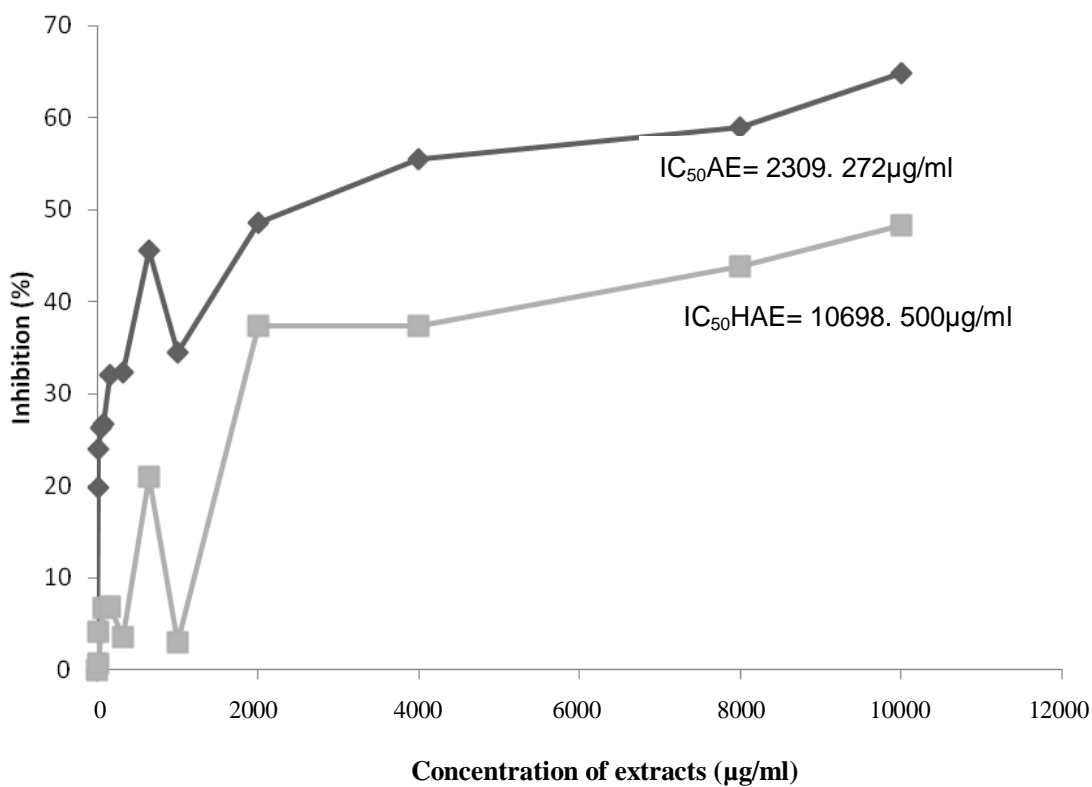
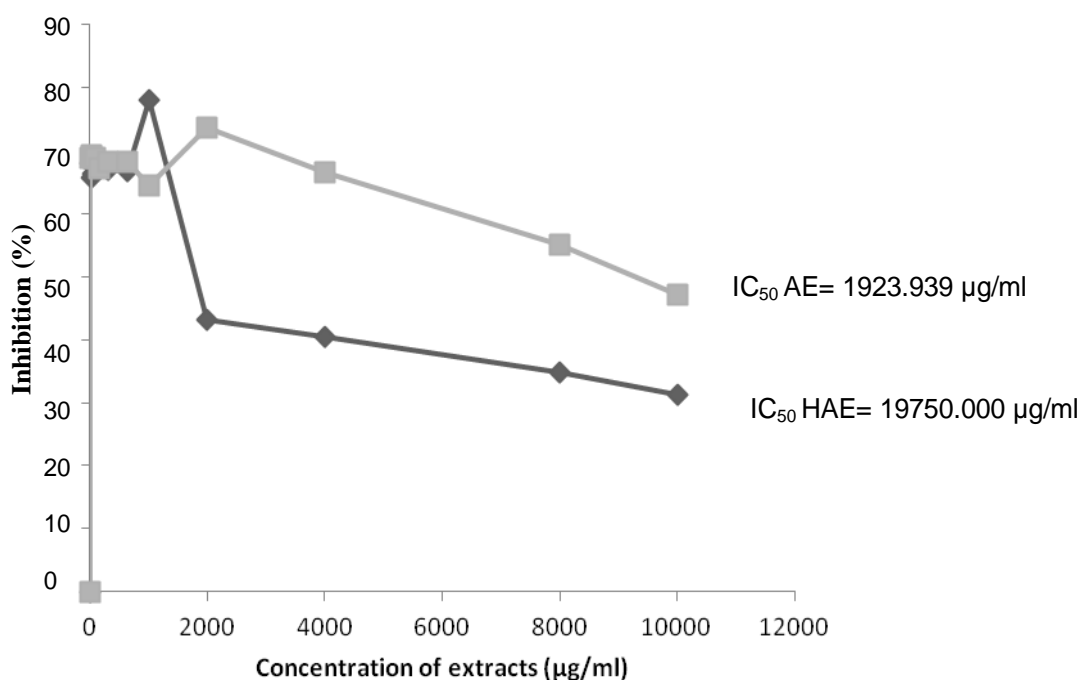**Figure 1.** Inhibitory effect of G. *tessmannii* on nitric oxide radical

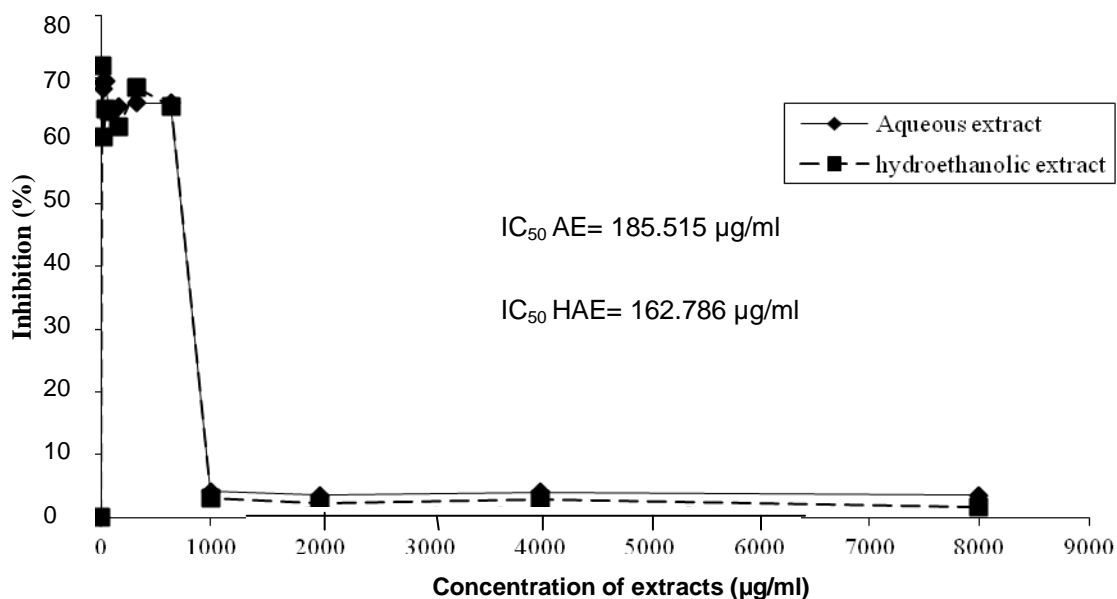**Figure 2.** Inhibitory effect of G. *tessmannii* on hydroxyl radical
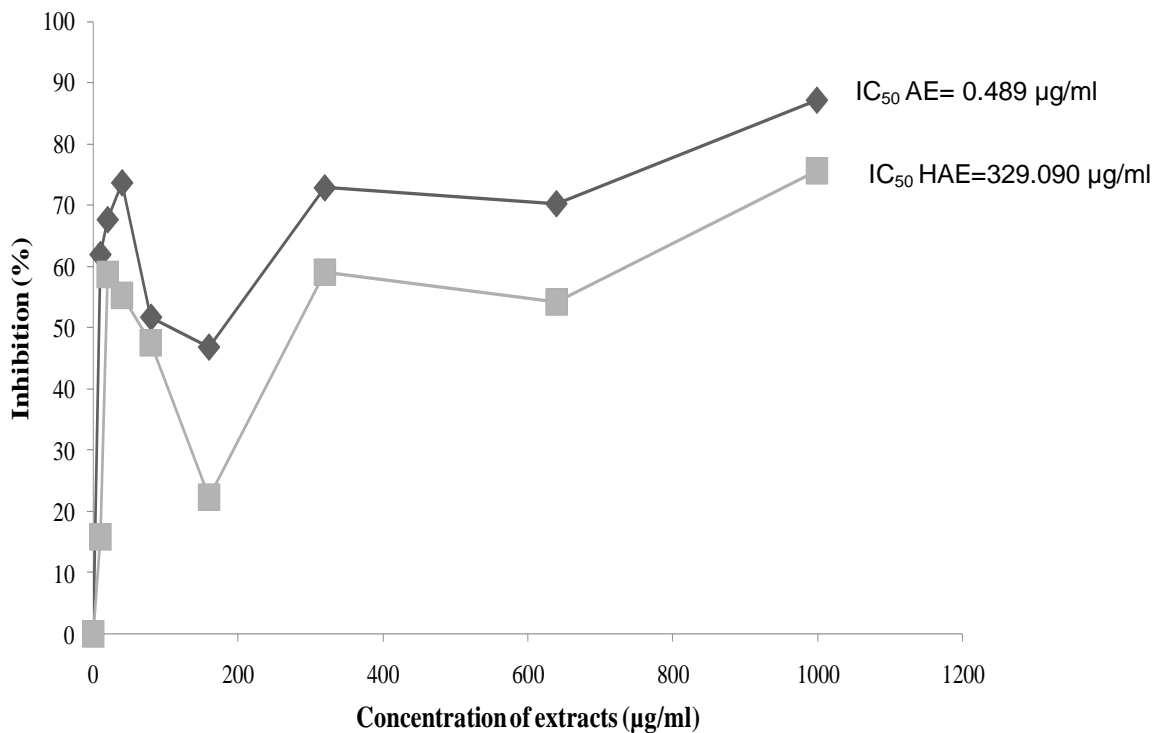
Figure 3. Metal chelating activity of G. *tessmannii*

IC_{50} AE= 185.515 µg/ml

IC_{50} HAE= 162.786 µg/ml

Figure 4. Inhibitory effect of G. *tessmannii* on thermal peroxidation of olive oil

IC_{50} AE= 0.489 µg/ml

IC_{50} HAE=329.090 µg/ml

bases of DNA and also with thiols leading to damages. Hydroethanolic extracts could better inhibit hydroxyl radicals (IC_{50}: 1923.939 µg/ml) compared to aqueous extracts (IC_{50}: 19750.000 µg/ml). In organism, metal ions react with hydrogen peroxide to produce OH° radical. Thus, inhibition of hydrogen peroxide and metal chelating
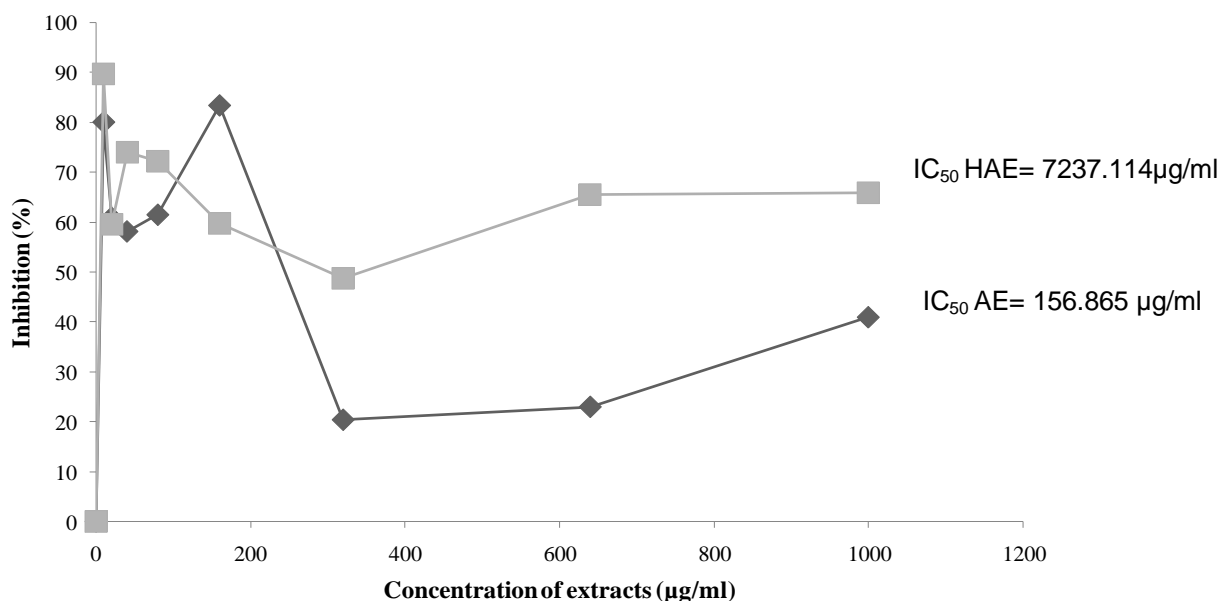
Figure 5. Inhibitory effect of G. *tessmannii* on chemical peroxidation of olive oil.
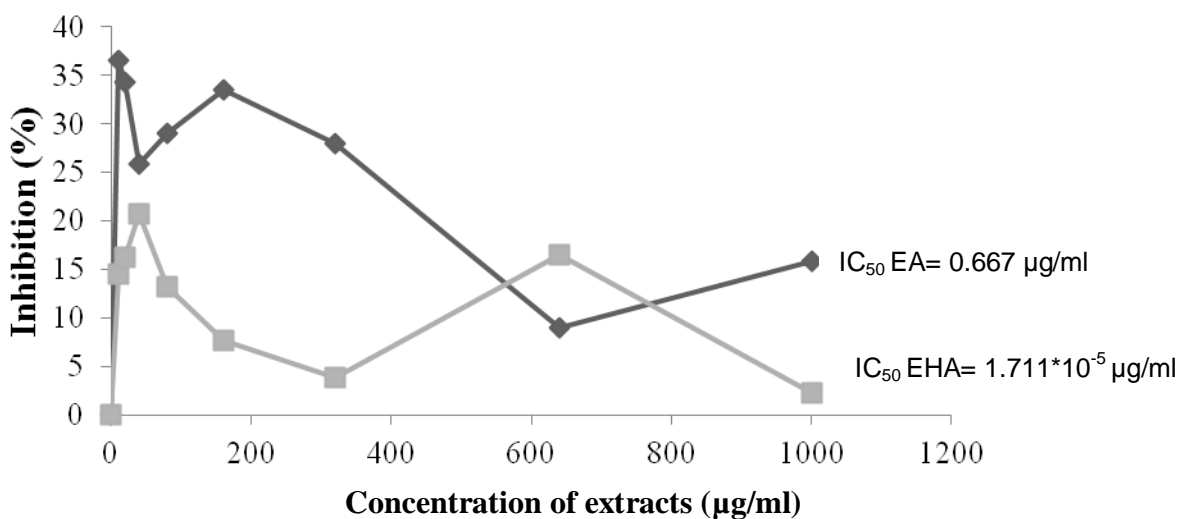

Figure 6. Anti-lipid peroxidation of G. *tessmannii*.

are important process of cells. Ferrozine forms a complex with Fe^{2+} ions producing a red coloration. Thus, the reduction of color intensity estimates chelating activity of plant (Yamaguchi et al., 2000). Metal chelating of plant is its ability to compete with ferrozine for Fe^{2+} ions. Thus ethanolic extract (IC_{50}: 162.786 µg/ml) had the best metal chelating activity than aqueous extract (IC_{50}: 185.515 µg/ml).

There is relation between antioxidant status and incidence of human diseases. Antioxidants are responsible of several antioxidant activities and can be isolated and used to prevent disorders due to free radicals (Eun et al., 2003). Aqueous extracts of *G. tessmannii* inhibit lipid peroxidation *in vitro* (polyunsaturated fatty acids of olive oil) and *ex vivo* (liver lipid peroxidation). This may be attributed to bioactive compounds like polyphenols. Antioxidant properties of polyphenols like inhibition of lipid peroxidation and free radical is crucial. In our study, extracts of *G. tessmannii* could inhibit lipid peroxidation by scavenging free radical, by linking metal ions like

copper and/or inhibiting enzymes implied in oxidation systems. The peroxidation of membrane lipids initiated by oxygen radicals may lead to cell injury. Initiation of lipid peroxidation by ferrous sulphate takes place either through ferryl-perferryl complex (Gutteridge, 1985) or through -OH radicals by Fenton reaction (Halliwell, 1978) thereby initiating a cascade of oxidative reactions. The results obtained in the present studies may be attributed to several reasons like the inhibition of ferryl-perferyl complex formation, scavenging of OH or superoxide radicals or by changing the ratio of $Fe^{3}+/ Fe^{2+}$; reducing the rate of conversions of ferrous to ferric or by chelating of the iron itself (Braugghler et al., 1986).

ABBREVIATIONS

TCA, Trichloroacetic acid; **TBA,** thiobarbituric acid; **EDTA,** ethylenediamine tetraacetic acid; **MDA,** malondialdéhyde; **TBARs,** thiobarbituric acid reactive substances; **PUFAs,** polyunsaturated fatty acids.

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