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Antioxidant and inhibitory effects of aqueous and ethanolic extract of *Tapinanthus bangwensis* leaves on Fe^{2+}-induced lipid peroxidation in pancreas *(in vitro)*

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Abstract

This study was designed to investigate and compare the aqueous (AQTB) and ethanolic (ETTB) extracts of *Tapinanthus bangwensis* leaves on the phenolic content, antioxidant activity and inhibitory effect on Fe^{2+}- induced lipid peroxidation in pancreas *(in vitro)*. Antioxidant properties (Inhibition of Fe^{2+}-induced lipid peroxidation in rat pancreas, total phenol and total flavonoid contents, ferric reducing antioxidant property, nitric oxide radical scavenging ability using *in vitro* model were evaluated. The result revealed that the ETTB had higher total phenol and total flavonoid contents than AQTB. Furthermore, ETTB exhibited strong antioxidant properties as observed by its nitric oxide (NO) radical scavenging ability and ferric reducing antioxidant property than AQTB.The result also revealed that incubation of rat pancreas in the presence of 25 µM Fe^{2+} caused a significant increase ($p < 0.05$) in malondiadehyde (MDA) contents of the rat pancreas (156.9%) when compared with the basal pancreas homogenate (100%). However, AQTB showed higher inhibitory effects on pancreatic MDA produced in a dose-dependent pattern (0 - 0.63 mg/mL) than the ETTB. The antioxidant activity and inhibitory effect of both extracts against lipid peroxidation could be attributed to the phenolic phytochemicals present in the extracts. It could therefore be concluded that both extracts protect the pancreas against oxidative damage but aqueous extract exhibited the better protection.

Introduction

The current surge of interests in medicinal plants has received much attention globally, as researchers are now looking for alternative sources to synthetic agents (Ekhaise *et al*., 2010). The alternative therapy of herbal medicine in health treatment and preventive strategies is also gaining considerable acceptability and popularity worldwide (Evans, 2005). Several medicinal plants have been worked upon and has been shown to elicit therapeutic functions owing to their intrinsic bioactive components and as such can scavenge free radical generation in the systems by acting as antioxidants agents in the body. Hence more focus is now on drugs that could be derived from plant based sources (Ekhaise *et al.*, 2010; Bassey *et al*., 2012)

African mistletoe *(Tapinanthus bangwensis)* is a semi-parasitic plant, growing on a host of evergreen and deciduous trees all year round. It is an obligate parasite (Kay, 1986; Evans, 2005; Osadebe and Uzochukwu, 2006; Egbewande *et al*., 2011). Extracts from the leaves are used by traditional medical practitioners to treat various ailments such as liver disorders. They are used as anticancer agents (Grossarth-Maticek and Ziegler, 2007), management of diabetes mellitus (Kafaru, 1993; Obatomi *et al*., 1994; Osadebe *et al*., 2004), as antihypertensive agent (Kafaru, 1993),incorporated into diets for broiler optimal performance (Egbewande *et al*., 2011) The mistletoe plant has been described "an all-purpose herb" because of its rich folkloric uses, magical and also at the same time a sacred plant of the European folklore (Obatomi *et al*., 1994; Osadebe *et al*., 2004).The phytochemical screening of *T. Bangwensis* leaves revealed that it contains bioactive principles such as flavonoids, tannins, saponins and steroidal glycosides (Ekhaise *et al*., 2010). Mistletoe can grow on either edible or non-edible trees.Those that grows on edible plants is used for medicinal purposes (Evans, 2005). Owing to its rich potentials, this study was designed to investigate and compare the aqueous (AQTB) and ethanolic (ETTB) extracts of *Tapinanthus bangwensis* leaves on the phenolic

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content, antioxidant activity and inhibitory effect on Fe^{2+}- induced lipid peroxidation in pancreas *(in vitro)*.

Materials and Methods

Sample collection

Fresh samples of *Tapinanthus bangwensis* leaves were purchased from the market in Otun-Ekiti, Ekiti State, Nigeria. The authentication of the leaves was carried out by a Plant Botanist at the Department of Plant Science, Ekiti State University, Ado-Ekiti Nigeria. All the chemicals used were of analytical grade.

Chemicals and reagents

The following chemicals and reagents: quercetin, gallic acid, Folin–Ciocalteau's reagent, potassium ferricyanide, ferric chloride, potassium acetate, trichloroacetic acid (TCA), methanol, disodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4), sulphanilamide, and sodium carbonate were purchased from Sigma -Aldrich Chemical Co. (St. Louis, MO), Chemie GmbH (Steinheim, Germany) and BDH Chemicals Ltd., (Poole, England).

Aqueous extract preparation

The aqueous extract of the leaves were prepared by soaking the Tapinanthus bangwensis in water for about 24 hours; the mixture were filtered, and the filtrate were evaporated under pressure at 40ºC. The flask containing the powdered dried extract was thoroughly washed with distilled water and the solution was kept in the refrigerator at 0ºC. The frozen extract solution was lyophilized and recovered as dried extract. The dried extracts were later reconstituted in water, stored at 4°C and used for subsequent analysis

Ethanolic extract preparation

The ethanol extract of the leaves were prepared by soaking the Tapinanthus bangwensis in alcohol for about 24 hours; the mixture was filtered, and the filtrate was evaporated under pressure at 40ºC. The extracts were stored in the refrigerator for subsequent analysis. The container containing the powdered dried extract was subsequently rinsed with distilled water and the solution was kept in the refrigerator at 0ºC. The frozen extract solution was lyophilized and recovered as dried extract. The dried extracts were later reconstituted in water, stored at 4°C and used for subsequent analysis.

Determination of total phenol content

The total Phenol content of the extract was determined by the method reported by Singleton *et al*. (1999). Appropriate dilutions of the extracts were oxidized with 25 L of 10% folin- ciocalteau's reagent (v/v) and neutralised by 2.0 L of 7.5% sodium carbonate. The reaction mixture was incubated for 40 minutes at 45ºC and the absorbance was measured at 765 nm with a spectrophotometer. The total phenol content was subsequently calculated and reported in percentage. Gallic acid was used as standard phenol.

Determination of total flavonoid content

The total flavonoid content of both extracts was determined using a slightly modified method reported by Meda *et al*. (2005). Briefly, 0.5 mL of appropriately diluted sample was mixed with 0.5 mL methanol, 50 µL of 10% $AlCl_3$, 50 µL of 1mol L−1 potassium acetate and 1.4 mL water, and allowed to incubate at room temperature for 30 min. Thereafter, the absorbance of the reaction mixture was subsequently measured at 415 nm. The total flavonoid was calculated using quercetin as standard.

Determination of reducing 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). A 2.5 mL aliquot was mixed with 2.5 mL of 200 mmol L−1 sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50^{0}C for 20 min and then 2.5 mL of 10% trichloroacetic acid was added. This mixture was centrifuged at 650 rpm for 10 min. 5 mL of the supernatant 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 property was subsequently calculated using ascorbic acid as standard

Nitric oxide scavenging activity determination

Varying concentration of the extracts were added to 5 mM of sodium nitroprusside and phosphate buffer was added to each test tube to make volume up to 1.5 ml. Solutions were incubated at 25ºC for 30 minutes. Thereafter, 1.5 ml of Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine dichloride and 3% phosphoric acid) was added to each test tube. The absorbance was read spectrophotometrically at 546 nm and percentage of scavenging activity was subsequently calculated

Animal ethics

All of the animals received humane care

Table 1. Total phenol, total flavonoid contents and ferric reducing antioxidant property of aqueous (AQTB) and ethanolic (ETTB) extract of *Tapinanthus bangwensis*

Antioxidant	Total phenol (mgGAE/g)	Total flavonoids (mgQE/g)	Ferric Reducing Property (mgAAE/g)
AQTB	2.88 ± 0.12[a]	1.32 ± 0.01[c]	2.65± 0.06[e]
ETTB	3.63 ± 0.17[b]	2.41 ± 0.17[d]	3.47± 0.10[f]

Values represent mean± standard deviation of triplicate readings, n = 3. Values with different superscript letters along the same column are significantly ($p < 0.05$) different.

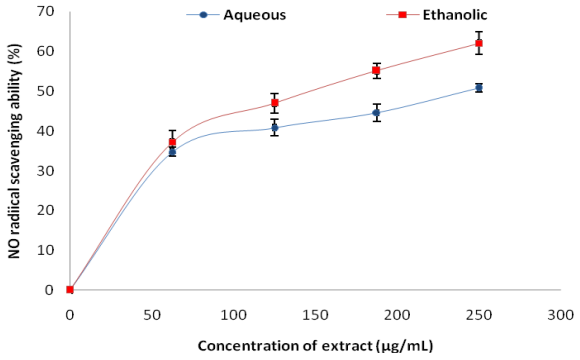

Figure 1. Nitric oxide radical scavenging ability of aqueous and ethanolic extract of *Tapinanthus bangwensis*.
Values represent mean± standard deviation of triplicate readings, n = 3

according to the criteria outline in the Guide for the Care and the Use of Laboratory Animals prepared by the National Academy Science and published by the National Institute of Health (USA). The ethic regulations have been followed in accordance with national and institutional guidelines for the protection of animals' welfare during experiments. The experiment was carried out at the Functional food, Nutraceuticals and Phytomedicine Laboratory, Department of Biochemistry, Federal University of Technology, Akure, Ondo State, Nigeria.

Lipid peroxidation assay

Preparation of tissue homogenate

The rats were decapitated under mild diethyl ether anaesthesia and the pancreas was rapidly dissected, placed on ice and weighed. This tissue was subsequently homogenized in cold saline (1;10,w/v) with about 1–up- and -down strokes at approximately 1200 rpm in a felon glass homogenizer. The homogenate was centrifuged for 10min at 3000xg to yield a pellet that was discarded and a low-speed supernatant (SI) containing mainly water proteins and lipids (cholesterol, galactolipids individual phospholipids, ganglioside) was kept for lipid peroxidation assay.

Lipid peroxidation and thiobarbituric acid reactions

The Lipid Peroxidation assay was carried out by the modified method of Ohkawa *et al*. (1979), 100 µl of supernatant fraction was mixed with a reaction mixture containing 30 µl of 0.1M Tric.HCl buffer (pH 7.4), extract (0 – 100 µl) and 30 µl of the proxidant (250 µl freshly prepared $FeS0_4$). The volume was made up to 300 µl by water before incubation at 37°C for 1 hour. The colour reaction was developed by adding 600 µl of 8.1 % sodium dodecyl sulfate (SDS) to the reaction mixture containing supernatant, followed by the addition of 600 mL of acetic acid/ HCL (pH 3.4) and 600 µl of 80% Thiobarbituric acid(TBA). This mixture was incubated at 100°C for 1 hour. The absorbance of thiobarbituric acid reactive species (TBARS) produced were measured at 510 nm. MDA (Malondialdehyde) produced was expected as % control.

Data analysis

The result of the three applicants where pooled and expressed as mean ± standard deviation (S.D). Student t-test and the least significance difference (LSD) were carried out. Significance was accepted at $p \leq 0.05$ (Zar, 1984)

Results

The results of total phenol and total flavonoid contents of AQTB and ETTB are shown in Table 1 and reported as gallic acid equivalent (GAE) for total phenol and quercetin equivalent antioxidant capacity (QEAC) for total flavonoids. The results revealed that there is significant ($p<0.05$) difference in total phenol content and total flavonoid content of both extracts. However, ETTB (2.41mgQE/g)had a significantly ($p<0.05$) higher total flavonoid content

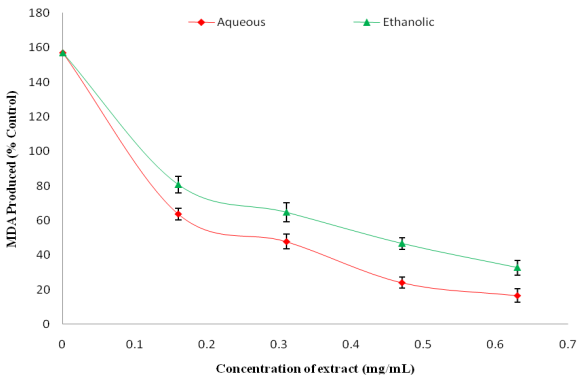

Figure 2. Inhibition of lipid peroxidation by aqueous and ethanolic extract of *Tapinanthus bangwensis*
Values represent mean± standard deviation of triplicate readings, n = 3.

than AQTB (1.32 mgQE/g). In addition, ETTB (3.63 mgGAE/g) had a significantly ($p<0.05$) higher total phenolic content than AQTB (2.88 mgGAE/g). The in vitro antioxidant indices (ferric reducing antioxidant property and nitric oxide scavenging property) of both extracts were presented in Table 1 and Figure 1 respectively. The trend of the result of the total phenol content of both extracts agrees with that of the total flavonoid content in that ETTB showed higher phenolic content than AQTB.

Furthermore, the ferric reducing antioxidant property of the extracts was assessed for their ability to reduce Fe (III) to Fe (II) and expressed as ascorbic acid equivalent (AAE).as shown in table 1. The result revealed that both extracts possess good reducing potentials. Moreover, ETTB (3.40 mgAAE/g) had significant difference ($p<0.05$) higher reducing power than AQTB (2.65 mg AAE/g).Also, the NO radical scavenging ability of both extracts is presented in Figure 1. Judging by the EC50 (extract concentration that caused 50% inhibition), the result revealed that there was significant difference ($p< 0.05$) between ETTB (EC50 = 153.8µg/ml) and AQTB (EC50 = 266.7 µg/ml). It is noteworthy that the phenolic contents of both extracts are consistent with their antioxidant properties in that ETTB exhibited higher phenolic contents and antioxidant properties than AQTB. Figure 2 showed that incubation of rat pancreas in the presence of ferrous sulphate (25 µM) caused a significant increase ($p < 0.05$) in MDA contents of the rat pancreas (156.9 %) when compared with the basal pancreas homogenates (100 %).

Discussion

Recent research has shown that most of the biological actions of plant foods are linked to their phenolic contents (Scalbert *et al*., 2005; Oboh and Rocha, 2007; Salas *et al*., 2010). Phenolic compounds exert their antioxidant properties by scavenging free radicals, chelating metals, reducing action and inhibition of lipid peroxidation (Alia *et al*., 2003; Adefegha and Oboh, 2011).In this study, the phenolic content, antioxidant properties and inhibitory effects of aqueous (AQTB) and ethanolic (ETTB) extracts of Tapinanthus bangwensis leaves on Fe^{2+}- induced lipid peroxidation in pancreas - *in vitro* were assessed.

Phenols and polyphenolic compounds, such as flavonoids, are ubiquitous in food products derived from plant sources, and they have been shown to possess significant antioxidant activities (Van Acker *et al*., 1986). It has been reported that green leafy vegetables, soft fruits and medicinal plants exhibited higher levels of flavonoid (Sultan and Anwar, 2009). From the result, the higher total phenolic and total flavonoid contents of the ethanolic extract compared with observed may be due to the presence of higher bioactive phenolics and flavonoids in ethanolic extract of Tapinanthus bangwensis than the aqueous extract. Therefore, extraction of plant constituents with ethanol solvents may enhance the chance of extracting more phenolics and flavonoid than water. Plants rich in antioxdants exhibit their defensive mechanistic actions of reducing power by electron and hydrogen atom transfer (Dastmalchi *et al*., 2007). Reducing powers of extracts were assessed based on their ability to reduce Fe^{3+} to Fe^{2+} and the results are presented in Table 1 as ascorbic acid equivalent (AAE). The significantly ($p<0.05$) higher ferric reducing antioxidant property of the ethanol extract compared to the aqueous extract (Table 1) may be directly linked to the phenolic released by the solvent (ethanol and water) used for extraction. The phenolics may in turn, favour electron-donation in the reaction mixture (Patel *et al*., 2010). Furthermore, Antioxidant activities of plant phenolics may also exhibit their radical scavenging ability and redox properties by acting as reducing agents, hydrogen donors and singlet oxygen quenchers (Amic *et al*., 2003). Figure 1 showed the ability of the extracts to scavenge nitric oxide (NO) radicals. NO is a very unstable species under the aerobic condition. It reacts with oxygen to produce the stable product nitrates and nitrite through the intermediates nitrogen (IV) oxide and other oxides. From the result, the significantly ($p<0.05$) higher NO radical scavenging abilities of EETB than AQTB revealed that the former has higher ability to decrease the amount of nitrous acid generated in the course of the reaction and revealed that EETB may be a better scavenger than AQTB. The result of this study supports the reports by several reports that plant phenolic phytochemicals are good

free radical scavengers (Gülçin *et al*., 2003; Alia *et al*., 2003, Oboh and Akindahunsi, 2004, Oboh *et al*., 2008, Patel *et al*., 2010; Adefegha and Oboh, 2012).

In biological systems, lipid peroxidation (oxidative degradation of polyunsaturated fatty acid in the cell membranes) generates a number of degradation products, such as malondialdehyde (MDA), and is found to be an important cause of cell membrane destruction and cell damage (Kalonia *et al.*, 2011). Lipid peroxidation in rat pancreas homogenate was induced with Iron II sulphate and the potential antioxidant effect of aqueous (AQTB) and ethanolic (ETTB) extract of Tapinanthus bangwensis was determined. There was statistically significant increase ($P < 0.05$) in the formation of MDA contents of the pancreas (in vitro) as shown in figure 2. MDA is regarded as an important diagnostic index of lipid peroxidation in several tissue injuries (Janero, 1990). The increased lipid peroxidation in the presence of Fe^{2+} could be attributed to the fact that Fe^{2+} can catalyze one electron transfer reactions that generate reactive oxygen species such as hydroxyl (OH) radical which is formed from hydrogen peroxide (H_2O_2) through Fenton reaction (Whittaker *et al*., 1997). The significantly ($p < 0.05$) reduction in MDA production by the extracts revealed that both extracts can mitigate and modulate the lipid components of the membrane during peroxidation. The reason for the higher inhibitory effects on pancreatic elevated MDA by AQTB when compared with ETTB cannot be categorically stated but may be due to the fact that the water extractable phenolic phytochemicals from the plant are more potent against lipid peroxidation in rat pancreas *(in vitro)* than the ethanolic extract. The potency of water extractable phytochemicals from the plant against lipid peroxidation in rat pancreas *(in vitro)* may have been influenced by the bilayer arrangement of lipids (hydrophilic head embedded in hydrophobic tail) in the biological membrane.

Conclusion

In conclusion, the ethanolic extract of *Tapinanthus bangwensis* had higher bioactive phenolics and flavonoids than the aqueous extract. Furthermore, the ethanolic extract showed higher scavenging abilities against NO and reduced Fe^{3+} to Fe^{2+} than the water extractable phenolic phytochemicals. Conversely, aqueous extract of *Tapinanthus bangwensis* showed better *in vitro* inhibition against Fe^{2+}-induced lipid peroxidation in pancreas. *Tapinanthus bangwensis* leaves possess the antioxidant substance which may be potentially responsible for the treatment of several ailments including liver disorders. This leaves a lot of scope to conduct numerous studies on antioxidative stress, antidiabetic and hepatoprotective activities of the plant leaves. Further work is on going to check the potency of the leaves by means of *in vivo* antioxidant studies.

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