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Slovak Journal of Food Sciences

Potravinarstvo Slovak Journal of Food Sciences
vol. 12, 2018, no. 1, p. 70-78
doi: https://doi.org/10.5219/825
Received: 23 August 2017. Accepted: 25 January 2018.
Available online: 11 February 2018 at www.potravinarstvo.com

ISSN 1337-0960 (online)

EVALUATION OF ANTIOXIDANT POTENTIALS OF DIFFERENT SOLVENT-FRACTIONS OF *DIALIUM INDIUM* (AFRICAN BLACK VELVET TAMARIND) FRUIT PULP – *IN VITRO*

Afolabi Olakunle Bamikole, Oloyede Omotade Ibidun, Obajuluwa Adejoke Ibitayo, Adewale Olusola Bolaji, Olayide Isreal Idowu, Balogun Bosede Damilola, Fadugba Abimbola, Obafemi Tajudeen Olabisi, Awe Obabiolorunkosi Joseph, Adewumi Funmilayo

ABSTRACT

Plant phytonutrients have been harnessed for their various curative properties both *in vitro* and *in vivo*. In this study African black velvet tamarind (ABVT) fruit pulp was evaluated for it antioxidant potentials using chloroform and hexane fractions through different antioxidant parameters. In the results; total phenolic contents quantified in mg GAE/dried sample in chloroform and hexane extracts were; 14.57 ±5.85 and 9.78 ±4.61, total flavonoid contents in chloroform and hexane extracts as; 48.58 ±0.00 and 27.35 ±0.00 while the FRAP (µg AAE.g^{-1} dried sample) was lower in chloroform (298.10 ±0.00) than hexane extracts (1029.81 ±0.00). More also, ability of varied concentrations of the extracts (with their IC50) to cause inhibition against Fe^{2+}-induced MDA that was determined by TBARS in rat's brain and liver tissue homogenates, Fe^{2+}-chelating ability and other antioxidant assays, showed an appreciable significant (p <0.05) difference. The various antioxidant properties showed by ABVT has indicated that, if the pulp is incorporated in diet, it could serve as an alternative in managing various ROS-induced degenerative ailments as it has been clearly demonstrated in the protection of brain and liver homogenates from Fe^{2+}-induced oxidative stress.

Keywords: Oxidative stress; cellular damage; antioxidants; *Dialium indium*; African Black velvet tamarind

INTRODUCTION

Oxidative stress induction through reactive oxygen species (ROS) has been studied explicitly and the roles play in promoting different diseases both in humans and animals directly or otherwise as a results of either a decrease in natural cellular antioxidant ability or an increased oxidants level cannot be over emphasized **(Afolabi and Oloyede, 2014a)**. Fe^{2+}, among series of metals has been implicated to play a crucial catalytic role in the generation/production of ROS which have the capacity to damage cellular lipids and other macromolecules resulting in wide range impairment in the cellular function and integrity through Fenton's reaction **(Afolabi, 2015; Britton et al., 2002)**. Several studies have shown that, when ROS are generated, several cellular protective pathways must have been disrupted by several mechanisms which could lead to cellular eruption and damage to enzymes localized in different cellular organelles **(Afolabi and Oloyede, 2014b; Akomolafe, 2013; Rani, 2013)**, which aftermath, would lead to the formation of several neurodegenerative, cardiovascular diseases and many other terminal ailments **(Oboh et al., 2013)**.

In recent times, attention has been shifted to exploiting plant-based products for the prevention and treatment of these different ROS linked diseases by various inhibition against its formation and different activities **(Raghuveer et al., 2011)**, also, in the body, several cellular defensive anti-oxidative mechanisms have been devised to fight against the initiation of ROS **(Britton et al., 2002)**. However, consumption of diets enriched in antioxidants may help in fighting the ROS-initiated diseases by strongly improving body's antioxidant status. *Dialium indium*, commonly known as African black velvet tamarind (ABVT) is a large tree found in many parts of Africa such as West Africa, Central African Republic and the Chad. The fruits are usually circular and frightened, usually has a sweet-Sour taste, black in colour with a stalk of about 6mm long, it belongs to the family *Fabaceae-caesalpiniodaea* **(Eziaku and James 2014; Okegbile and Taiwo, 1990)**. It is popularly called *Icheku* or *Nchichi* in Igbo, *Awin* among Yorubas and *Tsamiyar Kurm* in Hausa, Nigeria. The fruit is quite popular as a spice with belief

that fruit can bring down systemic cholesterol level. The velvet tamarind pulp is eaten in Southeastern Nigeria because of its refreshing properties and pleasant taste **(Nwaukwu and Ikechi, 2012)**. Black tamarind and its pulp has been found to be highly enriched in vitamin C and other vital essential nutrients needed in human diet which could also be used instead of synthetic drugs to treat stomach upset when soaked in water **(Nwaukwu and Ikechi, 2012; Dike, 2010)**.

Scientific hypothesis

It has been well elucidated in the current work that, when 0.1 mg.mL^{-1} of the various solvent extracts of ABVT was appraised, the trends that indicated various activities increasingly in concentration – dependent manner were observed. If 0.2 mg.mL^{-1} of the same extracts was subjected to the same assays as found in the current work, it could be scientifically assumed that same trend would be achieved, if and only if the same methods and procedures are engaged.

MATERIAL AND METHODOLOGY

Chemicals used

Chemicals and reagents used such as thiobarbituric acid (TBA), 1, 10-phenantroline, Folin-coicalteau's reagent, trichloroacetic acid (TCA), gallic acid with Diphenyl-2-picryl-hydrazyl (DPPH) from Sigma-Aldrich, Inc. (St Loius, MO), $FeCl_3$, Sodium carbonate, $AlCl_3$, Sodium dodecyl sulphate (SDS), $FeSO_4$ (Iron II Sulphate), potassium ferricyanide, ferric chloride acetic acid, hydrogen peroxide, methanol were procured BDH Chemicals Ltd., (Poole, England) and other chemicals used were of analytical grades and prepared in all-glass apparatus using distilled water.

Sample collection and preparation

African Black velvet tamarind (ABVT) fruits were obtained from a popular place in Ikare- Akoko in Ondo State, Nigeria. The voucher sample was taken to the herbarium of Plant Science Department of Ekiti State University, Ado-Ekiti, Ekiti State, where it was authenticated. Thereafter, the sample was treated and pulverized using a laboratory blender and the fine powders obtained stored at moderate temperature until further use.

Obtention of sample

Ethanolic extraction procedure

The blended ABVT pulp was air-dried to a constant weight in a ventilated place at ambient temperature of 30 ± 2 °C, pulverized using a laboratory blender and the fine powdery form obtained was stored at moderate temperature until further use. About 120 g of the sample was weighed and used for the extraction in 70% ethanol for 72 h.

Solvent Partitioning

About 5 g of the ethanolic extract of ABVT pulp was reconstituted in distilled water and then defatted with Pet-ether, the fat free aqueous residue was partitioned repeatedly with n-hexane in a separating funnel flask until exhaustion. The remaining marc was finally partitioned again with chloroform solvent until exhaustion. Thereafter, the aqueous residue was remove and the solvent fractions were concentrated in water bath at 50 °C.

Determination of total phenolic content

The total phenolic contents of the solvent extracts were determined by the method of **Singleton et al. (1999)**. 0.2 mL of the extract was mix with 2.5 mL of 10% folin-ciocalteau's reagent and 2 mL of 7.5% Sodium carbonate. The reaction mixture will be subsequently incubated at 45 ^{o}C for 40 min., and the absorbance was measured at 700 nm with garlic acid as standard and the result expressed in mg GAE.g^{-1} of the dried sample.

Determination of total flavonoid

The total flavonoid contents of the extracts were determined using a colorimeter assay developed by **Bao et al. (2005)**. 0.2 mL of each extract was added to 0.3 mL of 5% $NaNO_3$ at zero time. After 5 min, 0.6 mL of 10% $AlCl_3$ was added and after 6 min, 2 mL of NaOH was added to the mixture followed by the addition of 2.1 mL of distilled water. Absorbance was read at 510 nm against the reagent blank and flavonoid content was expressed as µg quercetin equivalent/gram dry sample (µg QE.g^{-1} dry sample).

Determination of ferric reducing property (FRAP)

The reducing properties of the extracts were determined by the described method of **Pulido et al. (2000)**. 0.25 mL of the extract was mixed with 0.25 mL of 200 mmol.L^{-1} of Sodium phosphate buffer pH 6.6 and 0.25 mL of 1% KFC. The mixture was incubated at 50 ^{o}C for 20 min, thereafter 0.25 mL of 10% TCA was also added and centrifuge at 2000 rpm for 10 min, 1 ml of the supernatant was mixed with
1 mL of distilled water and 0.2 mL of 1% ferric chloride and the absorbance was measure at 700 nm and ascorbic acid was used as standard, with the result expressed in µg ascorbic acid equivalent/g dried sample (µg AAE.g^{-1} dried sample).

Fe^{2+} Chelating assay

The *in vitro* Fe^{2+} chelating ability of the ABVT pulp solvent extracts were assayed according to the method of **Puntel et al. (2005)**. Briefly, 0.9 mL of aqueous 0.5 mmol.L^{-1} $FeSO_4$ and 0.15 mL of leaf extract were incubated for 5 min at room temperature. Then, 78 µL of ethanolic solution of 1,10-phenanthroline was added. The absorbance of the orange colour solution was read at 510 nm. The *in vitro* Fe^{2+} chelating ability of the samples was calculated by using the following formula:

Chelating ability (%) = (Abs $_{control}$ – Abs s_{ample}) / Abs $_{control}$ x 100

Abs control = The absorbance of the control (reaction mixture in the absence of sample)
Abs sample = The absorbance of the reaction mixture (with the sample).

Estimation of DPPH Radical Scavenging Ability

The free radical scavenging ability of the ABVT pulp extracts against DPPH was determined using the described method of **Gyamfi et al. (1999)**. 1 mL of the extract was

mixed with 1mL of the 0.4 mmol.L^{-1} methanolic solution of the DPPH, the mixture was left in the dark for 30 min before measuring the absorbance at 516 nm.

Determination of NO radical scavenging ability

The scavenging effects of the solvent extracts of ABVT pulp on nitric oxide (NO) radical were measured according to the method of **Mercocci et al. (1994)**. An amount of 100 – 400 µL of the aqueous extract was added in test tubes to 1 mL of SNP solution (25 mmol.L^{-1}) and the tubes were incubated at 37 °C for 2 h. An aliquot (0.5 mL) of the incubating mixture was removed and diluted with 0.3 mL of Griess reagent. The absorbance of the chromophore formed was immediately read at 570 nm against distilled water as blank. Results were expressed as percentage Nitric oxide radical-scavenging ability.

(%) Nitric oxide radical scavenging activity = (Abs_{Ref} – Abs_{sample} / Abs_{Ref}) x 100

Degradation of deoxyribose (Fenton's reaction)

The ability of the solvent fractions of ABVT pulp to prevent Fe^{2+}/H_2O_2 induced decomposition of deoxyribose was carried out using the method of **Halliwell and Gutteridge (1981)**. Briefly, freshly prepared aqueous extract (0 – 100 µL) was added to a reaction mixture containing 120 µL 20 mmol.L^{-1} deoxyribose, 400 µL 0.1 M phosphate buffer (pH 7.4), 40 µL 20 mmol.L^{-1} hydrogen peroxide and 40 µL 500 µmol.L^{-1} FeSO4, and the volume for made to 800 µL with distilled water. The reaction mixture was incubated at 37 ^{o}C for 30 min, and the reaction was stop by the addition of 0.5 ml of 2.8% trichloroaceticacid (TCA), this was followed by the addition of 0.4 mL of 0.6% TBA solution. The tubes were subsequently incubated in boiling water for 20 min. The absorbance was measured at measured at 532 nm in spectrophotometer.

$$\text{Hydroxyl radical scavenging ability (\%)} = \frac{Abs_{control} - Abs_{Test\ sample}}{Abs_{control}} \times 100$$

where $Abs_{control}$ = absorbance of the control (reacting mixture without the test sample) and $Abs_{test\ sample}$ = absorbance of reacting mixture with the test sample.

Lipid peroxidation by thiobarbituric acid reactive species assay

Preparation of Tissue homogenates

The rats were decapitated under mild di-ethyl ether anesthesia while the cerebral and liver tissues were rapidly extracted and placed on ice and weighed. This tissues were subsequently homogenized in cold 0.1 mol.L^{-1} Tris-HCl buffer pH 7.4 (1:10 w/v). The tissue homogenates were centrifuged for 10 min at 3000 g to yield a pellet that was discarded and the supernatant was used for the assay.

Thiobarbituric acid reactive species assay

The lipid peroxidation assay was carried out using the modified method of **Ohkawa et al. (1984)**. Briefly, the reaction mixture consisting 100 µL of tissue, 30 µL of 0.1 mol.L^{-1} pH 7.4 Tris-HCl buffer, different concentrations of the extracts were incubated with 50 µL of the freshly prepared 250 µmol.L^{-1} $FeSO_4$ with distilled water at 37 °C for 1 h. The color reaction was carried out by adding 200, 500 and 500 µl each of the 8.1 % sodium dodecyl sulphate (SDS), 1.33 M acetic acid (pH 3.4) and 0.6% TBA respectively. The reaction mixture was incubated at 100 °C for 1 h. The absorbance was read after cooling at 532 nm in an ultraviolet visible-spectrophotometer. The results were expressed in percentage inhibition of Malondialdehyde (MDA) produced.

Statistical analysis

All experiments were carried out in duplicate. Results were expressed as mean values ±standard deviation (SD) of duplicated samples. Differences and levels of significance were evaluated by one-way analysis of variance (ANOVA) followed by Duncan's multiple test **(Zar, 1984)**. Significance was accepted at $p < 0.05$.

RESULTS AND DISCUSSION

Plants are naturally endowed with natural antioxidants that exhibit cell's defensive mechanisms through scavenging the reactive oxygen species (ROS), with protective effects against degenerative diseases in humans **(Shalaby and Shanab, 2013)**. They exhibit antioxidant activities through electron donation and thereby neutralizing the damaging effects of free radicals, whose formation is associated with aerobic cells normal natural metabolic processes **(Amic et al., 2003)**. This work reports the antioxidant properties of different solvent extracts of ABVT fruit pulp by considering various parameters. Most plants that are rich in phenolic and polyphenolic acids exhibit strong antioxidant and anti-radical activities **(Mary et al., 2003)**, capable of removing free radicals, activate antioxidant enzymes, chelate metal catalysts, reduce alpha-tocopherol radicals and inhibit oxidases **(Amic et al., 2003)**. This group of compounds constitute the main class of natural antioxidants present in plants **(Andrea et al., 2003)**. As represented in Figure 1, the phenolic contents in the different solvent extracts of ABVT pulp considered, were clearly revealed, chloroform extract demonstrated higher content of phenol groups than that of hexane extract, which means that, the potential to exhibit antioxidant activities will be more in the chloroform extract than that of hexane extract.

Also, the flavonoid contents of ABVT pulp solvent extract as shown in Figure 2, reveal that, chloroform extract of the ABVT pulp showed higher flavonoid content than that of hexane extract. Howbeit, the plant flavonoids have been unfolded in several works recently to have antioxidant activity both *in vitro* and *in vivo* **(Shimoi et al., 1996; Geetha et al., 2003)**, by suppressing the reactive oxygen formation, chelating trace elements involved in free radical production, scavenging reactive species and protecting antioxidant defensive mechanism in the cell **(Agati et al., 2012)**. Figure 3, shows the ferric reducing ability (FRAP) of the extracts expressed in μg ascorbic acid equivalent/g dried sample, the reducing power is associated with antioxidant activity and may serve as a significant indicator of the antioxidant activity **(Meir et al., 1995)**. The results in the Figure 3, indicated that, hexane extract of ABVT pulp has higher reductive

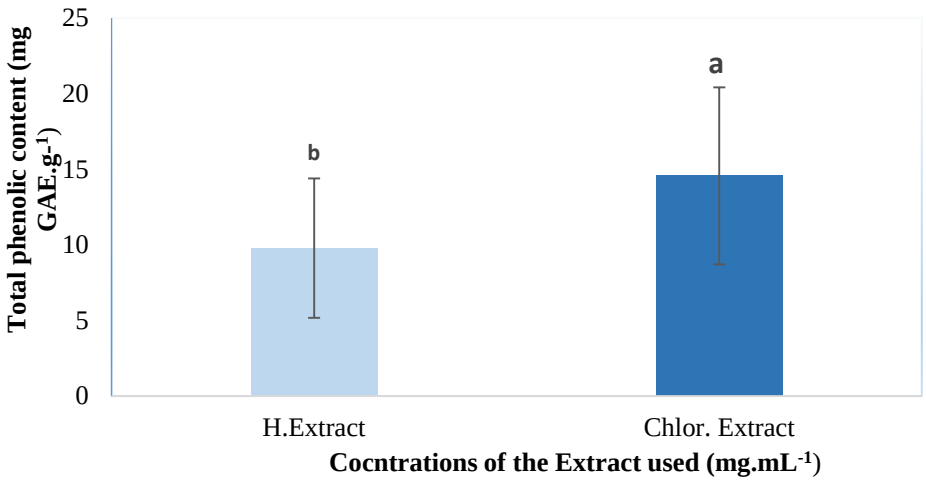

Figure 1 Total phenolic contents of hexane and chloroform fractions of ABVT pulp.
Key: H. Extract = Hexane Extract; Chlor. Extract = Chloroform Extract, GAE=Galic acid equivalent. The alphabets a and b show the levels of significant (p <0.05) difference between the extracts.

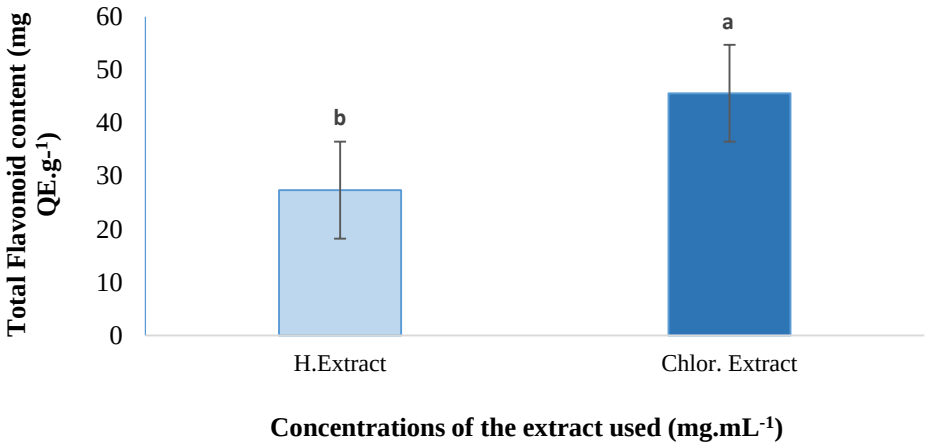

Figure 2. Total flavonoid contents of hexane and chloroform extracts of ABVT pulp.
Key: H. Extract = Hexane Extract; Chlor. Extract = Chloroform Extract, QE = Quercetin equivalent. The alphabets a&b show the levels of significant (p <0.05) difference between the extracts.

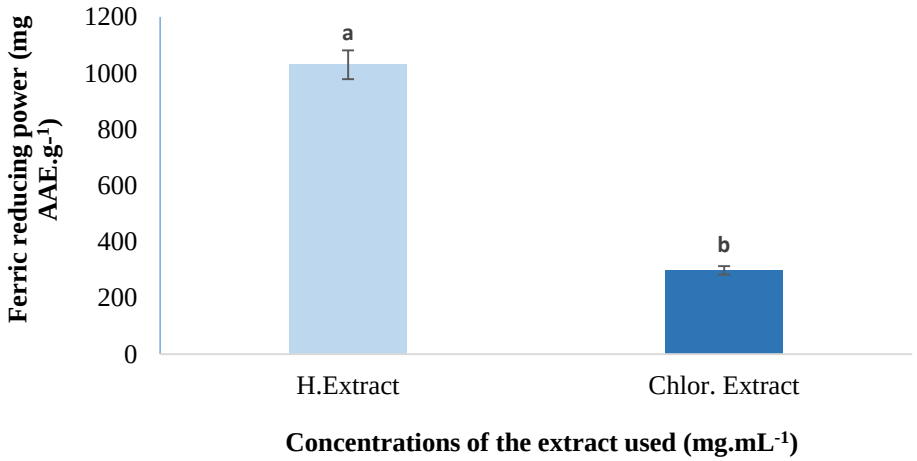

Figure 3. Ferric reducing abilities of hexane and chloroform extracts of ABVT pulp.
Key: H. Extract = Hexane Extract; Chlor. Extract = Chloroform Extract, AAE = Ascorbic acid equivalent. The alphabets a and b show the levels of significant (p <0.05) difference between the extracts.

capacity than that of chloroform extract. Whereas, in Figure 4, the disruption of *o*-phenanthroline-Fe^{2+} complex expressed as percentage inhibition in Figure 4, during incubation with both solvent extracts of ABVT fruit pulp, reveals that, H. Extract of ABVT pulp was also able to chelate Fe^{2+} considerably higher than Chlor. Extract. The ability of the H. Extract to chelate Fe^{2+} matched with the fact that, the H. Extract showed higher value for FRAP in

Figure 3. However, the chelating ability showed by these extracts could have been credited to the phenolic and flavonoid contents as reported by **Zhao et al. (2006)**. The possibility of an extracts/substances to chelate and deactivate transition metals by the antioxidant mechanism of action, has been said to prevent such metals from participating in the initiation of lipid peroxidation and oxidative stress through metal-based catalytic reaction **(Oboh et al., 2007)**.

The Figure 5 relays the bleaching capacity of 1,1-diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$) chromogenic radical by antioxidant/reducing compounds present in ABVT pulp solvent extracts to its corresponding hydrazine **(Boligon et al., 2014)**. DPPH is a free radical donor that accepts an electron or hydrogen to become a stable diamagnetic molecule **(Afolabi and Oloyede, 2014)**. As shown in Table 1, there was no significant difference in the IC_{50} of the various concentrations of the solvent extracts of ABVT

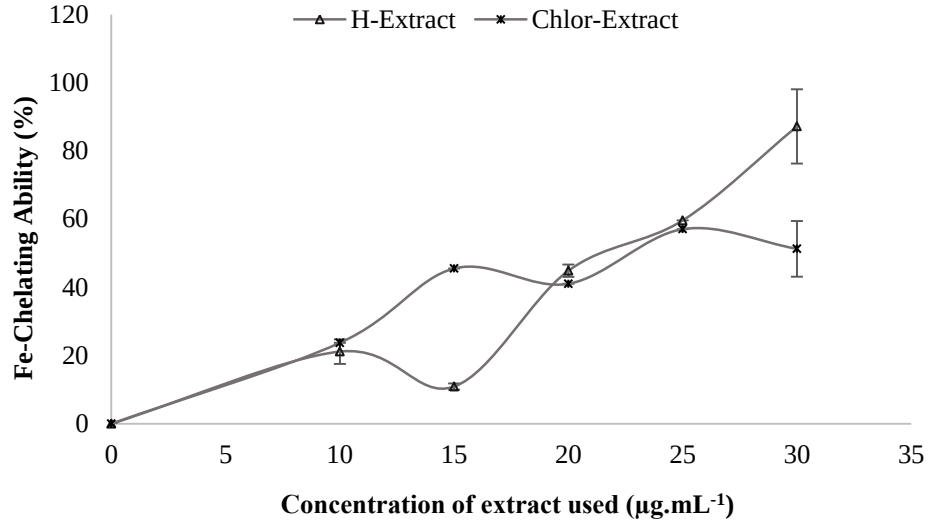

Figure 4 Fe^{2+} – chelating ability (%) of different solvent extracts of ABVT pulp.
Key: H. Extract = Hexane Extract; Chlor. Extract = Chloroform Extract.

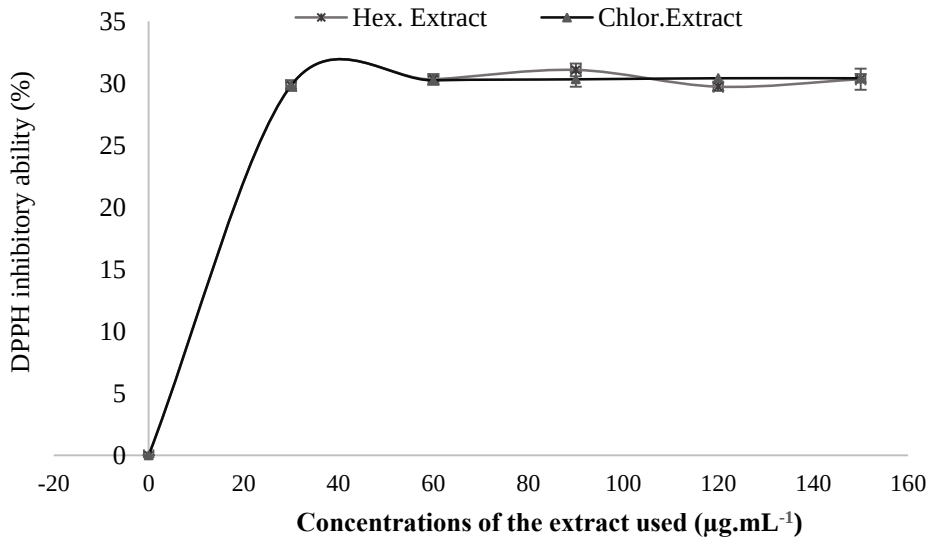

Figure 5 DPPH$^{\bullet}$ radical scavenging ability of different solvent extracts of ABVT pulp.
Note: Hex. Extract = Hexane Extract; Chlor. Extract = Chloroform Extract.

Table 1 The IC_{50} (µg.mL^{-1}) values (Concentration of the extracts that will cause 50 percent inhibition) of ABVT pulp solvent extracts calculated from a linear regression curve of the percentage (%) inhibitions against various concentrations of the extracts.

Assays(%)	Hexane extract	Chloroform extract
Fe^{2+}Chelation	30.16 ±2.16^{a}	23.94 ±1.35^{a}
DPPH Radical Scavenging	181.62 ±0.37^{a}	181.37 ±2.14^{a}
NO radical scavenging ability	107.20 ±2.79^{a}	73.24 ±0.63^{b}
Hydroxyl radical scavenging ability	131.61 ±13.55^{a}	131.61 ±0.49^{a}

Note: Results represent mean values (n = 2) ±SD. The values with the same superscript along the row are not significantly (p <0.05) different.

pulp examined. The inhibition of DPPH• radical exhibited by both extracts, could be attributed to their various phenolic and flavonoid contents.

Nitric oxide (NO) is an important physiological messenger that participates in inflammatory processes which can directly/independently induce toxicity in the tissues resulting in vascular damage and other disorders at an increased level **(Moncada et al., 1991)**. The cellular damage is heightened when NO react with superoxide radical to form a non-radical peroxynitrite (ONOO-), which is a powerful oxidant **(Halliwell and Gutteridge 1999; Balavoine and Geletti, 1999)**. As presented in Figure 6, ABVT pulp solvent extracts showed considerable inhibitions against generation of NO induced radicals. The mechanisms of inhibition exhibited by the extracts are not known but could be ascribed to the presence of antioxidant contents, as ability of plants to demonstrate antioxidant properties against generation of oxidants have been linked to the presence of array of important phenolic and non-phenolic phytochemicals which includes phenolic acids, flavonoids and alkaloids **(Cheplick et al., 2007)**, however, this agrees with Figure 1 and 2.

The ability of the solvent fractions of ABVT pulp to prevent Fe^{2+}/H_2O_2 from causing decomposition of deoxyribose to generate OH radical through Fenton reaction was also carried out. H. Extract showed higher inhibitory effect against the production of hydroxyl radical at the very lower concentration than Chlor. Extract, the results are presented in Figure 7, with various IC_{50} in Table 1. This inhibition possibly could have been responsible for by the antioxidant parameters as shown in Figure 1 and 2 as well.

Likewise, incubation of both the liver as well as brain tissue homogenates with Fe^{2+} to test for the ability of

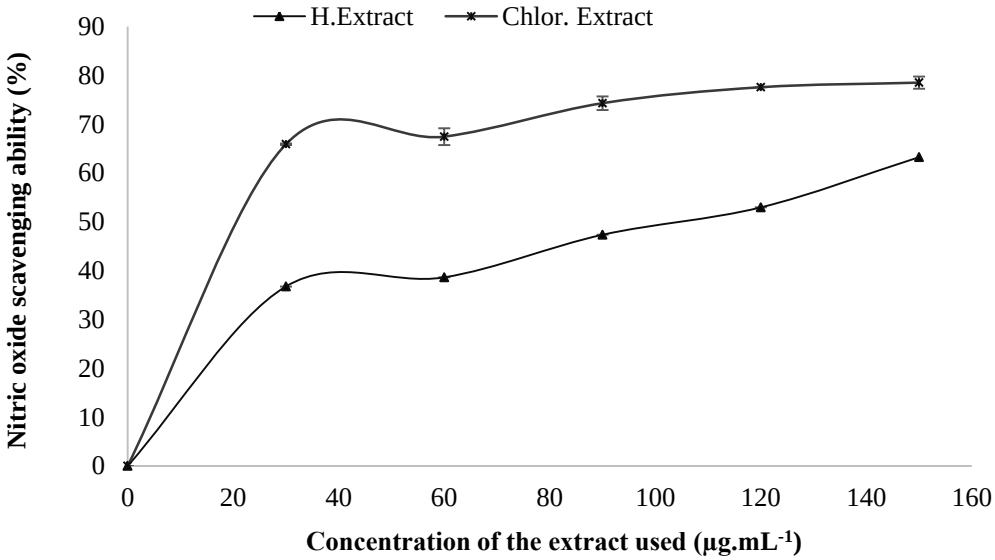

Figure 6 Nitric oxide inhibitory ability (%) of different solvent extracts of ABVT pulp.
Note: H. Extract = Hexane Extract; Chlor. Extract = Chloroform Extract.

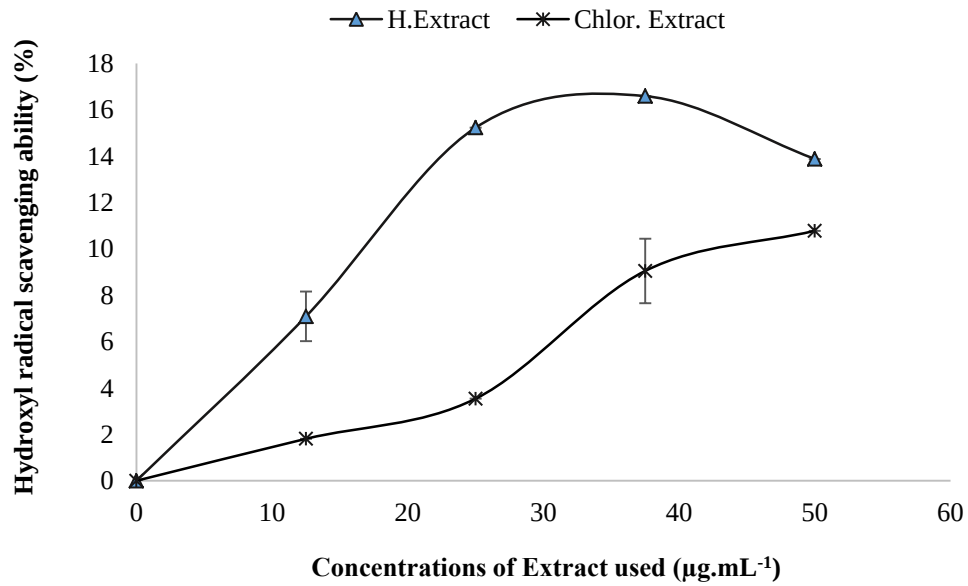

Figure 7 Hydroxyl radical scavenging ability (%) of different solvent extracts of ABVT pulp.
Note: H. Extra ct = Hexane Extract; Chlor. Extract = Chloroform Extract.

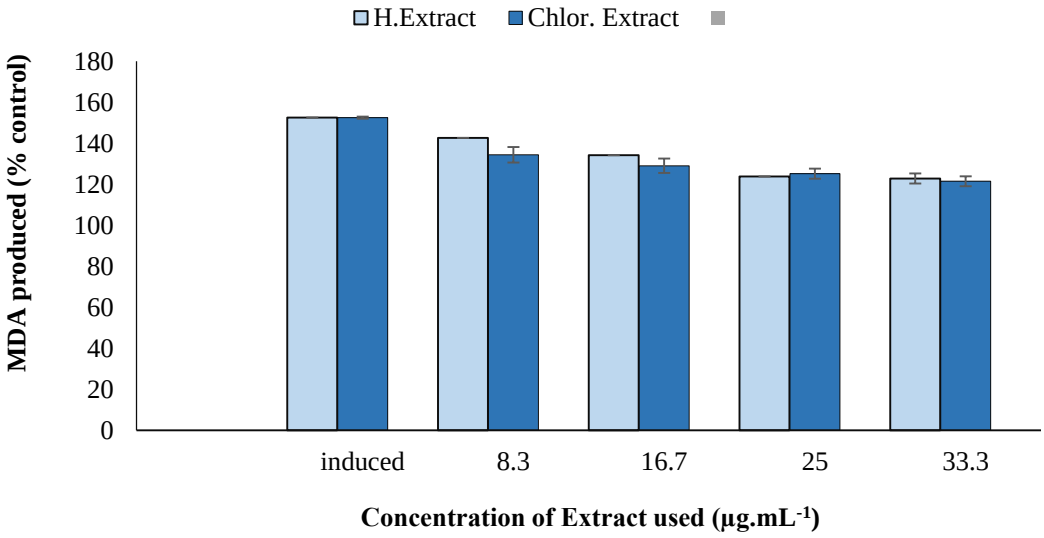

Figure 8a Inhibition of Fe^{2+} – induced lipid peroxidation in rat brain homogenate by different solvent extracts of ABVT pulp. **Key:** H. Extract = Hexane Extract; Chlor. Extract = Chloroform Extract.

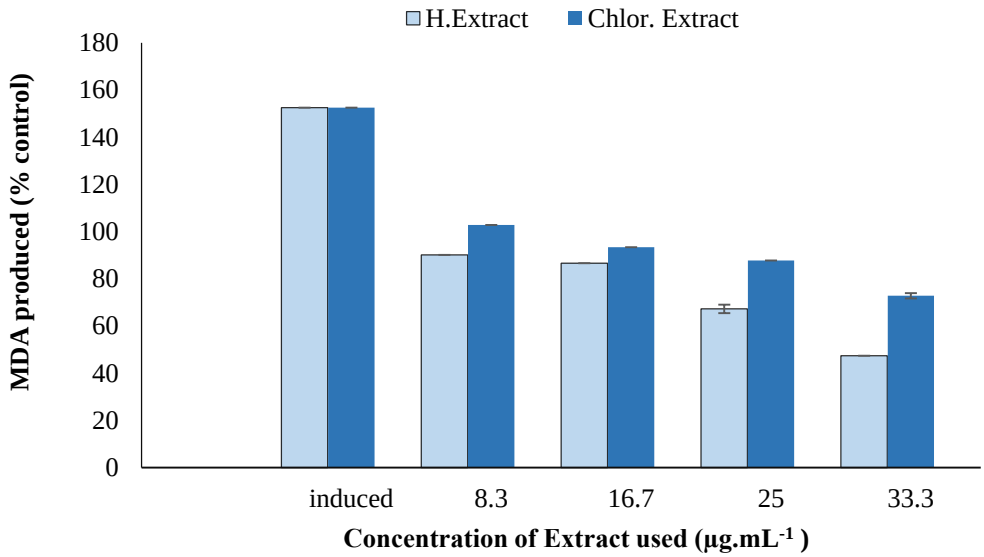

Figure 8b Inhibition of Fe^{2+} – induced lipid peroxidation in rat liver homogenate by different solvent extracts of ABVT pulp. Key: H. Extract = Hexane Extract; Chlor. Extract = Chloroform Extract.

ABVT pulp solvent extracts in inhibiting the formation of MDA was assayed for by lipid peroxidation reaction as shown in Figure 8 a and b. Lipid peroxidation is one of the major effects of ROS-mediated injury leading to the generation of a variety of relatively stable end products. ROS concentrations above the clearance capacity of the cell cause oxidative stress, mitochondrial dysfunction, cellular damage, in most cases, cell death **(Ferreiro et al., 2012)**. Several studies have implicated malondialdehyde (MDA), one of the byproducts as main indicator in monitoring the level of oxidative stress/damage caused by ROS in the tissues **(Aitken and Fisher, 1994)**. Incubation of both the liver and brain tissue homogenates with Fe^{2+} (iron II) induced oxidative stress, being perceptible by the increased MDA levels in homogenates in the absence of the extracts (Figure 8a and b). However, there were significant (p <0.05) reduction when the solvent extracts of ABVT pulp were introduced in the reaction with the various concentration considered in dose-dependent manner. The ability of the ABVT pulp solvent extract to inhibit formation of MDA could be traceable to the antioxidant properties of the extracts as being evidenced in Fe^{2+} chelation and/or scavenge free radicals produced by the Fe^{2+}-catalyzed production of reactive oxygen species (ROS), which is in agreement with Figure 4.

CONCLUSION

The potency of ABVT pulp solvent extracts having appraised, has indicated that the pulp, if incorporated in diet, could serve as an alternative in managing various

degenerative ailments as it has been clearly demonstrated in the protection of brain and liver from Fe^{2+}-induced oxidative stress. The mechanism for doing this has also been shown in Fe^{2+}-chelation, DPPH inhibition, nitric oxide inhibition and hydroxyl radical scavenging ability. All these could have been as a result of the antioxidant parameters in the form of phenolic/polyphenolic acid and flavonoids.

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Acknowledgments:

The authors hereby acknowledge the founding father and the President of Afe-Babalola University, Aare-Afe Babalola for the timely encouragement that facilitated this manuscript.

Contact address:

Afolabi Olakunle Bamikole, Phytomedicine and Toxicology Laboratory, Biochemistry Unit, Department of Chemical Science, College of Science, Afe-Babalola University. P.M.B 5454, Ado- Ekiti, Ekiti State, Nigeria, E-mail: afolabiob@abuad.edu.ng

Oloyede Omotade Ibidun, Department of Biochemistry, Ekiti State University, P.M.B 5363, Ado-Ekiti, Nigeria, E-mail: pjmoloyede@yahoo.com

Obajuluwa Adejoke Ibitayo, Department of Biological Sciences, College of Science, Afe-Babalola University. P.M.B 5454, Ado- Ekiti, Ekiti State, Nigeria, E-mail: joke_ibitayo@yahoo.com

Adewale Olusola Bolaji, Phytomedicine and Toxicology Laboratory, Biochemistry Unit, Department of Chemical Science, College of Science, Afe-Babalola University. P.M.B 5454, Ado- Ekiti, Ekiti State, Nigeria, E-mail: solaustine2003@yahoo.com

Olayide Isreal Idowu, Phytomedicine and Toxicology Laboratory, Biochemistry Unit, Department of Chemical Science, College of Science, Afe-Babalola University. P.M.B 5454, Ado- Ekiti, Ekiti State, Nigeria, E-mail: israrobinson95@gmail.com

Balogun Bosede Damilola, Department of Biochemistry, Ekiti State University, P.M.B 5363, Ado-Ekiti, Nigeria, E-mail: bose2k15@yahoo.com

Fadugba Abimbola, Department of Biological Sciences, College of Science, Afe-Babalola University. P.M.B 5454, Ado- Ekiti, Ekiti State, Nigeria, E-mail: falanaabimbola@yahoo.com

Obafemi Tajudeen Olabisi, Phytomedicine and Toxicology Laboratory, Biochemistry Unit, Department of Chemical Science, College of Science, Afe-Babalola University. P.M.B 5454, Ado- Ekiti, Ekiti State, Nigeria, E-mail: femi.bisi@yahoo.com

Awe Obabiolorunkosi Joseph, Phytomedicine and Toxicology Laboratory, Biochemistry Unit, Department of Chemical Science, College of Science, Afe-Babalola University. P.M.B 5454, Ado- Ekiti, Ekiti State, Nigeria, E-mail: omonigold@gmail.com

Adewumi Funmilayo, Chemistry Unit, Department of Chemical Science, College of Science, Afe-Babalola University. P.M.B 5454, Ado- Ekiti, Ekiti State, Nigeria, E-mail: adewumifunmilayo@yahoo.com