International Journal of Herbal Medicine
Available online at www.florajournal.com

E-ISSN: 2321-2187
P-ISSN: 2394-0514
IJHM 2018; 6(3): 20-24
Received: 14-03-2018
Accepted: 17-04-2018

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Antioxidant activity and inhibitory effect of ethanolic extract of *Ficus exasperata* leaves on pro- oxidant induced hepatic and cerebral lipid peroxidation in albino rats *in vitro*

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Abstract
Oxidation of lipid has been found to be a crucial step in the pathogenesis of several diseased conditions. A good number of pro-oxidants are known to cause oxidative damage to biomolecules such as proteins, lipids and DNA. This study was designed to evaluate the protective effect and other antioxidant potentials of ethanolic extract of *Ficus exasperata* leaves against pro-oxidant [Iron (Fe^{2+}),Hydrogen peroxide (H_2O_2), and Sodium nitroprusside (SNP)] induced lipid peroxidation in cerebral and hepatic tissues of albino rats. An aliquot of 100µl of clear supernatant of the tissue homogenates were incubated for 1hr at 37^0C in the presence of the extract with and without pro-oxidants, iron and hydrogen peroxide (final concentration 10µM), and sodium nitroprusside (final concentration 30µM) to determine the extent of lipid peroxidation. The phenol content, flavonoids content, ferric reducing ability, iron chelating properties, DPPH and ABTS scavenging abilities of the extract were also evaluated. The results shows that incubation of the tissue homogenates with the pro-oxidants caused significant ($P<0.05$) increase in levels of thiobarbituric acid reactive substances (TBARS), while administration of the extracts provided a significant protection to TBARS levels in the tissues, indicating a protective inhibitory potentials on the pro-oxidants. The extracts at different concentrations demonstrated significant antioxidant potentials, indicating their abilities to act as radical scavengers and chelators. Ethanolic extracts of *Ficus exasperata* could be a potential antioxidant candidate that could help in the management of degenerative diseases whose etiology has been linked to oxidative stress.

Keywords: Oxidative stress, pro-oxidants, lipid peroxidation, antioxidant, hepatic and cerebral

1. Introduction
Lipid peroxidation is a complex process known to occur in both plants and animals. It involves the formation and propagation of lipid radicals, the uptake of oxygen, a rearrangement of the double bonds in unsaturated lipids and the eventual destruction of membrane lipids, with the production of a variety of breakdown products, including.
alcohols, ketones, alkanes, aldehydes and ethers [1]. The formation of these reactive oxygen species (ROS) is a natural consequence of aerobic metabolism and is associated with oxygen homeostasis, i.e. the balance between constitutive oxidants and antioxidants [2]. Prooxidant refers to any endobiotic or xenobiotic that induces oxidative stress either by generation of ROS or by inhibiting antioxidant systems. It can include all reactive, free radical containing molecules in cells or tissues. Increased free radical production or reduced antioxidant defense response may give rise to increased oxidative stress, which has been associated with lipid peroxidation that causes food deterioration, a number of human chronic diseases such as cellular aging, carcinogenesis, coronary heart disease, diabetes, mutagenesis and neurodegenerative infections. Antioxidant compounds play a vital role to the body defense system against Reactive Oxygen Species (ROS), which are the harmful by-products engendered during normal aerobic cellular respiration [3]. *Ficus exasperata* is a medicinal plant whose parts are commonly used by Nigerians in the treatment of various diseases. The brain and nervous system are particularly vulnerable to oxidative stress due to limited antioxidant capacity [4]. The objective of the study is to evaluate the antioxidant effects of *Ficus exasperata* against free radicals and pro oxidant induced lipid peroxidation brain and liver using *in vitro* analysis.

2. Materials and methods
2.1. Materials
2.1.1. Collection of samples
Fresh samples of *Ficus exasperata* leaves were collected from the premises of Ekiti State

University, Ado-Ekiti, Ekiti State, Nigeria. The plants was identified and authenticated at the Herbarium of the Department of Plant Science and Biotechnology, Ekiti State University, Ado-Ekiti. Voucher specimens with reference number UHAE 2016/091 was deposited in the herbarium for reference purposes. The fresh leaves were air-dried at room temperature until it was completely dry, pulverized using an electrical blender and obtained powdery form were stored until further use.

2.1.2. Chemicals and Reagents
Trichloroacetic Acid (TCA), Tris-HCl buffer 7.4, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2'-azino-bis-3-ethylbenzthiazoline-6-Sulphonic acid (ABTS), Sodium chloride (NaCl), Sodium azide (NaN_3), Dimethyl sulfoxide (DMSO), Ouabain, Mecury Chloride ($HgCl_2$), Ammonium Molybdate, Vitamin C, Magnesium Chloride ($MgCl_{2)}$, Adenosine Monophosphate (AMP), Phosphate buffer, Calcium chloride ($CaCl_{2)}$ Trichloroacetic acid(TCA), Deoxyribose, Thiobarbituric acid(TBA), 2,2-diphenyl -1-picrylhydrazyl(DPPH) and 1, 10-phenanthroline were obtained from Sigma (St. Louis, MO). All other chemicals were of analytical grade and obtained from standard chemical suppliers.

2.1.3. Animals
Adult albino rats are used (200-250g) were used. The animals were used according to the standard guidelines of the committee on Care and Use of experimental Animal Resources.

2.2. Methods
2.2.1. Preparation of ethanolic extract
100g of the powdery form was dissolved in 500ml of 70% ethanol for 48hours. The crude extract was filtered with Whatman filter paper and evaporated under pressure at 60°C using a rotary evaporator.

2.2.2. Preparation of tissues
The tissues were all removed from decapitated adult Wistar rats. The tissues were quickly removed, placed on ice and homogenized in cold 50mM Tris-HCl pH 7.4. The homogenate was centrifuged at 4,000x*g* for 10minutes to yield the low-speed supernatant fraction that was used for the assays.

2.2.3. Determination of total phenol
The total phenol content of the extract was determined by the method of [5]. 0.1ml of the extract was mix with 0.1ml of distilled water followed by 0.5ml of 10% Folin-Ciocalteau's reagent and 2.5ml of 7.5% Sodium carbonate. The reaction mixture will be subsequently incubated at 45ºC for 40mins, and the absorbance was measure at 700nm in the spectrophotometer, garlic acid would be used as standard phenol.

2.2.4. Determination of Flavonoid
The total flavonoid content of the extract was determined using a colourimeter assay developed by [6]. 0.1ml of the extract was mixed with 0.1ml of distilled water, 1.5ml of 5% $NaNO_3$ at zero time. After 5min, 0.3ml of 10% $AlCl_3$ was added and after 6min, 1ml of 1M NaOH was added to the mixture. Absorbance was read at 520nm against the reagent blank and flavonoid content was expressed as mg quercetin equivalent.

2.2.5. Determination of ferric reducing property
The reducing property of the extract was determined by assessing the ability of the extract to reduce $FeCl_3$ solution as described by [7]. 0.25ml of the extract was mixed with 0.25ml of 200mM of Sodium phosphate buffer pH 6.6 and 0.25ml of 1% Potassium ferrocyanide (KFC). The mixture was incubated at 50ºC for 20min, thereafter 0.25ml of 10% TCA was also added and centrifuge at 2000rpm for 10min, 1ml of the supernatant was mixed with 1ml of distilled water and 0.1% of $FeCl_3$ and the absorbance was measure at 700nm.

2.2.6. Iron chelation determination
The ability of the extract to chelate Fe^{2+} was determined using a modified method of [8]. Briefly, 150mM $FeSO_4$(150µl) was added to a reaction mixture containing 168µl of 0.1M Tris-HCl pH 7.4, 218µl saline and extract(0-20µl) and the volume is made up 1ml with distilled water. The reaction mixture will be incubated for 5min, before the additional of 13µl of 1, 10-phenantroline the absorbance was read at 510nm. The Fe (II) chelating ability was subsequently calculated with respect to the reference which contains all the reagents without the leaf extract.

2.2.7. 2,2'-Azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) scavenging ability assay
2,2'-Azino-bis-3-ethylbenzthiazoline-6-sulphonic acid was generated by reacting 50ml 7mM ABTS with 50ml of 2.45mM $K_2S_2O_8$. The assay was performed as described by [9]. Different concentrations of the plant extract were prepared, to which the ABTS solution was added in the ratio 1:9 (100µL of plant extract with 900µL of ABTS solution). The resulting mixture was kept in the dark for 30mins, and the absorbance was read at 700nm wavelength on the spectrophotometer. The results was compared the scavenging ability of Vitamin C which was used as the standard.

2.2.8. 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging ability
The free radical scavenging activity was determined as described by [10]. 600µL each of the extract concentrations prepared was added to 600µL of DPPH solution. The reaction mixture was incubated in the dark for 30 minutes. The absorbance of the residual DPPH was determined at 518 nm in a spectrophotometer. One 600µL of distilled water was added to 600µL DPPH solution, incubated at 25 °C for 30 minutes and used as control. Vitamin C was used as a standard free radical scavenger. The absorbance decreases with increasing free radical scavenging ability. Each test was carried out using three replicates.

2.2.9. Lipid peroxidation
Rats were decapitated via cervical dislocation and the cerebral (whole brain) and hepatic (liver) tissues were rapidly dissected, placed on ice and weighed. Tissues were immediately homogenized in cold 50mM Tris-Hcl, pH 7.4. The homogenates was centrifuged for 10mins at 4000rpm to yield a pellet that was discarded and a clear supernatant (S1). An aliquot of 100µl of S1 was incubated for 1hr at 37^0C in the presence of the extract with and without pro-oxidants, iron (final concentration 10µM), and sodium nitroprusside (SNP) (final concentration 30µM). This was then used for lipid peroxidation determination. Production of thiobarbituric acid reactive species (TBARS) was determined as described by [11], except that the buffer of the color reaction has a pH of 3.4. The color reaction was developed by adding 200µl of 8.1%

sodium dodecyl sulphate (SDS) to S1 followed by sequential addition of 500µl of acetic acid(pH 3.4)and 500µl of 0.8% Thiobarbituric acid (TBA). This mixture was incubated at 100^0c for 30mins. The absorbance was measured at 532nm relative to that of the controls.

3. Results

Table 3.1: The flavonoid, total phenol content and ferric reducing property of the ethanolic extract of *Ficus exasperata*.

Antioxidant	Values(mg/g)
Total phenol	7.39 ± 0.38
Total flavonoid	7.44 ± 0.61
FRAP	1.17 ± 0.07

Results are means of three replicates of ethanolic extract of *Ficus exasperata* (mg/g) ± standard deviation.

Table 3. 2: Iron chelation property of *Ficus exasperata*.

Concentration (Mg/g)	Values
Reference	59.14 ± 0.00^{a}
5	36.34 ± 6.37^{b}
10	49.77 ± 2.59^{c}
15	45.53 ± 2.76^{d}
20	40.38 ± 4.36^{e}

Values represent Mean±SD of duplicate readings. Superscript with the same letter along the same column are not significantly different using T-test (P<0.05).

Table 3.3: DPPH radical scavenging ability of *Ficus exasperata.*

Concentration (mg/ml)	Standard (%)	*F. exasperate* (%)
Control	-	54.39 ± 3.40^{a}
0.313	$26.28\pm1.05^{a,c}$	36.81 ± 3.46^{b}
0.625	$30.32\pm5.0^{a,b}$	16.67 ± 8.27^{c}
1.250	31.68 ± 2.88^{b}	10.28 ± 0.22^{c}
2.500	$29.02\pm1.35^{a,b}$	10.98 ± 0.62^{c}
5.000	23.54 ± 0.40^{c}	11.79 ± 0.32^{c}

Values represent Mean ± SD of triplicate readings.Superscript with the same letter along the same column are not significantly different using T-test (P<0.05).

Table 3.4: ABTS radical scavenging ability of *Ficus exasperata.*

Concentration (mg/ml).	Standard (%)	*F. exasperata* (%)
Control	-	65.96 ± 2.96^{a}
0.313	0.56 ± 0.4^{a}	$61.40\pm1.29^{a,b}$
0.625	$1.61\pm0.4^{b,c}$	57.23 ± 2.37^{b}
1.250	1.89 ± 0.28^{c}	47.93 ± 3.59^{c}
2.500	$0.98\pm0.43^{a,b}$	21.12 ± 4.09^{d}
5.000	$1.58\pm0.53^{b,c}$	2.70 ± 0.53^{e}

Values represent Mean ± SD of triplicate readings.Superscript with the same letter along the same column are not significantly different using T-test (P<0.05).

Table 3.5: Inhibitory effect of extract on FE^{2+}- induced hepatic and cerebral lipid peroxidation.

Concentration(mg/ml)	Brain	Liver
Control Without Fe^{2+}	$87.10\pm29.41^{a,d}$	89.74 ± 28.47^{a}
Control With Fe^{2+}	198.70 ± 11.16^{b}	245.08 ± 10.26^{b}
0.313	161.37 ± 16.68^{c}	155.34 ± 10.99^{c}
0.625	148.93 ± 17.97^{c}	155.34 ± 18.73^{c}
1.250	138.00 ± 12.23^{c}	138.75 ± 22.06^{c}
2.500	108.21 ± 4.57^{d}	76.54 ± 16.79^{a}
5.000	62.58 ± 13.06^{a}	85.21 ± 1.31^{a}

Values represent Mean±SD of triplicate readings. Superscript with the same letter along the same column are not significantly different using T-test (P<0.05).

Table 3.6: Inhibitory effect of extract on H_20_2- induced hepatic and cerebral lipid peroxidation.

Concentration (mg/ml)	Brain	Liver
Control Without H_20_2	213.40 ± 27.58^{a}	$99.54\pm20.58^{a,b}$
Control With H_20_2	281.65 ± 35.97^{b}	101.80 ± 13.71^{a}
0.313	197.57 ± 28.65^{a}	82.19 ± 6.63^{b}
0.625	110.85 ± 13.34^{c}	49.02 ± 4.57^{c}
0.125	$86.72\pm9.62^{c,d}$	54.29 ± 2.99^{c}
2.500	$73.15\pm6.43^{c,d}$	38.84 ± 3.46^{c}
5.000	$64.47\pm12.23^{c,d}$	$27.52\pm1.31^{c,d}$

Values represent Mean±SD of duplicate readings. Superscript with the same letter along the same column are not significantly different using T-test (P<0.05).

Table 3.7: Inhibitory effect of extract on SNP- induced hepatic and cerebral lipid peroxidation.

Concentration(Mg/ml)	Brain	Liver
Control Without SNP	221.32±27.76[a]	89.36±18.69[a]
Control With SNP	279.39±49.45[b]	338.58±17.02[b]
0.313	210.01±18.60[a]	201.34±26.09[c]
0.625	90.49±7.84[c]	53.16±5.88[d]
0.125	61.83±13.11[c]	36.20±5.18[d]
2.500	55.80±3.27[c]	35.44±15.44[d]
5.000	54.29±0.00[c]	60.33±7.70[d]

Values represent Mean±SD of duplicate readings.Superscript with the same letter along the same column are not significantly different using T-test (P<0.05).

4. Discussion

Interest in medicinal plants has been revived in recent times because of their efficacy in providing cost effective therapy to several diseases due to the presence of secondary metabolites in their body parts. These compounds otherwise known as phytochemicals have been reported to exhibit anticancer, antimicrobial, antiplasmodic, anti-inflammatory, antiulcer, antidiabetic and antihypertensive properties [12]. Medicinal plants are rich in these polyphenols which make them versatile tool for the treatment of ailments in folkoric medicine. Many degenerative human diseases have been recognized as being a consequence of free radical damage, there have been many studies undertaken on how to delay or prevent the onset of these diseases. The most likely and practical way to fight against degenerative diseases is to improve body antioxidant status, which could be achieved by supplying the body with exogenous antioxidants. Medicinal plants and foods from plant origin usually contain natural antioxidants that can scavenge free radicals [13]. The leaves *Ficus exasperata* possess a good quantity of phenols and flavonoids as shown in Table 3.1. In fact, phenolics, such as flavonoids, have antioxidant capacities that are much stronger than those of vitamins C and E and have been found to possess antioxidant and free radical scavenging activity [14]. The use of iron chelation is a popular therapy for the management of Fe (II)-associated oxidative stress in brain. The ability of agents to chelate transition metals specifically iron (II) has been considered a defence mechanism of antioxidant agents. TABLE 3.2 showed that the ethanolic extract demonstrated a marked iron (II) chelating effect. This may be related to its high phenolic and flavonoid content since phenols and flavonoids have been reported to be a good chelator of iron [15]. The iron chelating ability of *Ficus exasperata* is also an indicator of the neuro-protective property of the plants as iron is involved in the pathogenesis of Alzheimer's disease and other diseases by multiple mechanisms. *Ficus exasperata* demonstrated marked radical scavenging activity against DPPH and ABTS (TABLE 3.3&3.4). In the present studies, it was observed that ethanolic extract of *F. exasperata* was able to significantly inhibit the formation of TBARS in the rat brain and liver homogenate subjected to iron (Table 3.5). Inhibition of lipid per-oxidation in brain homogenate is analogous to neuro-protection [16]. The brain is particularly vulnerable to oxidative damage because of its high oxygen utilization, its high content of oxidizable polyunsaturated fatty acids and the presence of redox-active metals. The result presented in Table 3.6 indicated that the extract exerted an antioxidant effect on *in vitro* SNP induction of lipid peroxidation in brain and liver homogenates, (P<0.05) at the highest concentration tested. Finally the results of this study demonstrated the high efficacy of the ethanolic extract of *Ficus exasperata* growing in Nigeria, in free radical scavenging, inhibition of reactive oxygen species and lipid per-oxidation which may be associated with their medicinal use in the treatment of diseases associated with oxidative stress in brain. *Ficus exasperata* can be considered as a source of plant antioxidants, with a potential use in food, cosmetics and pharmaceutical fields. However, more detailed studies are required to evaluate the neuro-protective ability of these plants.

5. Conclusion

The results of the present study showed that leaves extract of *Ficus exasperata* has antioxidant activity which could be attributed to its phenolic and flavonoid content. Hence, *Ficus exasperata* is a potential antioxidant candidate that could help in the management of degenerative diseases whose etiology has been linked to oxidative stress.

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