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Evaluation of the Antioxidant and Hepatoprotective Activities and Micronutrient Content of Diospyrospreussii seed Extracts.

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ABSTRACT
The antioxidant, hepatoprotective and the micronutrient in the seeds extracts of Diospyros*preussii* were evaluated in this study. Antioxidant related vitamins and mineral elements were determined by colorimetry and flame photometry. Hepatoprotective studies were done using carbon tetrachloride intoxicated rats; using levels of liver enzymes as biomarkers. Silymarin and vitamin E were the standards. The values of the micronutrients in the powdered seed were also determined. Phytocompounds in the extracts were a well determined and the acute toxicity test of the methanol extract was estimated in mice orally.The seed had a significant percentage of fats, protein, fiber, moisture, ash and carbohydrates. The vitamin composition of the methanol extract had vitamins E, C and β-carotene in abundance. The extracts were able to inhibit the affects of carbon tetrachloride on the livers of rats, as indicated by the levels of the biomakers. The approximate values of protein, carbohydrate, ash and fiber total lipids were 31.5%, while the methanol extracts yielded 20.0%. The mineral element composition in mg per 100 g of the powdered seed and methanol extract were determined. The phytochemical constituents were also determined.The antioxidant effect was determined spectrophotometrically. The extracts exhibited antioxidant and hepatoprotective activities, the effects were dose dependent and comparable with those of the standards.

KEYWORDS: *Diospyrospreussi,* antioxidant, hepatoprotection, methanol extract, mineral elements, vitamins

INTRODUCTION

Oxidative stress is related to pathogenesis of various important diseases, in which reactive oxygen species (ROS) and related agents such as superoxide, singlet oxygen, peroxynitrite, hydrogen peroxide, hydroxyl radical and alkoxyl radical play an important role [1-2]. The oxidation reactions *in vivo* by ROS are known to trigger oxidative stress. Superoxide is produced by one electron transfer in the mitochondrial electron transfer chain. Enzymes implicated in the production of superoxide include xanthine oxidase and cytochrome P450(s), with several enzymes like superoxide dismutase (SOD), glutathione peroxidase and catalase serving as protective antioxidants against oxidative stress [3].
Hydrogen peroxide is produced by a wide variety of enzymes including monooxygenases, and oxidases. These free radicals are generated in normal or pathological cell metabolism for fighting infection and elimination of other foreign matters. The production and regulation of the ROS in the body is monitored by the endogenous antioxidant.
Currently, the endogenous antioxidant may not regulate the production of ROS because of rapid increase in factors that increase the production of ROS, which include cigarette smoking, alcohol intake, microbial infections and intake of other xenobiotics [4]. ROS are very reactive and can easily lead to destruction of many cellular components, which results to some disorder like neurodegenerative disorder, cancer, arteriosclerosis, rheumatism, male reproductive tract disorder [5-8] as well as Alzheimer's disease, Parkinson's disease and aging [9-11]. Liver is the major centre for detoxication of chemicals in the body. In the process of detoxicating the toxin, liver damage may occur hence, hepatotoxicity. So many drugs and other chemicals have been found to induce hepatotoxicity [12]. Many agents including plant products are found to have antioxidant and

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hepatoprotective activities [13-15]. To compensate the deleterious effect of the oxidants and other effects in the body, the WHO recommends the use of natural antioxidants that can delay or inhibit the lipid or other molecule oxidation by inhibiting the initiation or propagation of oxidative chain, reactions [16].

*Diospyrospreusii*gruke (Ebenaceae) is dicotyledonous tropical and sub-tropical tree, with alternate leaves but has no stipulated. The fruit is a berry which is always surrounded at the base of the persistent and often enlarged calyx. It has large leaves which are glabrous and the veins form a very close raised network on both surfaces. It grows up to 30 feet high and 2 feet in girth, flowers between January – March and bears fruits between June and September. The fruit, leaf and stem bark of the plant are used for treatment of various communicable and incommunicable diseases such as chicken pox, and small pox. The leaf extract mixed with other plant extracts are used in management of many sexually transmitted diseases [17]. The seeds are eaten commonly among the natives during the season.

This study evaluated the antioxidant and hepatoprotective potentials of *Diospyrospreusii*seed extracts. The phytochemical constituents of the extracts were determined. Mineral element and antioxidant related vitamin compositions of the powdered seed and extracts were also determined. This is the first report so far on medicinal values of this plant.

MATERIALS AND METHODS

The reagent and solvents were sourced commercially

Chloroform, was obtained from Sigma-Adrichlaborchemikalien GmbH Riedel-dettaen Germany. Methanol and ethanol were obtained from SharlauChemie S.A European Union CE label, CE Etiquetta CE. Ferric chloride solution, ammonium solution, aluminum chloride and carbon tetrachloride were obtained from S.D fine chemical ltd; Boisar. Distilled water was obtained from Lion table water UNN Nsukka, Enugu State. Sulphuric acid was obtained from BDH chemicals Ltd Poole, England. Sodium hydroxide was obtained from schuchardt, 8011 HohenbrubrummbeiMunchen.

Thiobabituric acid was obtained from sigma chemical company, St.Louis, Mo, USA. SGOT and SGPT kits were obtained from Randox, U.K. Serum bilirubin kits were obtained from Boehringer Mannheim GmbH Diagnostica, Germany. Alkaline Phosphatase kits were obtained from QuimicaClinicaAplicada (QCA), Spain. All other chemical were obtained from local sources and were of analytical g

Plant material and extract preparation

The mature fruit of *Diospyrospreusii*was harvested in the month of September from Nsukka, Enugu State, Nigeria. The fruit was authenticated by A. Ozioko of the Centre for Ethnomedicine and Drugs Development, a subsidiary of Bioresources Development and Conservation Program (BCDP), Nsukka, Enugu State. The fruit was opened to obtain the seeds. The seeds were washed, dehusked and air-dried. Approximately 200 g of powdered seed was divided into two portions.Total lipids were extracted following a gravimetric method [18]. About 100 g of the powdered seed was mixed with 375 ml of chloroform: methanol (1:2), 125 ml of chloroform and 120 ml of water were added to the mixture, shaken vigorously and filtered. The filtrate was allowed to separate in a separating funnel. The lower layer was collected and concentrated *in vacuo* with a rotary evaporator to obtain a semi-solid extract (F1). Another quantity of the powdered seed (100 g) was macerated in absolute methanol for 24 hours at room temperature with occasional stirring. It was filtered and the filtrate concentrated in *vacuo* to obtain a dry extract (F2).

Animals

The animals used were Wistar albino rats (110-180 g) and albino (25 – 30 g) of either sex purchased from animal house of the Department of Pharmacology and Toxicology, University of Nigeria, Nsukka. The animals were fed with standard feed and water *ad libitum*. They were allowed to stay for 7 days in the laboratory before use to acclimatize.

Acute toxicity (LD_{50}) test

The median lethal dose of the methanol extract was determined orally in mice using standard method, outlined by Lorke [19]. Briefly, albino mice (25 – 30 g) of either sexes were divided into 3 groups of 3 animals per group. The extract dispersed in 1% sodium carboxymethyl cellulose was administered to the mice at doses of 10, 100 and 1000 mg/kg and the animals were monitored for 24 h for gross behaviour and mortality. From the result of the first phase, doses of 1500, 2500, 3500 and 5000 mg/kg were administered orally to 4 animals of 1 mouse per group. The animals were monitored for 24 h for mortality. The LD_{50} was calculated as the geometric mean of the maximum dose that caused 0% death and the minimum dose that caused 100% death.

Phytochemical tests.

Different classes of phytocompounds present in the seed powder, methanol extract and total lipids

extract were determined through standard procedures [20]. The classes tested for included alkaloids, flavonoids, glycosides, cardiac, cyanogenic and anthracene glycosides, proteins, carbohydrates, reducing sugars, saponins, steroidal aglycone, tannins, and acidic compounds. The solvents used for this work are of analytical grade.

2.5 Proximate analysis of the powdered seed

The parameters determined included total ash value, moisture and fiber contents. These were determined following official methods [21]. The total protein content was determined using the micro-Kjeldahl method [22], while total carbohydrate content was determined by differential method [23].

Determination of vitamins and mineral elements

Vitamin C was determined calorimetrically using 2, 6-dichlorophenol indophenol following a standard method [24]. β-Carotene and vitamin E were estimated according to the method of Paulo et al.,[25].

For the determination of mineral elements 1.0 g of the pulverized seed and methanol extract was digested by warming with 50 ml of a mixture of nitric acid and perchloric acid (5:1) in Kjeldahl flask. The content of the flask was heated until the mixture was nearly dried. The residue was extracted with diluted HCl and made up 10 ml with water. Standard solutions of the elements to be determined were prepared with pure salts of the elements. Other concentrations were obtained by serial dilution. Colorimetric methods using Dithizone, Phenathroline and Strontium Chloride solution were used in the determinations of zinc, iron and Calcium respectively following a Standard method (26).

Magnesium was determined through complexometric titration using standard solution of EDTA and masking agent, while Sodium and Potassium were determined by flame photometric method**.**

Antioxidant and hepatoprotective Screening

The lipid per-oxidation was determined by assessing the level of thiobarbituric acid reactive substances in the serum (27). A 0.4 ml of serum was mixed with 1.6 ml of 0.5 ml potassium chloride (KCl) buffer followed by addition of 0.5 ml of 2%trichloroacetic acid (TCA). This was followed by the addition of 0.5 ml of thiobarbituric acid (TBA) and the mixture was placed in water bath for 45 minutes at 90 ^{o} C. The blank was added the same volume of trichloroacetic acid, thiobarbituric acid and normal saline, 0.10 ml of distilled water was added instead of serum. The mixture was placed in a water bath for 45 minutes at 90^{o} C. After the incubation time, the mixture was allowed to cool and centrifuged at 3000 rpm for 10 minutes. The absorbance of the clear supernatant was measured against a reference blank of distilled water at 532 nm and 600 nm. The concentration was derived from the standard curve of lipid per-oxidation.

Glutamate – oxoloacetic transaminase (SGOT), glutamate-pyruvate transaminase (SGPT), alkaline phosphate, bilirubin in sera were determined (28). Briefly, 45 rats (which were given free access to drinking water *ad libitum*) were randomly divided into nine groups of five animals each. Group 1 was individually treated with 2 ml/kg of 1% sodium carboxymethylcellulose only. Groups II – IV received orally graded doses of F1 (200, 500 and 1000 mg / kg) respectively dispersed in 1% sodium carboxymethylcellulose.

Groups V – VII received orally graded doses of F2 (200, 500 and 1000 mg/kg) respectively. Group VIII received 1000 IU of vitamin E orally and group IX received 25 mg / kg dose of Silymarin orally. On the third and fourth day of treatment with extracts and standard drugs, the animals were given 0.75 ml/kg of carbon tetrachloride intraperitioneally. On the fifth day, the animals were sacrificed by anesthesia and dissected. 10 ml blood samples were collected from the animals through cardiac heat puncture with sterile syringes into properly labeled EDTA bottles. The blood samples were centrifuged and the sera were collect and use for the antioxidant and hepatoprotective screening.

Statistical analysis

The data obtained were analyzed statistically and reported as mean ± SEM for the number of animals in each group (n = 5). Data obtained from the vehicle (1% sodium carboxyl methylcellulose) were used as baseline values. The difference between the treated group data and that of control were subjected to student's *t-test* at 95% confidence interval ($P<0.05$).

RESULTS AND DISCUSSION

The total lipid and methanol extractions yielded 31.5 and 20.0%, respectively. These percentage yields are relatively considerable. In the approximate analyses, the powdered seed was found to contain the following (%): fat (31.50), protein (21.39), fibre (22.5), moisture (4.55), ash (1.81) and carbohydrate (18.26). The seed is rich in nutrient food components like proteins and fats that can help in cell repair and generation, while the fibre content aids digestion. In the preliminary acute toxicity test of the methanol extract, all the animals survived,

even at 5000 mg/kg, this suggests that the seed extract is relatively safe.

The antioxidant related vitamin compositions were in 10 g of the powdered seed as follows: vitamin E (7.0 mg), vitamin C (5.05 mg) and β-carotene (3.00 IU). The mineral elements compositions are listed in Table 1. The relative abundances of the phytocompounds in the seed powder and extracts are shown in Table 2. The liver protective effects of F1, F2 and the standard drugs are shown in Table 3.

Table 1: Mineral element composition in the seed powder and methanol extract

Mineral element	*Pulverized seed (mg/100 g)*	*Methanol extract (mg/100 g)*
Calcium	0.10	0.15
Magnesium	0.14	0.10
Sodium	1.00	1.25
Potassium	48.00	47.00
Zinc	0.40	0.31
Iron	1.32	1.24

Table 2: Classes of phytocompounds in the seed powder and extracts

Test	*Seed powder*	*(F1)*	*(F2)*
Alkaloids	+++	+	+
Flavonoids	+	+	+
Glycosides	+	+	+
Proteins	+	-	-
Carbohydrate	+	-	-
Saponins	+	+	+
Tannins	+	+	-
Reducing sugar	+	-	-
Steroidal aglcone	-	-	-
O-and C-glycosides	-	-	-

+ = present; - = absent.

Table 3: Levels of Antioxidant and Hepatoprotective parameters in Serum Liver Enzymes.

Treatment Group	*Dose (mg/kg, p.o.)*	*Total Bilirubin (mg/dl)*	*Direct Bilirubin (mg/dl)*	*SGDT (iμ/L)*	*SGPt (iμ/L)*	*Alkaline Phosphate (iμ/L)*
Control (1 % sodium carboxyl methylcellulose	2 ml/kg	0.44±0.03	0.14±0.03	132.5±0.67	31.5±0.82	54.0±1.01
F_1	200	0.59±0.02	1.25±0.55	197.5±0.95*	58.0±0.31	102.5±0.55
F_1	500	0.48±0.03	0.18±0.04	185.5±0.60	57.0±0.24	88.0±0.36
F_1	1000	0.37 ±0.03	0.12 ±0.08	158.0 ±0.98	50.0 ±1.12	83.0±0.29
F_2	200	0.76±0.01	0.43±0.62	159.0±0.15	52.0±0.09*	48.0±0.35
F_2	500	0.59±0.06	0.16±0.14	142.5±0.31	45.5±0.11	45.0±0.86
F_2	1000	0.44±0.04	0.75±0.41*	103.0±0.22	33.0±0.31	55.3±0.94
Vit. E	1000 IU	0.35±0.07	0.11±0.01	158.0±0.55	36.0±0.71*	45.3±1.03
Silymarin	25.0	0.34±0.08	0.10±0.02	138.3±0.93*	34.6±0.34	113.3±1.27

Values are mean ± SEM, n = 6. Statistically significant different in respect to negative control: P<0.05.

Table 4: Results of Antioxidant Screening

Groups	Agents	Treatment/doses	TBARS
Group i	Chloroform-methanol extract	1000 mg/kg	8.86 ± 2.38
Group ii		500 mg/kg	9.75 ± 0.83
Group iii		200 mg/kg	12.36 ± 1.63
Group i	Methanol extract	1000 mg/kg	10.75 ± 1.92
Group ii		500 mg/kg	8.25 ± 1.50
Group iii		200 mg/kg	7.00 ± 2.75
Group iv	CCl4	0.75 mg/kg	14.17 ± 2.81
Group v	Vitamin E	1000 IU	9.67 ± 1.06
Group vi	Normal	-	6.25 ± 0.00

CONCLUSION

The extracts have different classes of compounds which are known to complex or trap metal ion, thereby inhibiting chain of reactions capable of being induced by metal ions.

The methanol extract (F2) and the seed powder are rich in antioxidant vitamins, though the concentration in 100 g of the samples was more in the seed powder. In fact, many medicinal plants contain large amounts of antioxidants such as polyphenols. Many of these phytochemicals possess significant antioxidant capacities that are associated with lower occurrence and lower mortality rates of several human diseases (29, 30). The results strongly suggest the presents of polyphenolic compounds like flavonoids, glycosides and vitamins C, E and β-carotene as the important components of this plant, and some of its pharmacological effects could be attributed to the presence of these valuable constituents. The seed and F2 were moderately rich in mineral element composition, like magnesium and zinc which are useful in endogenous antioxidant enzyme production. The seed is rich in potassium which indicates diuretic property and also has a high concentration of iron suggesting that it may be helpful in management of anaemia.Zinc and some of the element affect both non-specific and specific immune function as they participate in major biochemical pathways involved in the perpetuation of genetic materials.

From the result, the extracts F1 and F2 protected the liver from the effect of carbon tetrachloride in a dose dependent manner. F1 in doses of 200 and 500 mg/kg had little protection. The hepatoprotective effect of the extract was more pronounced in the methanol extract (F2). The reason may be that the total lipid extraction procedure could not extract the antioxidant phenolic compounds, mostly hydrophobic in nature and are known free radical scavenge. The results of F2 were comparable to that of silymarin and much better than that of vitamin E. The methanol extract had significant antioxidant and hepatoprotective activities, which may be useful therapeutic agents for treating radical-related pathological damage. The result suggests that methanol extract of *Diospyrospreussii*seed has antioxidative activity table 4 and can protect the organs from peroxidation damage. This could be due to the presence of antioxidant related vitamins, mineral and phytochemical constituents present in the seed. Further studies are ongoing to narrow down these activities to their particular components.

Conflict of interest declaration

The authors declare no conflict of interest.

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