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Studies on *In Vitro* and *In Vivo* Antioxidant Evaluation and Total Phenolic, Flavonoidal ContentEstimation of *Beta vulgaris* root

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ABSTRACT

Beta vulgaris root extracts are traditionally used as a folk medicine to treatailments such ashypertension, hypoglycemic, inflammatory, and hepertocellular disorder. In this study the antioxidant potential of root extract of *B. vulgaris* were mentored invitroagainst 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and modulatory effect on rat brain enzymes.Total phenolic, flavonoid contents, and antioxidant potential against DPPH of the root extracts at varying concentrations were evaluatedusing ethyl acetate. Thus concentration of root extracts of *B. vulgaris* root at100, 200, and 400 mg/kg body weights were administered orally for (7)days to albino rats followed byevaluated of oxidative stress markers such as thiobarbituric acid reactive substances (TBARS), Superoxide Dismutase (SOD), Catalase (CAT), and Glutathione (GSH) in the animals brain homogenate.The percentage inhibition and IC50 value ofthe root extracts were significantly ($P < 0.01$) dose dependent compared to ascorbic acid standard. Similarly SOD, CAT, and GSHincreased significantly ($P < 0.01$) in test animals administered200 and 400 mg/kg of ethyl acetate extract of *B. vulgaris* root. The results obtained here suggest thatB. vulgaris roots extracts suggests a neuroprotective potential of this plant roots.

KEYWORDS

Beta vulgaris, Flavonoids, 2, 2-diphenyl-1-picrylhydrazyl, Oxidative stress, Antioxidant

INTRODUCTION

Oxidation is very essential to many living organisms for the production of energy to fuel biological processes and lead to production of both free radicals and Reaction Oxygen Species (ROS). These ROS and free radicals are byproduct of normal cellular metabolism of the body and are produced during irradiation (Ultraviolet [UV] light, X-rays, and γ-rays), inflammation, pollution, and mitochondria-catalyzed electron transport reactions [1]. ROS may contribute to oxidative damage resulting to loss of function, cellular death including various numbers of disorders such as coronary atherosclerosis, ischemia, ageing, diabetes, cancer, immunosuppression, and neurodegenerative disorders [2]. The interruption of the normal cellular function of lipids, proteins, carbohydrates, enzymes, and nucleic acids is due to an imbalance between pro-oxidants and antioxidants giving rise to oxidative stress [3]. However, the body can form defense against harmful effect of oxygen and nitrogen reactive species using exogenous and endogenous antioxidant enzymes such as Catalase (CAT), glutathione peroxidase, and Superoxide Dismutase (SOD); and Non-enzymatic Systems as Thiol Reduced (Glutathione [GSH]), vitamins, minerals, and polyphenols [4]. Antioxidants are vital substances that possess the ability to protect the body from damage caused by free radical induced oxidative stress.

Beta vulgaris (beet) is a plant which is included in Betoideae subfamily in the Amaranthaceous family. It is the economically most important crop of the large order Caryophyllales. It has several cultivar groups such as: the root vegetable known as the beetroot or garden beet; the leafy vegetables chard, spinach beet; and mangelwurzel, which is a fodder crop [16]. Three subspecies are typically recognized. All cultivars fall into the subspecies *B. vulgaris* subsp. *vulgaris*. The wild ancestor of the cultivated beets is the sea beet (*B. vulgaris* subsp. *maritima*). The claimed therapeutic use of beetroot includes its antitumor, carminative, emmenagogue, and hemostatic and renal protective properties and is a potential herb used in cardiovascular conditions[8]. The juice of beetroot is also consumed as a natural remedy for sexual weakness

and to expel kidney and bladder stones. In recent years, beetroot has gained popularity to be a natural food to boost the energy in athletes [8]. Recent reports indicate that *B. vulgaris* extracts (root) possess antihypertensive, hypoglycemic, antioxidant [9], anti-inflammatory, and hepatoprotective activities [6]. Previously, red beetroot extract has been demonstrated to be an effective multiorgan tumor suppressing agent in laboratory animals [7].

Recent studies have also postulated that renal inflammation, which is characterized by infiltration of inflammatory cells such as monocytes/macrophages and subsequent release of pro-inflammatory cytokines and activation of NF-κB(nuclear factor kappa-light-chain-enhancer of activated B cells) in response to oxidative stress, is involved in this process phe. This research is therefore aim atevaluate the *invitro* and *invivo* antioxidant and total Phenolic, Flavonoidal content estimation of *B. vulgaris* root.

MATERIALS AND METHODS

Plant Materials and Preparation of Extract

Fresh roots of *Beta vulgaris* (1 kg) was macerated and soaked in70% ethanol (1 litre)for three (3)days. The obtained root extract wasconcentrated using rotary evaporator before complete drying. The extract was then resuspended in distilled water(1L).

Experimental Animals

Thirty six (36) weaned male albino rats of the same stock were obtained from the animal farm of Imo StateUniversity Owerri. The animalswere takentothe laboratorywheretheywerehousedin a plasticcage,placed on commercial rat and allowed free access to water *ad libitum*. The animals were of the same sex, age and weighed about160 on average. The animals were subsequently allowed to acclimatize for two (2) weeks before commencement of experiment.

Acute Oral Toxicity Study

Acute toxicity study of *B. vulgaris* root extract was performed on the albino rats according Lorke'smethod [15].

Preliminary Phytochemical Screening

The preliminary phytochemical screening were carried out onof *B. vulgaris* root as described by[18].

Estimation of total phenolic content [19] and total flavonoid content [20]

The Total Phenolic Content (TPC) of extracts was measured using the Folin-Ciocalteu method[20].

In vitro antioxidant by 2, 2-diphenyl-1-picrylhydrazyl scavenging assay [21]

In vitro antioxidant by 2, 2-diphenyl-1-picrylhydrazyl scavenging activity assay was monitored using [21] method.

In vivo Antioxidant

In vivo antioxidant activity was determined by [38].

Lipid Peroxidation Assay (Thiobarbituric Acid Reactive Substances)[22]

Lipid Peroxidation Assaythiobarbituric acid reactive substances (TBARS)[22].

Superoxide Dismutase Assay[23]

This was estimated by the reaction mixture which contained 0.1 mL of phenazinemethosulfate (186 µL), 1.2 mL of sodium pyrophosphate buffer (0.052 mL; pH 7.0), 0.3 mL of the supernatant after centrifugation (1,500 × g for 10 min followed by 10,000 × g for 15 min) of homogenate was added to the reaction mixture. Enzyme reaction was initiated by adding 0.2 mL of NADH (780 µM) and stopped after 1 min by adding 1 mL of glacial acetic acid. The amount of chromogen formed was measured by recording color intensity at 560 nm. Results were expressed in units/mg protein.

Catalase Assay[24]

It was determined with reaction solution contained 2.5 mL of 0.05 M phosphate buffers (pH 8.3), 0.7 mL of 0.2 M H2O2 and 0.1 mL of tissue homogenate. Changes in absorbance of the reaction solution at 570 nm were determined after 1 min. Results were expressed in units/mg protein.

Reduced Glutathione Assay[25]

This was estimated by using dithiobisnitro-benzoate as a substrate. The yellow color developed and read immediately at an absorbance of 412 nm and expressed as µM GSH/g protein.

Statistical Analysis

The values were expressed in mean ± standard error of the mean. Statistical analysis was done by one-way ANOVA followed by Dunnett's multiple comparison test versus control. $P < 0.05$ and $P < 0.01$ were considered as significant.

RESULTS

Qualitative Phytochemical Screening

Phytochemical analysis of extracts of *B. vulgaris* root revealed the presence of alkaloids, glycosides, flavonoids, coumarins, lignins, tannins, terpenoids, carbohydrates, protein, fatty acids, and phenolic compounds.

Estimation of Total Phenolics and Flavonoids Contents

TPC of CEBVR, MEBVR, and EABVR was observed at 8.43 ± 1.03 mg, 11.59 ± 1.31 mg, and 15.64 ± 1.31 mg of GAE/100 g, respectively, whereas the TFC of CEBVR, MEBVR, and EABVR was observed at 0.75 ± 0.10 mg, 1.31 ± 0.01 mg, and 1.97 ± 0.47 mg of QUE/100 g, respectively, as shown in Figure 1. The results suggest that EAMP had a higher level of phenolic and flavonoidal contents as compared to other extracts of *B. vulgaris* extracts root.

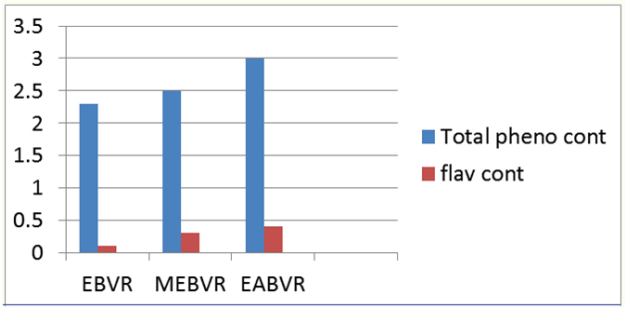

Figure 1: Comparative study of Total Phenolic content and Total Flavonoid content of various extracts of *Beta vulgaris* root

The values were expressed as mean ± standard error of mean; *P < 0.05, **P < 0.01 versus control. *ns*: Not significant

In Vitro Antioxidant Activity against 2, 2-Diphenyl-1-Picrylhydrazyl

The percentage of scavenging effect of DPPH on different extracts of Beta vulgaris rootwith a concentration of 1, 5, 10, 25, 50, and 100 µg/mL was compared with ascorbic acid as shown in Figure 2 and found dose-dependent inhibitory antioxidant potential against DPPH. Positive DPPH test suggests that the samples were free radical scavengers. The IC50 values of CEBVR, MEBVR, EABVR, and ascorbic acid in DPPH assay were 85.97, 67.85, 46.06, and 23.74 µg/mL, respectively, as shown in Figure 3. Among all these extracts, the ethyl acetate extract was found better DPPH scavenging activity with a minimum IC50 value of 46.06 µg/mL as compared to other extracts.

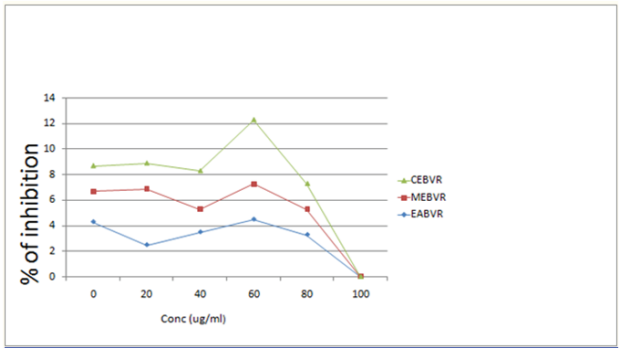

Figure 2: Effect of *Beta vulgaris* root extracts on percentage of inhibition against 2, 2-diphenyl-1-picrylhydrazyl.

The values were expressed as mean ± standard error of mean (n = 6). CEBVR, MEBVR, and EABVR were the extracts of chloroform, methanol, and ethyl acetate extract of Beta vulgaris extracts root respectively.

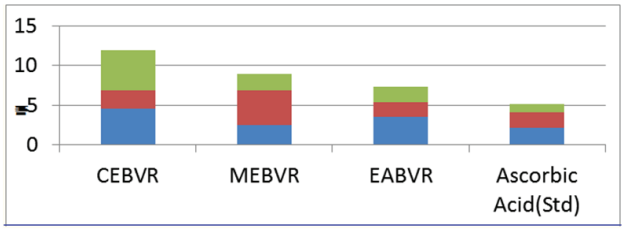

Figure 3: Estimation of IC_{50} value of various extracts of *B. vulgaris* root leaves against 2, 2-diphenyl-1-picrylhydrazyl.

The values were expressed as mean ± standard error of mean (*n* = 3). ***P* < 0.01 versus standard. CEBVR, MEBVR, and EABVR were the extracts of chloroform, methanol, and ethyl acetate extract of *B. vulgaris* root, respectively.

In Vivo Antioxidant Activity of Beta vulgaris Root Leaves on Rat

Effect of *B. vulgaris* root leaves on body weight and brain weight. No significant changes were observed in body weight of treated and control rats before, and after the administration of the ethyl acetate extract of *B. vulgaris* root for 7 days. Similarly, no considerable differences in the wet weight of the whole brain between control and EAMP treated rats as shown in Table 1.

The oxidative stressmarkers as lipid peroxidation (TBARS), SOD, CAT, GSH in rat brain homogenate was evaluated. EABVR at 200 and 400 mg/kg showed significantly ($P < 0.01$) and dose-dependently increased the level of antioxidant enzymes such as SOD, CAT, and GSH in brain tissue as compared to control rats as shown in Figure 3. However, EAMP (100 mg/kg) was produced no significant changes in antioxidant enzymes in rat brain tissue. In addition, EABVR at 400 mg/kg dose was significantly ($P < 0.01$) reduced the lipid peroxidation that is TBARS level to 4.85 ± 0.76 as compared to control rats of 2.51 + 0.45nM/min/mg protein. No significant change in TBBVR level was observed for EABVR (100 and 200 mg/kg) treated rat.

DISCUSSION

Natural antioxidants that are ubiquitous in fruits, vegetables, and medicinal plants have received great attention in recent times since they are effective and lesser toxic than synthetic antioxidants [26]. Polyphenols and flavonoids act as an antioxidant agent by the property of hydrogen atom donators, singlet oxygen scavengers, and free radical scavenger [27,28]. Again, flavonoids have been reported as inhibitors of lipid peroxidation[29] and prevent oxidative damage [30]. The present investigation observed that the TPCs of crude extracts of *B. vulgaris* root varied from 8.43 ± 1.03 to 15.64 ± 1.31 mg GAE/g, whereas the TFCs varied from 0.75 ± 0.10 to 1.97 ± 0.47 mg QUE/g. All the extracts of *B. vulgaris* root have a significant amount of phenolic and flavonoid components. However, the EABVR extract possessed the higher amount of phenolic and flavonoid content as compared to other extracts. They can be ranked as EABVR > MEBVR > CEBVR. The presence of flavonoids and phenolic components in ethyl acetate extract of B. vulgaris root substantiate the claim for its free radical scavenging activity and further attenuate the progression of oxidative stress induced diseases.

Antioxidants may offer resistance against the oxidative stress by scavenging the free radicals, inhibiting the lipid peroxidation, and by many other mechanisms and thus prevent diseases. Antioxidant activity is expressed in terms of IC50, and a lower IC50 value corresponds to a larger scavenging activity [31]. DPPH is a compound consists of a nitrogen free radical, which is easily quenched by a free radical scavenger. DPPH radicals are reduced into a non-radical form (DPPH-H) in the presence of a proton radical scavenger or hydrogen donating antioxidants [32]. In fact, DPPH radical has an absorbance at 517 nm, which disappears after acceptance of an electron or hydrogen radical from an antioxidant compound to become a stable diamagnetic molecule[33]. The effect of free radical scavenging activity of *B. vulgaris* root extracts on DPPH radicals is thought to be due to their hydrogen donation ability of polyphenols. In the present study, the extracts showed a dose dependent elevation in DPPH scavenging activity of which EABVR showed lower IC50 than others. A similar study revealed that the aqueous extract of *B. vulgaris*

Table 1: Effect of EABVR on Body Weight and Brain Weight in Rats

Group	Treatment	DOSE mg/kg	Before Treatment	After Treatment	Wet Brain Weight (g)
I	Control	D.W	178.1 ± 3.1	181.1 ± 1.0*	1.76 ± 0.03
II	EABVR	100	180.2 ± 2.4*	188.2 ± 1.0 *	177 ± 0.02
III	EABVR	200	181.1 ± 3.6*	186 ± 1.8*	1.71 ± 0.02*
IV	EABVR	400	182.1 ± 3.1*	189.6 ± 0.7	1.61 ± 0.03

All value were expressed as mean + SEM (n = 6), where *P<0.05 versus control. EABVR : Ethyl acetate extract of B.V; SEM: Standard Error of Mean. DW: Distilled water

Table 2: Effect of *Beta vulgaris* Root on Oxidative Markers in Rat Brain

Treatment	Control	100 ($mgml^{-1}$)	200 ($mgml^{-1}$)	300 ($mgml^{-1}$)	400 ($mgml^{-1}$)
TBARS mol/min/mg protein	2.51 + 0.45	4.85 ± 0.76	4.81 ± 1.04	4.46 ± 1.11	4.16 ± 1.2
SOD U/mg protein	12.3 + 21	15.1 ± 4.5	23.7 ± 9.6[a, b]	12.1 ± 2.9	12.3 ± 0.98
CAT (U/mm)	1.89 ± 0.55[a]	2.54 ± 0.77	1.67 ± 0.87	1.2 ± 0.89	2.05 ± 0.98
GSH (Umol/g tissue)	2.61 ± 0.92**	15.4 ± 1.6	15.6 ± 2.5	14.3 ± 2.2	15.4 ± 1.6

root showed a significant IC50 value (136 ± 0.003) but lower than ascorbic acid [34].

Lipid peroxidation is a complex process occurring in aerobic cells which reflects the interaction between molecular oxygen and polyunsaturated fatty acids. The radicals which are responsible for lipid peroxidation, that also causes food deterioration, ageing of organisms, and cancer promotion. These free radicals associated with other relative species cause the oxidation of biomolecules (e.g., Protein, amino acids, lipid, and DNA), which leads to cell injury and death [3]. Lipid peroxidation in biological systems has long been thought to be a toxicological phenomenon that can lead to various pathological consequences [35]. The end products of lipid peroxidation are reactive aldehydes, such as 4-hydroxyl non-enal and Malondialdehyde(MDA), many of which are highly toxic to cells [36]. In addition, the MDA can react with biomolecules and exert cytotoxic, genotoxic, and neurodegenerative disorders. However, the endogenous antioxidants such as SOD and CAT are susceptible to oxidative changes. The enzyme SOD removes the superoxide anion, while CAT,[37] a heme protein catalyses the reduction of H_2O_2 [38] and protects the tissues from highly reactive hydroxyl radicals that could be generated from H_2O_2. Indeed, GSH provides the first line of defense for the body by scavenging ROS. Oxidative stress readily oxidizes GSH non-enzymatically to glutathione disulfide by electrophilic substances such as free radicals and ROS[39] and causes depletion of GSH level by inhibiting glutamate-cystineantiporter[40]. Our results showed that inhibition of MDA formation increases with increase in dose of EAMP that is a reduction in TBARS level. In addition, there is a significant increase of antioxidant enzymes such as SOD and CAT and non-enzymatic GSH level in rat brain homogenate, which is due to the linkage between phenolics and antioxidant enzymatic and non-enzymatic activity [40,41] Moreover, literature reveals that *B. vulgaris* rootshowed the presence of mimosine, terpenoids, flavonoids, glycosides, alkaloids, quinines, phenols, tannins, saponins, coumarins, d-xylose and d-glucuronic acid 4-O-(3,5-dihydroxybenzoic acid)-β-D-glucoronide,[11] might have contributed to the antioxidant activity. Several studies were demonstrated by *in vitro* and *in situ* models that, certain flavonoids e.g., quercetin and catechins are capable of penetrate through the blood-brain barrier. Earlier reports found the presence of phenolics, flavonoids, and tannins were found to possess the antioxidant property and attenuate cell death induced by oxidative stress, which supported our present findings [40-48].

In summary, the present study revealed that the EABVR showed the highest level of total phenolic, as well as flavonoid compounds, and were capable of initiating, quenching free radicals to terminate the radical chain reaction, and acting as reducing agents. Moreover, a significant and linear relationship was found between the antioxidant activity and phenolic content, indicating that phenolic compounds could be major contributors to antioxidant activity. In addition, it has demonstrated that EABVR had a greater potential of the inhibitory effect on cell lipid peroxidation and improved the antioxidant enzymes in rat brain homogenate. The antioxidant and biological activities might be due to the synergistic action of bioactive compounds present in them. The observation of this current investigation strongly suggests the potential antioxidant activity of EABVR leaves potentiates the neuroprotective effect by stimulating brain enzymes. Hence, it may be used as a weapon in various neuroinflammatory and neurodegenerative diseases. Furthermore, studies are warranted for the isolation and the identification of individual phenolic compounds; and also in vivo studies are needed for understanding their mechanism of action as an antioxidant better.

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