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Antioxidant and Free Radical Scavenging Activity of Various Extracts of *Boerhavia diffusa* Linn. (Nictaginaceae)

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Abstract The main objective of this work is to make the phytochemical screening to evaluate the phenolic composition and anti-radical activity of various extracts of *Boerhaavia diffusa* Linn. that will explain his therapeutic effects. It will characterize the chemical groups, to identify the best solvent extraction of total polyphenols, flavonoids and condensed tannins by testing and modifying the extraction solvents and evaluate *in vitro* antioxidant activity of n-Hexane, dichloromethane, ethyl acetate, methanol and ethanol extracts from *Boerhaavia diffusa* trapping method according to the free radical DPPH. *Boerhavia diffusa* Linn. a plant of the Beninese pharmacopoeia used in traditional medicine in Benin. In this work n-Hexane, dichloromethane, ethyl acetate, methanol and ethanol extracts were prepared from the stem leaves of *Boerhavia diffusa* Linn. The quantitative estimation of total phenols, tannins and flavonoids by the colorimetric method showed that the extracts are rich in these compounds. Evaluation of antioxidant power was performed using the method of DPPH free radical trapping.

Keywords *Boerhavia diffusa* Linn; Free radical scavenging activity; DPPH; total phenols; flavonoids; tannins

Introduction

Boerhavia diffusa Linn. (Nictaginaceae) has been widely studied for its chemical constituentsand therapeutic activities. The roots are the source of a novel class of isoflavonoids known as rotenoids, flavonoids, flavonoid glycosides, xanthones, purine nucleoside, lignans, ecdysteroids, and steroids. Various animal studies and trials have confirmed the presence of activities, for example, immunomodulation, hepatoprotection, antifibrinolysis, anticancer activity, antidiabetic activity, anti-inflammation, and diuresis. In this paper, traditional uses, chemical constituents,

and reported pharmacological activities have been summarized to present the chemical and therapeutic potential of this plant.

This study focuses on *Boerhavia diffusa* Linn. (Nictaginaceae), a plant of the Beninese pharmacopoeia and is part of the search and recovery of biologically active substances such as natural substances with biological activity of interest in the field of biopharmaceuticals.

In this paper we report the antioxidative potential of *Boerhavia diffusa* Linn. from Calavi, department of Atlantic (Benin) by measuring the 2, 2- diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, total phenolic content, flavonoids and and condensed tannins in the extracts. It is reported to possess Diuretic, anti-inflammatory, antifibrinolytic, anticonvulsant, antibacterial, antihepatotoxic, antidiabetic.

There is our days, an increasing interest in the measurement and use of plant antioxidants for scientific research as well as industrial (dietary, pharmaceutical and cosmetic) purposes. There are many cellular biochemical pathways and environmental toxins which produce reactive oxygen species (ROS) [1] and contribute to the development of diseases such as cancer, cardiovascular disorders, diabetes, cataracts and many neurodegenerative diseases [2].

Ordinarily, the levels of free radicals in living organisms are controlled by a complex set of antioxidant defenses, which minimize oxidative damage to important biomolecules, but in Oxidative stress circumstances, the endogenous antioxidants are not enough to deal with the increased levels of ROS [3, 4]. In contrast, the accumulation of excessive ROS, mainly due to external influences such as radiation, ultraviolet light, cigarette smoke, pathogens, drugs, etc., can inflict damage upon cellular macromolecules such as DNA, proteins and lipids [5]. This concept is supported by increasing evidence indicating that oxidative damage plays a role in the development of many chronic diseases. Further, oxidative stress may be associated with nearly 200 diseases, such as cardiovascular diseases, cancer, atherosclerosis, hypertension, ischemia, diabetes mellitus, neurodegenerative diseases (Alzheimer's and Parkinson's), rheumatoid arthritis, and aging, but it should not be considered the primary cause of these diseases [6]. Thus, in order for the level of excessive ROS to be reduced, and so the oxidative damage can be suppressed, the need for additional intake of exogenous antioxidants can be suggested [7]. Antioxidants are substances that when present in low concentrations, compared to those of an oxidisable substrate significantly delay or prevent oxidation of that substance [8].

Many studies have confirmed that plants and foods rich in polyphenolic content are effective scavengers of free radicals, thus helping in the prevention of these diseases through their antioxidant activity [9]. Antioxidants which are present in plants, herbs and dietary sources help in preventing vascular diseases in diabetic patients [10]. Tannins and flavonoids are the secondary metabolites in plants considered to be the natural source of antioxidants which prevent destruction of β-cells and diabetes-induced ROS formation [11]. Thus, it is a good strategy to manage diabetes as a whole with plants which show good enzyme inhibitory and antioxidant activities [12].

Natural antioxidants or phytochemical antioxidants are secondary metabolites of plants which produce a very impressive array of antioxidant compounds that includes carotenoids, flavonoids, cinnamic acids, benzoic acids, folic acid, ascorbic acid, tocopherols, tocotrienols, *etc*. [9] to prevent oxidation of the susceptible substrate. Natural products especially from plants sources have the ability to reduce oxidative stress by acting as antioxidants [13].

Materials and Methods

Plant material

The stem leaves of *Boerhavia diffusa* Linn. were used in this study.Fresh stem leaves of *Boerhaavia diffusa* were collected from Calavi, Department of Atlantic, South Bénin. The samples of *Boerhaavia diffusa* were submitted in Abomey-Calavi University Herbarium, Department of Botany and voucher specimen deposited for authentification under the reference AA 6716/HNB. The collected material was dried for two weeks in laboratory (22°C) and ground to a fine powder using an electric grinder (Excella mixer grinder).

Chemicals

2,2-Diphenyl-2-picrylhydrazyl (DPPH), potassium hexacyanoferrate [$K_3Fe(CN)_6$], trichloroacetic acid, gallic acid, ascorbic acid, quercetin, and $FeCl_3$ were purchased from Sigma Chemical; Folin-Ciocalteu phenol reagent,

anhydrous sodium carbonate (Na_2CO_3), aluminium chloride, potassium acetate and solvent methanol were obtained from Merck Chemical Supplies (Darmstadt, Germany). All the chemicals used, were of analytical grade.

Extraction Procedure

Two hundred and fifty grams (250 g) of finely ground plant material were successively extracted by maceration with hexane, dichloromethane, ethyl acetate, ethanol and methanol for 72 h stirring. A second extraction of fifty grams (50 g) of dry powder was carried out with a mixture of ethanol-water (80:20). Each extraction is repeated three times. The macerates were filtered and concentrated using a rotary evaporator (BUCHI Rotavapor RII, Switzerland) at 40-50 °C. The obtained extracts were stored at 4°C until biological assay.

Quantification of Some Bioactive Molecules

Total Phenolics Content

Total phenolics of each extract were estimated by Folinciocalteu reagent method [14]. This method is based on the reduction in alkaline media of phosphotungstic mixture (WO42-) phosphomolybdic (MoO42-) of Folin reagent by the oxidizable group of phenolic compounds, leading to the formation of blue reduction products. Latter have a maximum absorption at 765 nm whose intensity is proportional to the amount of polyphenols present in the sample. Then, 200 µl of diluted sample were added to 1 ml of 1:10 diluted Folin–Ciocalteu reagent. After 4 min, 800 µl of saturated sodium carbonate (75 g/l) was added. After 2 h of incubation at room temperature, the absorbance at 765 nm was measured. The standard calibration curve was plotted using gallic acid ($y = 0{,}043x - 0{,}051$; $R^2 = 0{,}994$). The mean of three readings was used and the results expressed as mg of Gallic Acid Equivalents (GAE)/100 mg of extract.

Total Flavonoid Content

The determination of flavonoids was performed according to the colorimetric assay described previously [15]. To 1 ml of extract (100 µg mL^{-1}), 3 ml of methanol, 0.2 ml of 1 M potassium acetate, 0.2 ml of 10% aluminium chloride and 5.6 ml of distilled water was added and left at room temperature for 30 minutes. Absorbance of the mixture was read at 415 nm using UV spectrophotometer. Quercetin was used as reference compound to produce the standard curve ($y = 0{,}325x - 0{,}363$; $R^2 = 0{,}995$) and the results were expressed as mg of quercetin equivalent (QE)/100 mg of extract.

Condensed Tannins

Condensed tannins are determined by the method of vanillin in acidic medium described by Ba *et al*. (2010) [16]. Vanillin reagent was prepared by mixing equal volume: 8%, methanol at 37% and 4% of vanillin in methanol. The mixture was maintained at 30 °C before the assay. Two hundred (200) µl of each extract to be analyzed were added to 1000 µl of reagent of vanillin; the mixture was stirred and incubated in darkness at 30 °C for 20 min. The absorbance was measured at 500 nm by a spectrophotometer UV (Perkin Elmer) against white consisting of a mixture of methanol (37%) and HCl (8%) with equal volume.

DPPH Radical Scavenging Activity

The free radical scavenging capacity of the extracts was determined using DPPH [17].

In the presence of antioxidant which is typical for DPPH free radical decays, the change in absorbency at 517 nm is followed spectrophotometrically. The antioxidant activity was determined according to the method previously described [18]. All tests were performed in triplicate. Radical scavenging activity of extracts is expressed as radical scavenging percentage (RSP) and was calculated using the following equation [19]

$$RSP\ (\%) = (\%) = [(A_B - A_A)/ A_B] \times 100,$$

Were A_B is the absorbance of the blank (t = 0min) and A_A is the absorbance of the tested extract solution (t = 15min).

Statistical analysis

Data were presented as mean ± standard deviation (SD) of three determinations. Statistical analyses were performed using a one-way analysis of variance. The IC_{50} values were calculated by linear regression analysis. The difference was considered statistically significant when the $p < 0.05$.

Results

Performance of Extraction

The yields of n-Hexane (Hex.), dichloromethane (Di-Me.), ethyl acetate (Et-Ac.), methanol (MetOH.) and ethanol (EtOH.) extracts of *Boerhaavia diffusa* Linn. Ethanol and methanol extracts gave the highest yields of extraction respectively (Fig. 1). Ethanol and methanol favored the extraction of metabolites in stem leaves of *Boerhaavia diffusa* Linn.

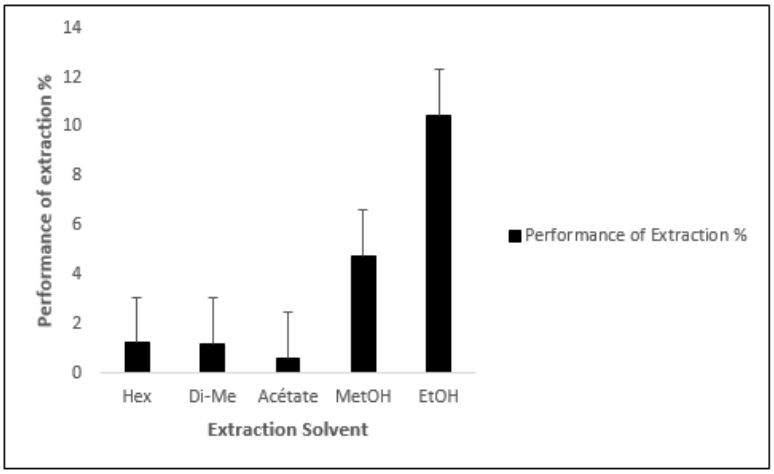

Figure 1: Yields of different extracts of stem leaves of Boerhavia diffusa Linn.

Hex: n-Hexane; Di-Me: dichloromethane; Et-Ac: ethyl acetate; MetOH: methanol; EtOH: ethanol. Values are mean ± SE (n =3).

Quantification of Some Bioactive Molecules Family

Total Phenolics Content

The amount of total phenolics measured by Folin-Ciocalteu method dependent on solvents of extraction and ranged from 4.807 to 8.766 mg GAE (Fig. 2). The highest content of total phenolics was detected in methanol extract with 8.766 mg GAE followed respectively by methanol (7.440 mg GAE), dichloromethane (6.575 mg GAE) and ethyl acetate (5.912 mg GAE). The lowest total phenolics were obtained in n- Hexane (4.807 mg GAE).

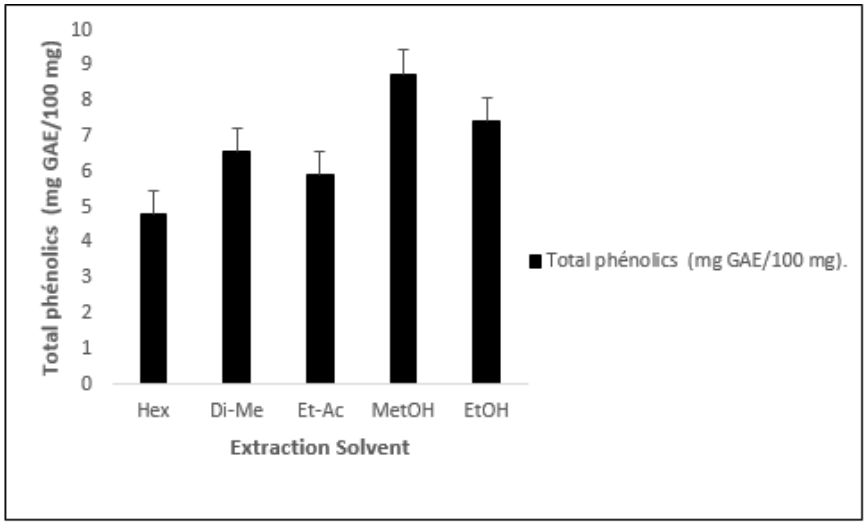

Figure 2: Content of total phénolics in different extracts of stem leaves of Boerhavia diffusa Linn.

Hex: n-Hexane; Di-Me: dichloromethane; Et-Ac: ethyl acetate; MetOH: methanol; EtOH: ethanol. Values are mean ± SE (n =3).

Total Flavonoid Content

The total flavonoid content among the various extracts was expressed in term of quercetin equivalent using respectively the standard curves equations ($y = 0.325x - 0.363$; $R^2 = 0.995$). The total flavonoid content in various extracts from *Boerhaavia diffusa* showed different results ranging from 4.751 to 6.243 mg QE/100 mg. Dichloromethane extract had the highest total flavonoid content (6.243 mg QE/100 mg) and ethanolic extract the lowest (4.751 mg QE/100 mg) (Fig. 3)

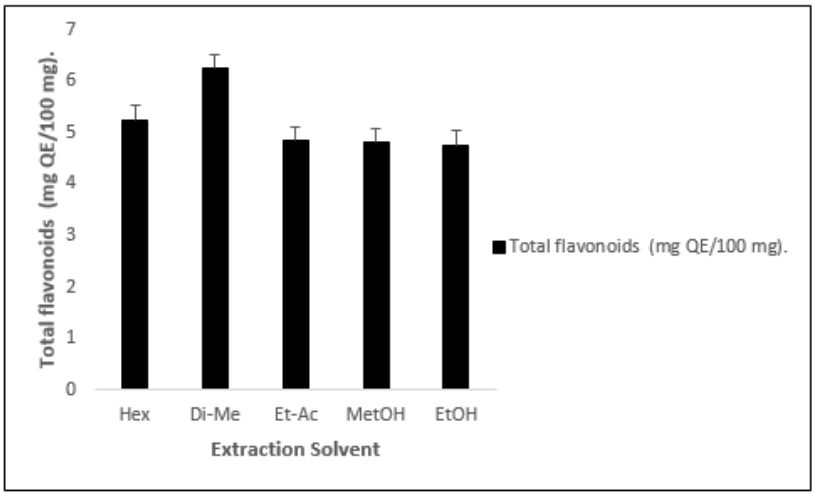

Figure 3: Total flavonoids content in different extracts of stem leaves of Boerhavia diffusa Linn.

Hex: n-Hexane; Di-Me: dichloromethane; Et-Ac: ethyl acetate; MetOH: methanol; EtOH: ethanol. Values are mean ± SE (n =3).

Condensed Tannin Content

All tests were carried out in triplicate. A standard calibration curve was plotted using catechins (Y=0,181X-0,073; R^2=0,942). The results were expressed as the condensed tannin content is expressed in equivalent mg of catechins (mgEC/100 mg).The estimation of Condensed tannins contents in the different extracts was showed Fig. 4.

Similarly, the highest content of Condensed tannins was obtained in dichloromethane extract (6.022 mg EC/100 mg) while the lowest (5.046 mg EC/100 mg) was given by methanol extract.

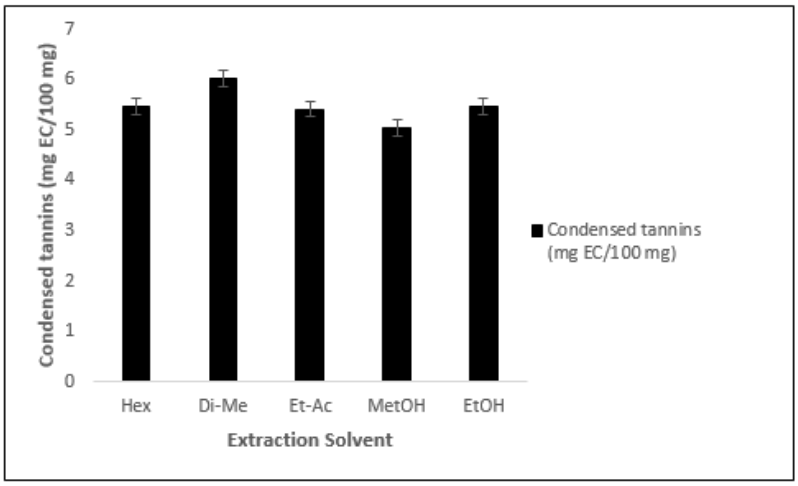

Figure 4: Condensed tannins content in different extracts of stem leaves of Boerhaavia diffusa Linn.

Hex: n-Hexane; Di-Me: dichloromethane; Et-Ac: ethyl acetate; MetOH: methanol; EtOH: ethanol. Values are mean ± SE (n =3).

Antioxidant activity DPPH Radical Scavenging Activity

The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity of *Boerhaavia diffusa* Linn. is given in Fig. 5. The reduction of DPPH radical by antioxidants is evaluated by the decrease in absorbance at 517 nm. The decrease in absorbance of DPPH radical caused by antioxidants is due to the reaction between antioxidant molecules and radical progress which results in the scavenging of the radical by hydrogen donation [20]. It is visually noticeable as a change in colour from purple to yellow. Hence, DPPH is usually used as a substance to evaluate the antioxidant potential of medicinal plants [21]. In this study, the DPPH radical scavenging activities of extracts therefore increased gradually in a dose concentration dependent manner (0.0078-1mg/ml).

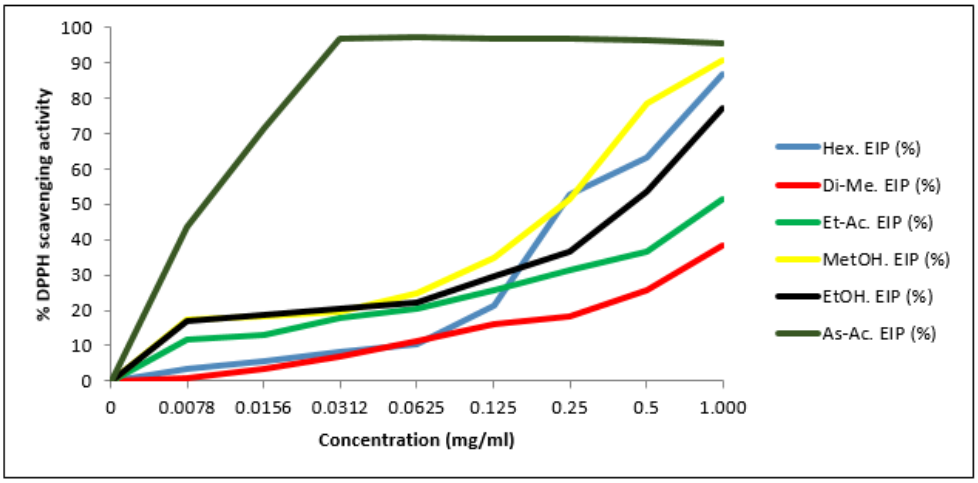

Figure 5: Radical scavenging activity of different extracts of stem leaves of Boerhaavia diffusa Linn

Hex: n-Hexane; Di-Me: dichloromethane; Et-Ac: ethyl acetate; MetOH: methanol; EtOH: ethanol. Values are mean ± SE (n =3).

A variation in antioxidant activities (Concentration inhibiting 50% of reaction) ranging from 0.238 to 0.950 mg/ml (Fig. 6).The results show that all extracts except dichloromethane (Di-Me.) showed significant activity (0.238≤ IC50≤0.950). IC_{50} = Concentration inhibiting 50% of reaction. The n-Hexane (Hex.) extract of *Boerhaavia diffusa* (IC50 = 0.238 mg/ml, IC_{50} = Concentration inhibiting 50% of reaction) manifest the greatest anti-radical capacity compared to extract methanol (MetOH.) (IC_{50} = 0.240 mg/ml), the ethanolic (EtOH.) extract (IC_{50} = 0.446mg/ml) and ethyl acetate (Et-Ac.) (0.950). It has been shown that the reference antioxidant ascorbic acid was IC_{50} = 0,010 mg/ml.

Discussion

Ethanol and methanol gave the highest yields in stem leaves of *Boerhavia diffusa* Linn. It allowed us to obtain a yield of 10.480 and 4.748 after extraction using 100g of *Boerhavia diffusa* Linn. powder. Phytochemical analysis confirmed the presence of important phenolic compounds such as tannins and flavonoids which were known to be natural bioactives substances and antioxydant properties.

Medicinal plants used in folk medicine are particularly interesting for investigation of their antioxidant effects. Some authors reported that the therapeutic benefit of medicinal plants is usually attributed to their antioxidant properties and oxidative stress is a prominent feature of these diseases [22].

The antioxidant n-Hexane, dichloromethane, ethyl acetate, methanol and ethanol extracts of *Boerhaavia diffusa* Linn and standard antioxidant (ascorbic acid) towards the DPPH radical was evaluated spectrophotometrically by following the reduction of this radical which is accompanied by itspassage of the purple colour (DPPH•) to the colour yellow (DPPH-H) measurable to 517nm (Fig. 6). This capacity reduction is determined by a decrease in absorbance induced anti-radical substances.

DPPH is a stable radical commonly used to determine the antioxidant activity of various compound. It is a stable free radical because of its spare electron delocalization over the whole molecule. This method is based on the

reduction of DPPH in the presence of a hydrogen-donating antioxidant, inducing a color change from purple to yellow at 517 nm. The degree of reduction in absorbance measurement indicates the radical scavenging (antioxidant) power of the extract. In the current study, the results revealed that at the same concentration, the inhibitory percentage of DPPH radical was not the same. At each concentration, n-Hexane, methanol and ethanol extracts gave the highest percentage inhibition followed by dichloromethane and ethyl acetate extracts.

These results show that there is a correlation between antioxidant activities of extract and extraction solvents. Similar observations have been reported in previous studies [23-24]. The solubility of the antioxidant compounds was found to have a significant effect on the recovery of compounds during extraction. Thus, the polarity of solvents has an indirect function in the extraction process, because it can raise the solubility of antioxidant compounds [25]. All extracts showed different percentages of inhibition of the DPPH scavenging activity on the concentration-dependent approach. Several studies have shown that the scavenging effects on the DPPH radical increases sharply with the increasing concentration of the samples and standards to a certain extent [26-27] and hence are said to be strongly dependent on the extract concentration. The results obtained in this study indicate that n-Hexane, methanol and ethanol extracts of *Boerhaavia diffusa* Linn. have a remarkable potency to donate electron to reactive free radicals, converting them into more stable non-reactive species, reduce the oxidized intermediates and act as primary antioxidant substances.

From the results presented above, it is evident that the extracts contained phenolic compounds at different levels in the following order: methanol >ethanol>dichloromethane > ethyl acetate > n-hexane. It has been reported that the solvents such as alcohols (methanol and ethanol), acetone, ethyl acetate, have been used for the extraction of phenolic compounds from plant materials [28].

The flavonoids content is expressed in mg QE/100 mg. Among all tested samples, extract with dichloromethane showed the highest value of flavonoids and condensed tannins content. In addition, the extracts with Hexane, methanol and ethanol also showed strong radical scavenging activities. It is reported that phenolics compounds and flavonoids are natural products which have been shown to possess various biological properties related to antioxidant mechanisms [29]. Polyphenols have the function to scavenge the free radicals in human body and to help maintain healthy body by scavenging or removing the reactive oxygen species (ROS) [8].

The strong inhibition of DPPH radical displayed by the extracts could be linked to polyphenolic compounds which are capable of donating electrons or transferring hydrogen atom to neutralize free radicals. Thus, it could be a promising therapeutic agent to treat stress induced by pathological conditions.

The antioxidant activities of *Boerhaavia diffusa* Linn. extracts may also be related to their total flavonoid content. The flavonoid content of the extracts was in the following order: dichloromethane > n-hexane >ethyl acetate> methanol >ethanol while the Condensed tannins content was in the order of: dichloromethane > n-hexane >ethyl acetate >ethanol>methanol.

Several studies have reported the biological activity of flavonoids [30-31]. But the best-described property of almost every group of flavonoids is their capacity to act as antioxidants. As antioxidants, flavonoids have been reported to be able to interfere with the biochemical pathways involved in the generation of reactive oxygen species (ROS), quenching free radicals, chelating transition metals and rendering them redox inactive in the Fenton reaction [32,33]. Therefore, the strong inhibition of DPPH radical displayed by the dichloromethane extract of *Boerhaavia diffusa* Linn. could be related to its flavonoids content that are able to donate electrons or transfer hydrogen to neutralize free radicals. It has been shown that the antioxidant molecules such as Ascorbic acid, flavonoids and tannins reduce and discoloured DPPH due to their ability to yield hydrogen, [34]. Whatever the nature of the radical-scavenging power of our plant extracts, it is to see that there is a correlation between the polyphenolics compounds and the antioxidant activities of the extracts were complex. Several reasons could be the different extraction solvent resulted in the differences of the extracts in their compositions, and consequently their antioxidant activities [35, 36]. The antioxidant methods used were based on different mechanisms and conditions. Mechanism of DPPH that was electron transfer method. So they may present differing results, each only partially reflecting the antioxidant activity [37-38]. The Folin Ciocalteu Reagent method to measure the polyphenolics content could be disrupted by other

soluble components in extracts such as proteins, peptides, polysaccharides, and pigments. It has been also shown that these compounds may be responsible for the antioxidant activity partly [39].

We conclude that, the results presented indicate that *Boerhaavia diffusa* Linn. extract attenuated oxidative stress via its antioxidant properties. The antioxidant profile of this plant can be harnessed to treat radicals related to pathological conditions. It has been also shown that the scavenging effects on the DPPH radical increased with the increasing concentration of the samples to a certain extent and hence are said to be strongly dependent on the extract concentration. The antioxidant activity exhibited by the extracts of *Boerhaavia diffusa* Linn. could justify the ethnotherapeutic usage of this plant by the traditional healers. However, further investigations on phenols, flavonoids, condensed tannins active principle, their *in vivo* antioxidant activity, and the different antioxidant mechanism are warranted.

DPPH• + antioxidant ® DPPH-H + antioxidant•

Purple color Yellow color

Figure 6: DPPH radical was evaluated spectrophotometrically by following the reduction of this radical which is accompanied by its passage of the purple colour (DPPH•) to the colour yellow (DPPH-H) measurable to 517nm.

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Competing Interest

All of the authors have nothing to declare as far as the conflict of interest is concerned.

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