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Antioxidant activity and total phenolic, flavonoid and flavonol contents of the bark extracts of *Acacia ataxacantha*

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Abstract
Acacia ataxacantha is a medicinal plant used in south Benin for various diseases. The current investigation was conducted to estimate the Total Polyphenols Compounds (TPC), flavonoids and flavonols contents and to evaluate antioxidant capacities in different extracts from *A. ataxacantha* barks. Total phenolic content was determined by using the Folin-Ciocalteu method while total flavonoid and flavonol were estimated using standard procedures. The antioxidant capacities in the forms of DPPH (2,2-diphenyl-1-picrylhydrazyl) and FRAP (Ferric Reducing Antioxidant Power) were evaluated by spectrophotometric methods. The results showed that TPC, flavonoids and flavonol values were higher in ethyl acetate extract: 74.18 mg GAE/100mg of Dry Plant, 26.65 mg QE/100mg DP and 23.14 mg QE/100mg DP. The inhibition percentage of DPPH radical scavenging activity ranged from 0.66 to 92.62%. Ethyl acetate extract had the highest FRAP capacity (1273.63 µmol AAE g^{-1}). Hence, *A. ataxacantha* represents a source of potential antioxidants that could be used in pharmaceutical and food preparations.

Keywords: *Acacia ataxacantha*, antioxidant, total phenolic, flavonoids, flavonol.

Introduction
Electron acceptors, such as molecular oxygen react rapidly with free radicals to become radicals themselves, also referred to as Reactive Oxygen Species (ROS). These ROS include free radicals with unpaired electrons, such as superoxide anions (O^{2-}), the hydroxyl radicals ($\cdot OH^{-}$) and non-radical, hydrogen peroxide (H_2O_2) [1]. ROS are essential cellular components, enzymatically generated in aerobic living organisms, which play an important role in different physiological and pathological processes. Particularly, at low levels, ROS take part in signal transduction, gene transcription and regulation of soluble guanylate cyclase activity [2, 3]. 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 [4, 5]. 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 [6]. 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 [7]. 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 [8]. Antioxidants are substances that when present in low concentrations, compared to those of an oxidisable substrate significantly delay or prevent oxidation of that substance [9]. Antioxidants have been shown to be effective in the treatment of various health problems, including neurodegenerative, systemic and infectious diseases [10].
Apart from their role as health benefactors, antioxidants are also added to food to prevent or delay its oxidation, normally initiated by free radicals formed during the food's exposure to environmental factors such as air, light and temperature [11]. Several synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert-butylhydroquinone (TBHQ) are commercially available and are currently in use. Unfortunately, new data indicating that their use is now restricted due to their side effects [12],

which involves an intense search for new, natural and efficient antioxidants. Plants are a potential source of natural antioxidants. 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*. [13] to prevent oxidation of the susceptible substrate. Natural products especially from plants sources have the ability to reduce oxidative stress by acting as antioxidants [14].

Fabaceae (also known as Leguminosae) is a cosmopolitan family, represented by 730 genera including *Stylosanthes*, *Tamarindus*, *Caesalpinia*, *Acacia* and over 19400 species [15]. The genus *Acacia* includes about 1200 species and the highest density and the greatest diversity is found in tropical and subtropical regions, but also found throughout the world [16]. Many uses of *Acacia* species are mentioned in the traditional pharmacopoeia of numerous countries [17-21]. One of these species is *Acacia ataxacantha*.

Acacia ataxacantha is a spiny plant in the Fabaceae family. All parts of this plant have medicinal properties and have been used as folk medicine in many regions of Africa. Used over a period of 3 to 5 days, *A. ataxacantha* allows treating headaches, pneumonia and bleeding [22]. Pods and seeds of this plant are used to treat dysentery [23]. The dough of the leaves is used in local application in dealing with abscesses while the decoction of these leaves is used per os in hyperthermic convulsions [24]. The sap of the stem bark fight against chickenpox [25]. The whole plant is used in the treatment of excessive coughing and yellow fever [26]. Earlier studies on this plant reported the antidiabetic activity [27]. In recent years, reports have revealed that several species of *Acacia* are rich in antioxidants [28-30]. In this paper we report the antioxidative potential of barks of *Acacia ataxacantha* from Ouidah, department of Atlantic (Benin) by measuring the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, Ferric Reducing Antioxidant Power (FRAP), total phenolic content, flavonoids and flavonols in the extracts.

Materials and Methods 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.

Plant material

Fresh bark samples of *A. ataxacantha* were collected from Ouidah, Department of Atlantic, South Bénin. The samples of *A. ataxacantha* were submitted in Abomey-Calavi University Herbarium, Department of Botany and voucher specimen deposited for authentification under the reference AA 6509/ 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).

Extraction procedure

Two hundred and fifty grams (250 g) of finely ground plant material were successively extracted by maceration with hexane, dichloromethane, ethyl acetate 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.

In vitro antioxidant potential DPPH Radical-Scavenging Activity

The ability of the extract scavenge the 2,2-diphenyl-1-picrylhydrazyl radical was evaluated. 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 [31]. Briefly, 1.5 ml of a freshly prepared methanolic solution of DPPH (2%) was mixed with 0.75 ml of extract solution (1 – 0.007 mg/ml). After 15 min of incubation in the dark, at room temperature, absorbencies were read at 517 nm against a blank sample consisting of a 1.5 ml of methanol and 0.75 ml of extract solution. All tests were performed in triplicate. DPPH radical inhibition percentage was calculated according to the following formula: inhibition (%) = [(AB – As)/ AB] x 100 were as is the sample (tested extract solution) absorbance and AB is the blank absorbance.

Ferric-Reducing antioxidant power (FRAP) assay

The reducing power was based on iron (III) to iron (II) transformation in the presence of the extracts. The iron (II) can be monitored by measuring the formation of Perl's blue at 700 nm. Reducing ability was performed using the procedure method described by Saeed *et al*. [32] with some little modifications. Briefly, 2 ml of extracts (100 µg/ml) were mixed with 2 ml of phosphate buffer (0.2 M, pH 6.6) and 2 ml of potassium ferricyanide (10 mg/ml). The mixture was incubated at 50°C for 20 min followed by addition of 2 ml of trichloroacetic acid (100 mg/l). The mixture was centrifuged at 3000 rpm for 10 min to collect the upper layer of the solution. A volume of 2 ml from each of the mixture earlier mentioned was mixed with 2 ml of distilled water and 0.4 ml of 0.1% (w/v) fresh ferric chloride. After 10 min reaction, the absorbances were read at 700 nm. Ascorbic acid was used to produce the calibration curve ($y = 0.028x-0.024$; $R^2 = 0.995$). The iron (III) reducing activity determination was performed in triplicate and expressed in µMol Ascorbic Acid Equivalent (AAE)/g of extract.

Estimation of Total Phenolic, Total Flavonoid and Total Flavonol Contents Total phenolic content

Total phenolics of each extract were estimated by Folin-ciocalteu reagent method [33]. This method is based on the reduction in alkaline media of phosphotungstic mixture ($WO4^{2-}$) phosphomolybdic ($MoO4^{2-}$) 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 [34]. 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.

Total flavonol content

The total flavonol content was determined by the colorimetric method using aluminum chloride [35]. Aliquots were prepared by mixing of 0.75 mL ethanolic extract solutions (0.1 mg/mL) and 0.75 mL aqueous $AlCl_3$ (20% w/v). The aborptions were read at 425 nm after 10 min of incubation against a blank (mixture of 0.75 mL ethanolic extract solutions and 0.75 mL ethanol) on a UV/visible light spectrophotometer. All tests were carried out in triplicate. A standard calibration curve was plotted using Quercetin (y = 0,211x – 0,193; R^2 = 0,996). The results were expressed as mg of Quercetin Equivalents (QE)/100 mg of extract.

Statistical analysis

Data were presented as mean ± SD. The graphical representation of the data was performed using the Graph Pad Prism 5.0 software (Microsoft, USA). The difference was considered statistically significant when the $p < 0.05$.

Results *In vitro* antioxidant activity DPPH Radical Scavenging Activity

DPPH is stable free radical that accepts an electron or hydrogen to become a stable diamagnetic molecule. Antioxidant on interaction with DPPH, transfer electron or hydrogen atom to DPPH and thus neutralizing its free radical character and convert it to 1 - 1 diphenyl- 2 - picryl hydrazine and the degree of discoloration indicates the scavenging activity of the drug [36]. 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 [37]. 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 [38]. In this study, the DPPH radical scavenging activities of extracts therefore increased gradually in a dose concentration dependent manner (7.81-1000 µg/ml). A variation in antioxidant activities ranging from 0.66 to 92.62% (Fig.1). The results show that from 250 µg/ml to 1000 µg/ml, all extracts except hexane showed significant activity (74.56≤IP%≤92.62) in comparison with the quercetin (88.86≤IP%≤94.08). At 125 µg/ml, three (03) extracts out of six (06) showed a percent inhibition greater than 55%.

At 62.5 µg/ml, the dichloromethane (IP% = 65.09) and ethyl acetate (IP% = 54.11) extracts showed considerable activity compared with the control (quercetin, IP% = 80.03). From 7.81 to 31.25 µg/ml, the extracts showed an inhibition percentage (IP%) less than 50%.

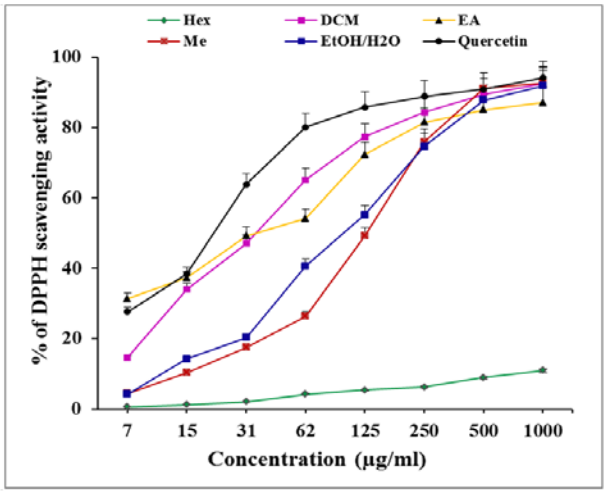

Fig 1: Radical scavenging activity of *Acacia ataxacantha* extracts. Hex: n-Hexane; DCM: dichloromethane; EA: ethyl acetate; Me: methanol; EtOH/Eau: hydroalcoholic. Each value represents a mean ± SE (n = 3).

Reducing power of *A. ataxacantha*

The reducing capacity of the extracts is another significant indicator of antioxidant activity. The presence of antioxidants in the sample would result in the reduction of Fe^{3+} to Fe^{2+} by donating an electron which is an important mechanism of phenolic antioxidant action [39]. In this assay, the compounds with reduction potential react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanides (Fe^{2+}), which then react with ferric chloride to form ferric ferrous complex that is greenish in colour [40]. In the current study, Ferric Reducing Antioxidant Power (FRAP) of the extracts varied from 120.33 to 1273.63 µmol AAE g^{-1} (Fig. 2). The strongest ferric reducing ability was found in ethyl acetate extract (1273.63 µmol AAE g^{-1}) followed by methanol (849.08 µmol AAE g^{-1}) and hydroalcoholic (816.73 µmol AAE g^{-1}). The lowest activities were obtained with dichloromethane (489.44 µmol AAE g^{-1}) and n-Hexane (120.33 µmol AAE g^{-1}).

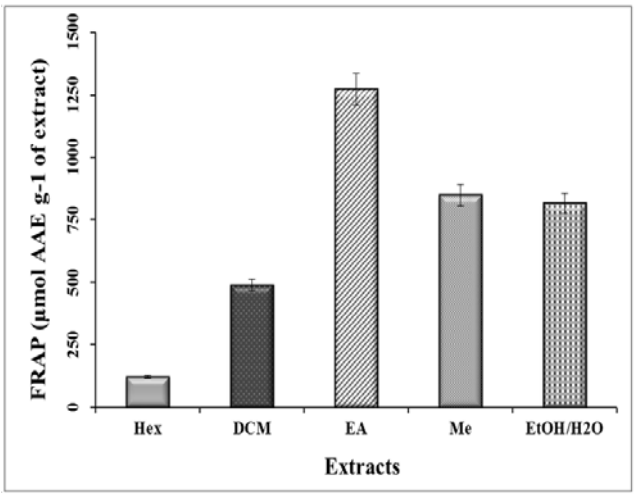

Fig 2: Antioxydant activity obtained using the FRAP method on *Acacia ataxacantha*. Hex: n-Hexane; DCM: dichloromethane; EA: ethyl acetate; Me: methanol; EtOH/Eau: hydroalcoholic. Each value represents a mean ± SE (n = 3).

Total phenolic, flavonoid and flavonol contents Total phenolic contents

The amount of total phenolics measured by Folin-Ciocalteu method dependent on solvents of extraction and ranged from 18.06 to 74.18 mg GAE (Table 1). The highest content of total phenolics was detected in ethyl acetate extract with 74.18 mg GAE followed respectively by methanol (36.51 mg GAE), hydroalcoholic (33.48 mg GAE) and dichloromethane (28.14 mg GAE). The lowest total phenolics were obtained in n-Hexane (18.06 mg GAE).

Total flavonoid and flavonol contents

The estimation of total flavonoids and total flavonol contents in the differents extracts was showed in table 1.The total flavonoid and flavonol content among the various extracts was expressed in term of quercetin equivalent using respectively the standard curves equations ($y = 0{,}325x - 0{,}363$; $R^2 = 0{,}995$) and ($y = 0{,}211x - 0{,}193$; $R^2 = 0{,}996$). The total flavonoid content in various extracts from *A. ataxacantha* showed different results ranging from 12.4 to 26.65 mg QE/100 mg. Ethyl acetate extract had the highest total flavonoid content (26.65 mg QE/100 mg) and hexane extract the lowest (12.4 mg QE/100 mg). Similarly, the highest content of total flavonol was obtained in ethyl acetate extract (23.14 mg QE/100 mg) while the lowest (9.90 mg QE/100 mg) was given by Hexane extract.

Table 1: Total phenolics, flavonoids and flavonols content in *Acacia ataxacantha* extracts

Extracts	Total phenolics (mg GAE/100 mg)	Total flavonoids (mg QE/100 mg)	Flavonols (mg QE/100 mg)
Hex	18.06±0.58	12.14±0.06	9.90±0.14
DCM	28.14±0.61	20.31±0.32	15.07±0.08
EA	74.18±0.40	26.65±0.68	23.14±0.28
Me	36.51±0.93	24.63±0.36	22.40±0.14
Hy/Et	33.48±0.61	15.51±0.02	15.46±0.44

Hex: n-Hexane; DCM: dichloromethane; EA: ethyl acetate; Me: methanol; Hy/Et: hydroalcoholic. Values are mean ± SE (n = 3)

Discussion

Since ancient times, many plants have been used for the treatment and prevention of many ailments and diseases and have shown a tremendous resource for the development of new drugs. 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 [41]. Several previous studies [42-44] have reported the antioxidant capacity of some *Acacia* species. However, there were no studies regarding antioxidant potential of different polarities extracts from *Acacia ataxacantha.* Several methods were used to determine the antioxidant activity of plants. Thus, our study involved two different methods to assess the antioxidant activity of *Acacia ataxacantha* (bark), namely, DPPH scavenging activity and ferric reducing/antioxidant power (FRAP) analysis. Among the most widely used procedures for measurement of antioxidant activity capacity, the DPPH radical scavenging analysis is one of the best known, accurate, and frequently employed to measure the electron donating ability of the plant [45, 46]. 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, ethyl acetate and dichloromethane extracts gave the highest percentage inhibition followed by methanolic and hydroalcoholic extracts. These results show that there is a coorélation between antioxidant activities of extract and extraction solvents. Similar observations have been reported in previous studies [47-49]. 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 [50]. 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 [51-53] and hence are said to be strongly dependent on the extract concentration.

Reducing power of Fe^{3+} to Fe^{2+} is another test to measure the antioxidant potential of the plant extract. The reducing capacity is a significant reflection of the antioxidant activity in assessing potential antioxidants [54]. The reducing power of extracts decreased in the order of ethyl acetate >methanol > hydroalcoholic > dichloromethane > n-hexane. In FRAP assay, the presence of antioxidants in the samples would result in reducing Fe3+ to Fe2+ by donating an electron which cause subsequent changing of reaction solution from yellow to green color. The degree of color change is proportional to the power and to concentration of antioxidant in sample. Thus, the Fe^{2+} can be monitored by measurement of the formation of Perl's Prussian blue at 700nm. It is suggested that there is a direct correlation between antioxidant activity and reducing power of components of some plants [54]. The results obtained in this study indicate that ethyl acetate, methanol and hydroalcoholic extracts of *A. ataxacantha* 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: ethyl acetate > methanol > hydroalcoholic > dichloromethane > 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 [55]. Among all tested samples, extract with ethyl acetate showed the highest value of total phenolic compounds, flavonoids and flavonols content. In addition, the extracts with dichloromethane, methanol and hydroalcoholic also showed strong radical scavenging activities. The phenolic compounds present in these extracts could justify their marked antioxidant 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 [56]. 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) [57]. 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 ataxacantha Acacia extracts may also be related to their total flavonoid content. The flavonoid content of the extracts was in the following order: ethyl acetate > methanol > dichloromethane > hydroalcoholic > n-hexane while the flavonol content was in the order of: ethyl acetate > methanol > hydroalcoholic > dichloromethane > n-hexane.

Several studies have reported the biological activity of flavonoids [58-60]. 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 [61-63]. Therefore, the strong inhibition of DPPH radical displayed by the dichloromethane extract of *A. ataxacantha* could be related to its flavonoids content that are able to donate electrons or transfer hydrogen to neutralize free radicals.

The relationship between the polyphenolics compounds and the antioxidant activities of the extracts were complex. Several reasons could be provided for this observation: (i) the different extraction solvent resulted in the differences of the extracts in their compositions, and consequently their antioxidant activities [64, 65]; (ii) the antioxidant methods used were based on different mechanisms and conditions. Mechanism of DPPH that was electron transfer method and FRAP was redox essays. So they may present differing results, each only partially reflecting the antioxidant activity [66-69]; (iii) 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 [70].

Conclusion

This study revealed that the barks of *A. ataxacantha* contain appreciable amounts of polyphenolic compounds that are capable of eliciting potent antioxidant activities. 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 *A. ataxacantha* barks could justify the ethnotherapeutic usage of this plant by the traditional healers. The results from the present study indicate that it would be highly economical for the production of potential antioxidant supplement(s).

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