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EVALUATION OF ANTIOXIDANT ACTIVITY AND PROTECTIVE EFFECT OF *CASSIA AREREH* (CAESALPINIACEAE) EXTRACTS AGAINST FREE RADICAL-INDUCED OXIDATIVE HAEMOLYSIS

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ABSTRACT

In this study, the cellular and non cellular antioxidant and free radical scavenging properties of the methanol and ethyl acetate extract of *Cassia arereh* leaves were evaluated. The antioxidant and free radical scavenging activity of the plant extracts and the standard (ascorbic acid and gallic acid) were assessed through DPPH radical, hydroxyl radical, nitric oxide scavenging activity and reducing power at various concentrations ranging from 1.62 to 200 µg/ml. For cellular antioxidant assay, we evaluated the haemolysis and the haematoprotective effect of *Cassia arereh* against hydrogen peroxide-mediated cytotoxicity of red blood cells (RBCs) induced with $CuSO_4/H_2O_2$. Total polyphenol and flavonoid content of extracts were also determined using colorimetric methods. DPPH assay showed IC_{50} values of 4.27 ± 0.9 and 3.21 ± 0.55 µg/ml respectively for methanol and ethyl acetate extract, instead of 12.5 ± 2.99 µg/ml for standard. Methanol extract (IC_{50} = 41.597 ± 7.46 µg/ml) had a significantly higher reducing power than the ethyl acetate one (IC_{50} = 238.89 ± 26.56 µg/ml). The reducing power of the methanol extract was found to be not significantly different from standards. Our results showed a non significant decrease in the nitric oxide radical production due to the extracts compared to ascorbic acid or gallic acid. It was also found that, the methanol extract (IC_{50} = 17.06 ± 0.82 µg/ml) was significantly more effective than that of ethyl acetate (IC_{50} = 76.34 ± 3.4 µg/ml) as hydroxyl radical scavenging activity. Both extracts induced haemolysis of sheep RBCs in a dose dependant-manner. Incubation of RBCs with extracts before exposing them to $CuSO_4/H_2O_2$ reduced the hydrogen peroxide-mediated cytotoxicity of RBCs respectively by 30.71 and 34.75 %, for methanol and ethyl acetate extracts after one hour. The antihaemolytic activity decreased with time for all the tested products. These results demonstrate the antioxidant properties of *Cassia arereh* and reveal that methanol is the best extractant for antioxidant agents of this plant.

Keywords: antioxidant activity, free radical scavenging activity, cytotoxicity, *Cassia arereh*.

INTRODUCTION

A growing amount of evidence indicates a role of reactive oxygen species (ROS) such as peroxyl radicals ($ROO^•$), hydroxyl radical ($HO^•$), superoxide anion ($O2^•$) and singlet oxygen ($^1O_2$) in the pathophysiology of aging and different degenerative diseases such as cancer, cardiovascular diseases, Alzheimer's disease and Parkinson's disease [1-2]. Living cells possess a protective system of antioxidants which prevents excessive formation and enables the inactivation of ROS. The antioxidants protect from the potentially damaging oxidative stress, which is a result of an imbalance between the

formation of ROS and the body antioxidant defense. Antioxidants have also been used in food industry to prevent deterioration, nutritional losses and off-flavoring in various foods, especially those containing polyunsaturated fatty acids. Recently, interest has increased considerably in finding naturally occurring antioxidants for use in foods because of their potency in health promotion and disease prevention, and their high safety and consumer acceptability [3].

In search of novel sources of antioxidants in the last years, medicinal plants have been extensively studied for their antioxidant activity. There are many reports in the literature about the antioxidant properties of medicinal plants [4-7], but generally, there is still a demand to find more information concerning the antioxidant potential of plant species. Polyphenols are the most significant compounds for the antioxidant properties of plant raw materials. The antioxidant activity of polyphenols is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers, metal chelators and reductants of ferryl hemoglobin [8-10].

Cassia arereh is a small tree which belongs to the family Caesalpiniaceae. It is found in northern Nigeria, Cameroon, Ethiopia and Eritrea. It is used by traditional medicine practitioner in the management of several ailments including parasitic infections, diarrhoea, dysentery, malaria, dermatitis, and skin infections [11-12]. It has been shown to possess anti-trypanosomiasis [13] as well as antimicrobial properties [14].

In view of the fact that there are no published information regarding the use of *C. arereh* as an antioxidant agent, this study is designed to evaluate whether *C. arereh* have some active principles that could be used for chemotherapeutic purposes. It has been recognized that the extraction solvent may significantly alter the antioxidant activity estimation [15]. Thus, the current study was to investigate the antioxidant and antiradical activities of *Cassia arereh* on a model of free radical-induced membrane damage and the influence of the extraction agent on the extractability of polyphenol components. The results from this study will lead to a better characterization of the antioxidant properties and will reveal the possible mechanisms of the antioxidant activity of the medicinal plant investigated.

MATERIALS AND METHODS

Chemicals: Folin-Ciocalteu reagent, gallic acid, rutin, ascorbic acid and 2,2-Diphenyl-2-picryl-hydrazyl (DPPH) were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Hydrogen peroxyde (H_2O_2 , 30 % v/v, 8.9 M) and copper sulfate pentahydrate ($CuSO_4$,$5H_2O$) were purchased from Merck (Darmstadt, Germany). All other chemicals and solvents used were of analytical grade and were purchased from Merck.

Plant Materials: The leaves of *Cassia arereh* (Caesalpiniaceae) were collected in the north region of Cameroon in September 2013. The botanical identification of the plant samples was carried out by a botanist and the voucher specimens (N° 39931/HNC.) are conserved at the National Herbarium Yaoundé. The plant material was then air dried at room temperature and ground into a fine powder.

Red blood cells suspension preparation: Blood was obtained by veinpuncture from healthy sheep, and collected into EDTA-containing tubes. Red blood cells (RBCs) were then separated from plasma by centrifugation at 3000 g for 15 min and washed three times with 5 volumes of phosphate buffer saline (PBS; 100mM pH 7.4) to obtain a packet cell preparation. The packed RBCs were finally suspended in PBS so as to produce an RBC suspension of hematocrit 10 % or 10^{8} cells/ml.

Preparation of extracts: Plant extracts were prepared by soaking the dried powdered plant (500 g) in polyester plastic bottle with 2500 ml of the appropriate solvent (methanol or ethyl acetate) while shaking vigorously for 3 to 5 min after every 2 hours and kept for 72 hours. The extract was then filtered and concentrated in a rotary vacuum evaporator. The extract was further concentrated by allowing it to stand overnight in an oven at 30°C. The dried material was stored at -20°C until use. The yield of each extract was determined.

Preliminary phytochemical screening of extract: A portion of each extract that was subjected for the biological screening was used for the identification of the major secondary metabolites employing the protocols described by Matos [16]. Briefly, the extract (1 mg/ml) was submitted to the following identification reactions: The characterization for tannins was performed with gelatin reactions. Triterpenoids and sterols were investigated by Liebermann-Burchard reagent and the alkaloids analysis was done by precipitation reactions with the reagents of Dragendorff and Mayer. For the search for flavonoids, the reactions using some magnesium chips and concentrated hydrochloric acid drop wise were employed and the presence of saponins was determined by the formation of foam. The presence of polyphenols and phenols was

determined by the formation of blue and green precipitates using $FeCl_3$ and $K_3Fe(CN)_6$ drop wise.

Estimation of total phenolic content: Total phenolic content was measured using Folin–Ciocalteu colorimetric method described previously by Ebrahimzadeh et al. [17]. 0.5 ml of the extract samples (1 mg/ml) were mixed with 2.5 ml of 0.2 N Folin-Ciocalteau reagent for 5 min and 2.0 ml of 75 g/l sodium carbonate were then added. The absorbance of the reaction was measured at 760 nm with a spectrophotometer (Thermo scientific BioMates™ 3S) after 2 h of incubation at room temperature. The standard curve was prepared using 25 to 500 µg/ml solutions of gallic acid in methanol-water (1:1, v/v). All determinations were carried out in triplicate. Total phenol values were expressed in terms of gallic acid equivalent (GE) mg/g of dry mass sample, which is a common reference phenolic compound.

Estimation of total flavonoid content: Flavonoid content of each extract was determined following colorimetric method [18]. Briefly, 0.5 ml of each plant extracts (1 mg/ml) were separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate, and 2.8 ml of distilled water, and left at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm with a spectrophotometer (Thermo scientific BioMates™ 3S). The standard curve was prepared using 25 to 500 µg/ml solutions of rutin in methanol. All determinations were carried out in triplicate. Total flavonoid content was expressed in terms of rutin equivalent (RE) mg/g of dry mass sample.

Determination of antioxidant activity of using chemical assays

DPPH radical Scavenging assay: The free radical-scavenging capacity of the extracts was determined using DPPH [19]. Various concentrations of plant extracts (1.62 to 200 µg/ml) were added to 1ml of the 0.004% methanol solution of DPPH, and the mixture was vortexed vigorously. The tubes were then incubated at room temperature for 30 minutes in dark, and the absorbance was taken at 517 nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. Ascorbic acid was taken as known free-radical scavengers. All tests were performed in triplicate. The percentage inhibition activity was calculated using the following formula, % Inhibition = $[1 - (A_1) / A_0] \times 100$, Where A_0 was the absorbance of the control (without extract) and A_1 was the absorbance of the extract or standard. IC_{50} (inhibitory concentration), which was defined as the concentration (µg/ml) of sample required to inhibit the formation of DPPH radicals by 50%, was determined.

Reducing power assay: The reducing power ability of the extract was determined using the method of Adesegun et al. [20] by mixing 2.5 ml of extracts solution of various concentrations (1.62 to 200 µg/ml) , with 2.5 ml of phosphate buffer (0.2 M, pH 6.6), 2.5 ml potassium ferricyanide (1%) and incubated at 50°C for 30 min. Trichloroacetic acid (2.5 ml, 10 %) was added and centrifuged (2000 rpm for 10 min). The supernatant (2.5 ml) was mixed with equal volumes of distilled water and ferric chloride (0.5 ml, 0.1 %).The absorbance was measured at 700 nm against a blank reagent. Ascorbic acid and gallic acid were used as reference materials. All tests were performed in triplicate. A higher absorbance indicated a higher reducing power. EC_{50} (effective concentration) values (µg/ml) were calculated and indicate the effective concentration at which the absorbance was 0.5 for reducing power.

Nitrogen oxide (NO) scavenging assay: Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be measured at 550 nm by spectrophotometer in the presence of Griess reagent. Nitric oxide radical inhibition was estimated by the use of Griess Illosvoy reaction [21]. In this investigation, Griess Illosvoy reagent was modified by using naphthyl ethylene diamine dihydrochloride (0.1% w/v) instead of using 1-naphthylamine (5%). The reaction mixture (3 ml) containing sodium nitroprusside (10 mM, 2 ml), phosphate buffer saline (0.5 ml) and the extract (1.62 to 200 µg/ml) were incubated at 25°C for 150 minutes. After incubation, 0.5 ml of the reaction mixture was mixed with 1 ml sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for completion of the reaction process of diazotization. Further, 1 ml of the naphthyl ethylene diamine dihydrochoride was added, mixed and was allowed to stand for 30 min at 25°C. The absorbance was taken at 550 nm. Gallic acid and ascorbic acid at the same dilution (1.62 to 200 µg/ml) were used as standards. All tests were performed in triplicate. Inhibition of nitrite formation by the plant extracts and standards were calculated relative to the control (% Inhibition = $[1 - (A_1) / A_0] \times 100$, Where A_0 was the absorbance of the control (without extract) and A_1 was the absorbance of the extract or standard). IC_{50} which is an inhibitory concentration of each extract required to

reduce 50% of the nitric oxide formation was determined.

Hydroxyl radical scavenging assay: The scavenging activity for hydroxyl radicals was measured with Fenton reaction [22]. Reaction mixture contained 60 µl of 1.0 mM $FeCl_2$, 90 µl of 1mM 1,10-phenanthroline, 2.4 ml of 0.2 M phosphate buffer (pH 7.8), 150 µl of 0.17 M H_2O_2 , and 1.0 ml of extract at various concentrations (1.62 to 200 µg/ml). The reaction started when H_2O_2 was added. After incubation at room temperature for 5 min, the absorbance of the mixture at 560 nm was measured with spectrophotometer (Thermo scientific BioMates^{TM} 3S). Gallic acid (1.62 to 200 µg/ml) was used as a standard. All tests were performed in triplicate. The percentage inhibition of hydroxyl scavenging activity was calculated using the following formula; % Inhibition = $[1 - (A_1) / A_0] \times 100$, Where A_0 was the absorbance of the control (without extract) and A_1 was the absorbance of the extract or standard.

Cellular antioxidant assay: For this purpose, we measured the potency of *Cassia arereh* to induce RBCs membrane damage and to manage hydrogen peroxide-mediated cytotoxicity of RBCs induced with $CuSO_4/H_2O_2$ at non-haemolytic concentration using haemoglobin (Hb) released as indicator.

Haemolysis test: Haemolysis test was employed to determine cellular toxicity of the extract as previously described [23]. 500 µl of the plant extracts at concentrations ranging from (0.25 to 32 mg/ml) were incubated with an equal volume of 10 % hematocrit sheep red blood cells in phosphate buffered saline (10mM PBS, pH 7.4) at 37°C for 3 h. Non-hemolytic and 100% hemolytic controls were the buffer alone and the buffer containing 1% Triton X-100, respectively. Cell lysis was monitored by measuring the release of hemoglobin from red blood cells spectrophotometrically (Thermo scientific BioMates^{TM} 3S) at 540 nm.

Antiradical assay of Cassia arereh on $CuSO_4/H_2O_2$ free radical-induced membrane damage: The results obtained from the haemolysis test above, showed that, extracts at low concentration (≤ 0.25mg/ml) have a similar effect as the non hemolytic control. Hence, the concentration of 25 µg/ml was considered as a non-haemolytic concentration and used for the study of the haematoprotective effect of extracts and standard against lysis solution on red blood cells. $CuSO_4/H_2O_2$ was used to initiate erythrocyte haemolysis. 10 % hematocrit sheep erythrocyte suspension in phosphate buffer saline (PBS, 100 mM) were preincubated for 0.5 minutes with an equal volume of a non-haemolytic concentration of plant-extracts or standards (25 µg/ml). The same volume of the lysis solution $CuSO_4/H_2O_2$ (100 µM/25 µM in 100 mM PBS) was then added to the various mixtures. Under these conditions, hydroxyl radicals were generated from the H_2O_2-Cu^{2+} mixture. The different combinations of reaction mixtures were incubated at 37 °C for 3 h with continuous shaking. After incubation, the reaction mixture was diluted with 8 volumes of PBS and then centrifuged at 3000 g for 5 min. The optical density of the supernatant fraction at 540 nm was recorded in a spectrophotometer (Thermo scientific BioMates^{TM} 3S) before and after 1, 2, or 3 h of incubation in order to monitor the cell lysis through hemoglobin release. Standards were vitamin E, butylated hydroxytoluene (BHT) and gallic acid. The absorbance of tested samples (RBCs + lysis solution + extracts or standards) was compared with controls (RBCs, without extract, standard or lysis solution) and (RBCs + lysis solution).

Statistical analysis: All the experiments for determination of total phenols, total flavonoids and antioxidant properties using DPPH, hydroxyl radical , nitric oxide, reducing power and cellular antioxidant assay were conducted in triplicates. The values are expressed as the mean ± standard deviation (S.D.). Data were statistically evaluated using the analysis of variance following the Duncan's test. The differences between groups were considered significant at $p < 0.05$.

RESULTS

Phytochemical screening: Phytochemical analysis revealed the presence of alkaloids, flavonoids, saponins, polyphenols, saponins and tannins in both the two extracts. Only ethyl acetate extract showed the presence of triterpenoids (Table 1).

Determination of phenolic Content: Table 2 shows the total phenolic content in the tested samples. Among the two plant-extracts, the highest phenolic content was found in methanolic extract (17.456 ± 0.157 mg GE/g of dry mass sample) followed by the ethyl acetate extract (12.039 ± 1.709 mg GE/g of dry mass sample). The difference in phenolic content was found statistically significant for the two extracts ($P < 0.05$).

Determination of flavonoid Content: The total flavonoid content in different extracts is shown in Table 2. As for the phenolic content, the highest amount of flavonoid content was found in

methanolic extract (6.268 ± 0.447 mg (RE) mg/g of dry weight) followed by the ethyl acetate extract (2.574 ± 0. mg (RE) mg/g of dry weight). However, the difference in flavonoid content of the two extracts was observed to be statistically insignificant.

Determination of antioxidant activity using chemical assays

DPPH radical Scavenging activity: Antioxidant activity of *C. arereh* using 2,2-diphenyl-1-picrylhydrazyl (DDPH) assay is shown in Figure 1 and Table 3. Antioxidant activity in terms of radical scavenging activity, using DPPH assay, ranged between 98.57 and 36.91 % in methanol extract, whereas, it ranged between 100.00 and 48.33 % in that of ethyl acetate extract. The maximum antioxidant activity was observed at high concentrations of extract. Both the two extracts were found more active compared to ascorbic acid, as standard. The IC_{50} values of methanol and ethyl acetate extract were 4.27 ± 0.9 and 3.21 ± 0.55 µg/ml respectively instead of 12.5 ± 2.99 µg/ml for standard (Table 3).

Reducing power activity: The reducing power of methanol and ethyl acetate extracts of *C. arereh* are summarized in (Figure 2). From the figure, reducing power increased with an increase in extracts concentration. Methanol extract had a higher reducing power than that of ethyl acetate. Reducing power values, ranged between 41.81 and 0.00 in the ethyl acetate extract whereas, in methanol, it ranged between 100.00 and 7.56. Ascorbic acid and gallic acid had the highest ability to reduce Fe (III) with IC_{50} values of 12.27 ± 0.11 and 3.42 ± 0.25 respectively (Table 3). These values were significantly different from the one of ethyl acetate extract (238.89 ± 26.56) ($P < 0.05$) but not from that of methanol (41.597 ± 7.46) (Table 3).

Nitrogen oxide scavenging activity: Nitrogen oxide (NO) scavenging activity of methanol and ethyl acetate extracts of *C. arereh* are summarized in (Figure 3). From the figure, we noticed a non significant decrease in the NO radical due to the scavenging ability of extract compared with ascorbic acid or gallic acid. For methanol extract, the antioxidant activity in terms of nitric oxide scavenging activity ranged between 15.72 and 0.00 %, whereas it ranged between 11.28 and 0.00 % in that of the ethyl acetate extract. Table 3 shown IC_{50} values of 74.37 ± 1.13 and 53.85 ± 1.42 µg/ml respectively for ascorbic acid and gallic acid. It is to be noted that methanol extract of *Cassia arereh* shows a greater inhibition of nitrite production compared to that of ethyl acetate with an IC_{50} value of 217.88 ± 22.25 in contrast to 828.57 ± 119.04 µg/ml (Table 3).

Hydroxyl radical scavenging activity: Activity of the extracts on hydroxyl radical is shown in Figure 4. Both the two extracts exhibited concentration dependent scavenging activity against hydroxyl radical generated in a Fenton reaction system. It was also found that the methanol extract is more effective than the ethyl acetate extract as far as hydroxyl radical scavenging activity is concerned. The antioxidant activity in terms of hydroxyl radical scavenging activity, ranged from 100 to 15.41 % for the methanol extract, and from 99.63 to 10.1 % for the ethyl acetate extract. The IC_{50} values were found to be 17.06 ± 0.82 and 76.34 ± 3.4 µg/ml respectively for ethyl acetate and methanol extract while IC_{50} value for gallic acid was 1.23 ± 0.1 µg/ml (Table 3).

Cellular antioxidant activity

Haemolysis activity: To assess the haemolytic activity of *Cassia arereh*, RBCs were exposed to this plant-extract without Cu^{2+} for 3 h as described in the experimental section. Both extracts induced haemolysis of sheep RBC in a dose dependent-manner (Figure 5). The dose effect is non linear and the highest doses (≥ 2 mg/ml) had similar haemolytic effect as Triton x 100. It can be deduced from Figure 5 that *Cassia arereh* at 0.25 mg/ml has no significant haemolytic activity as compared to the non haemolytic control. Thus, the concentration of 25 µg /ml was used in the following experiments to assess the antioxidant effect of *Cassia arereh.*

Haematoprotective activity: Treatment of RBC with hydrogen peroxide in the presence of $CuSO_4$ considerably increases release of haemoglobin (Figure 6) compared to the negative control (RBCs alone). Thus, a combination of $CuSO_4$ and H_2O_2 induces RBC cytotoxicity mediated by hydrogen peroxide. Incubation of RBCs with plant-extracts (25 µg/ml) before exposing them to $CuSO_4$ and H_2O_2 reduced the cytotoxicity of RBCs. As indicated in figure 6, methanol and ethyl acetate extracts of *Cassia arereh* (25 µg/ml) diminish respectively by 30.71 and 34.75 %, hydrogen peroxide-mediated cytotoxicity of RBC after a 1-h incubation. In the same experiment, 25 µg/ml of standards (vitamin E, butylated hydroxytoluene and gallic acid) diminished by 36.53, 33.80 and 42.81% respectively, hydrogen peroxide-mediated cytotoxicity of RBC (Figure 6). It is worth noting that, the antihaemolytic activity decreases with time for all the tested products (Figure 6). The most potent antihaemolytic products were gallic acid, vitamin E, ethyl acetate extract of

Cassia arereh followed by butylated hydroxytoluene (BHT) and methanol extract of *Cassia arereh.*

DISCUSSION

Plants are potential sources of natural antioxidants. Fruits and vegetables in the diet have been shown in epidemiological studies to be protective against several chronic diseases associated with aging such as cancer, cardiovascular diseases, cataracts, and brain and immune dysfunction [24-26]. The total antioxidant activity of the extracts is constituted by individual activities of each of the antioxidant compounds. Moreover, these compounds render their effects via different mechanisms such as radical scavenging, metal chelating activity, inhibition of lipid peroxidation, quenching of singlet oxygen, and so on, to act as antioxidant. Even if a sample exhibits high activity with one method, it does not always show similar good results with all other methods. Therefore, it is essential to evaluate samples accurately by several methods. In the present paper, we have evaluated the antioxidant activity and free radical scavenging activity of the methanol and ethyl acetate extracts of *Cassia arereh* leaves through DPPH radical scavenging activity, hydroxyl radical scavenging activity, nitric oxide scavenging activity, reducing power and cellular antioxidant assay.

The result obtained from DPPH assay showed an outstanding antioxidant activity of *Cassia arereh* (figure 1). Both the two extracts were more active compared to ascorbic acid, as standard when we considered the IC_{50} values (Table 3). DPPH assay is very convenient for the screening of large numbers of samples of different polarity because of its high throughput. It evaluates the ability of antioxidants to scavenge free radicals. These antioxidants donate hydrogen to free radicals, leading to non-toxic species and therefore to inhibition of the propagation of lipid oxidation [27].

Reducing power is to measure the reductive ability of antioxidant, and it is evaluated by the transformation of Fe (III) to Fe (II) in the presence of the sample [28]. The ability to reduce Fe (III) may be attributed from hydrogen donation from phenolic compounds [29] which is also related to the presence of reducing agents [30]. Reducing agents exhibited antioxidative potential by breaking the free radical chain, and by donating a hydrogen atom. Our results show that, Methanol extract had a higher reducing power than that of ethyl acetate (Table 3). The IC_{50} values obtained with methanol extract was found to be not significantly different from standards. The results found using this assay suggested that compounds present in methanol extract of *Cassia arereh* were good electron and hydrogen donors, and could terminate the radical chain reaction by converting free radicals into more stable products.

NO is generated in biological tissues by specific nitric oxide synthesis (NOSs), which metabolizes arginine to citralline with the formation of NO via a five electron oxidative reaction [31]. These compounds are responsible for altering the structural and functional behavior of many cellular components. Incubation of solutions of sodium nitroprusside in PBS at 25°c for 150 mn resulted in linear time dependent nitrite generation, which can be reduced by antioxidant principles, which compete with oxygen to react with nitric oxide thereby inhibiting the generation of nitrite. Our results showed a non significant decrease in the NO radical production due to the extracts compared to ascorbic acid or gallic acid.

Both the two extracts exhibited concentration dependent scavenging activity against hydroxyl radicals generated in a Fenton reaction system. It was also found that the methanol extract is more effective than the ethyl acetate extract as hydroxyl radical scavenging activity. Hydroxyl radical is a highly reactive oxygen centre radical formed from the reaction of various hydroperoxides with transition metal ions. It attacks proteins, DNA, polyunsaturated fatty acid in membranes, and most biological molecules it contacts [32-33] and is known to be capable of abstracting hydrogen atoms from membrane lipids and bringing about peroxidic reaction of the lipids.

Antioxidative properties of plants have been attributed to various components such as carotenoids, vitamin C, E, and phenolic and thiol (SH) compounds [34]. Many studies have focused on the biological activities of phenolics which are potent antioxidants and free radical scavengers [35-36]. The antioxidant activity of phenolics is mainly due to their redox properties, which allows them to act as reducing agents, hydrogen donors and singlet oxygen quenchers [8-10]. Phenolic compounds are also known to play an important role in stabilizing lipids against peroxidation and inhibiting various types of oxidizing enzymes [37-38]. Polyphenolic compounds such as flavonoids and tannins found in the extracts (Table 1) are considered to be the major contributors to the antioxidant activity of this medicinal plant. In addition to the qualitative estimation using phytochemical screening, we have quantified the total flavonoid and phenolic contents of plant-extracts using colorimetric methods. The results obtained show that the flavonoid and phenolic contents of plant-extracts were found to be 2.574 ± 0.211 and 6.268 ± 0.447 mg (RE) mg/g;

12.039 ± 1.709 and 17.456 ± 0.157 mg (GE)/g dry weight respectively for ethyl acetate and methanol extracts of *Cassia arereh* (Table 2). This result once more showed an outstanding antioxidant activity of *Cassia arereh* since flavonoids are a group of polyphenolic compounds with known properties which include free-radical scavenging, inhibition of hydrolytic and oxidative enzymes, and anti-inflammatory actions [39-40].

The results obtained for the partitions of different extractants showed that methanol, nature of most antioxidant compounds, as methanol extracts are largely polar compounds. The very good activity of the polar extracts is probably due to the presence of substances with an available hydroxyl group [41-43]. In summary, it was clear that the polarity of the extractants markedly influences the antioxidant activity.

Anti-haemolytic activity: Previous studies on RBC partitioning of relatively small organic cationic, anionic, and non-electrolytic molecules have shown that lipophilicity, molecular size, and chiral characteristics of molecules influence their penetration into RBC [44]. Thus, lipophilic compounds penetrate the RBC by being dissolved in lipid bilayer membranes while small size hydrophilic compounds enter the RBC through aqueous channels. Saponins are known to increase cell membrane permeability [45-46]; one may suggest that the presence of saponins in *Cassia arereh* allow the penetration of extract components into RBC particularly the hydrophilic moieties, since *Cassia arereh* extracts contain polyphenol compounds. This may explain, at least partially, the antiradical activity of *Cassia arereh* against cellular membrane damage. H_2O_2 induced haemolysis activity was assayed to measure the potency of plant-extracts to scavenge oxidative metal ions (Cu^{2+}) or free radicals deriving from H_2O_2 . Since free copper or iron are a crucial catalyst of oxygen centered radical formation in biological systems, their removal or their intracellular sequestration may result in diminished cellular sensitivity to oxidant damage [47-48]. Thus, it cannot be excluded that *Cassia arereh* extract may act as chelators of transition metals (Cu^{2+}).

CONCLUSION

The present investigations revealed that *Cassia arereh* was observed to be a potential source of antioxidant agents. It was clear that the polarity of the extractants markedly influences the antioxidant activity. The results obtained for the partitions of different extractants showed that methanol, the more polar one exhibited the best antioxidant results.

Present findings encourage further evaluation by isolation, characterization and identification of antioxidant molecules in *Cassia arereh.*

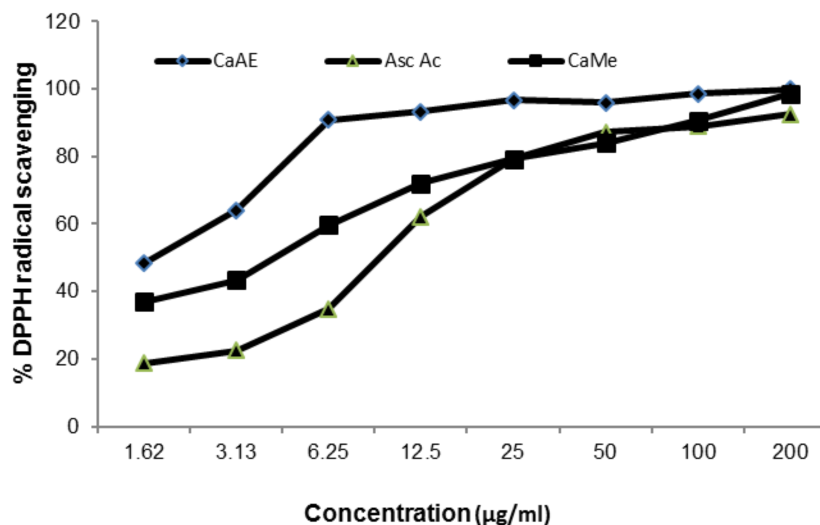

Figure 1: DPPH free radical scavenging activity of standard ascorbic acid (Asc Ac) and *Cassia arereh* extracts. Methanol extract of *Cassia arereh* (CaMe), Ethyl Acetate extract of Cassia *arereh* (CaAE).

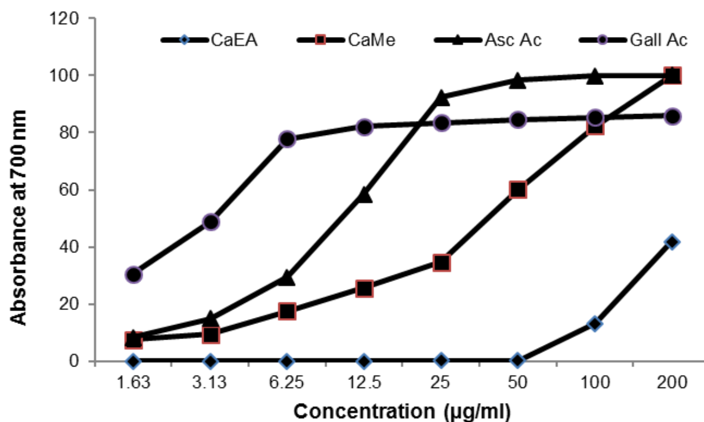

Figure 2: Reducing power of standards ascorbic acid (Asc Ac), gallic acid (Gall Ac) and *Cassia arereh* extracts. Methanol extract of *Cassia arereh* (CaMe), Ethyl Acetate extract of *Cassia arereh* (CaAE).

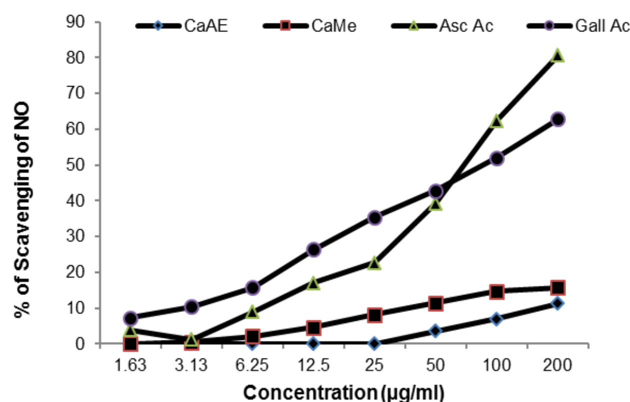

Figure 3: Nitric oxide free radical scavenging activity of standard ascorbic acid (Asc Ac), gallic acid (Gall Ac) and *Cassia arereh* extracts. Methanol extract of *Cassia arereh* (CaMe), Ethyl Acetate extract of *Cassia arereh* (CaAE).

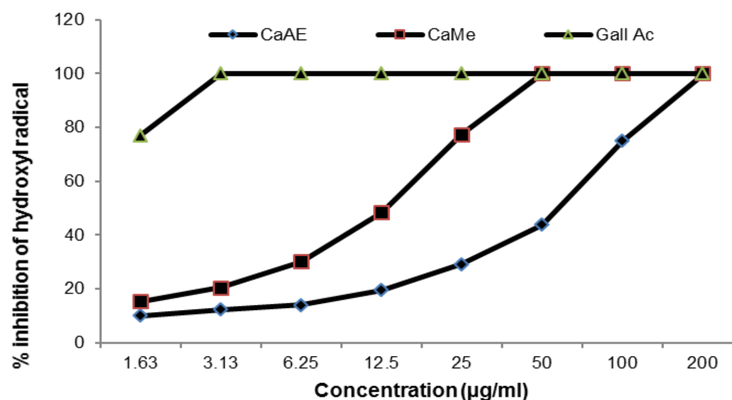

Figure 4: hydroxyl radical scavenging activity of standard gallic acid (Gall Ac) and *Cassia arereh* extracts. Methanol extract of *Cassia arereh* (CaMe), Ethyl Acetate extract of *Cassia arereh* (CaAE).

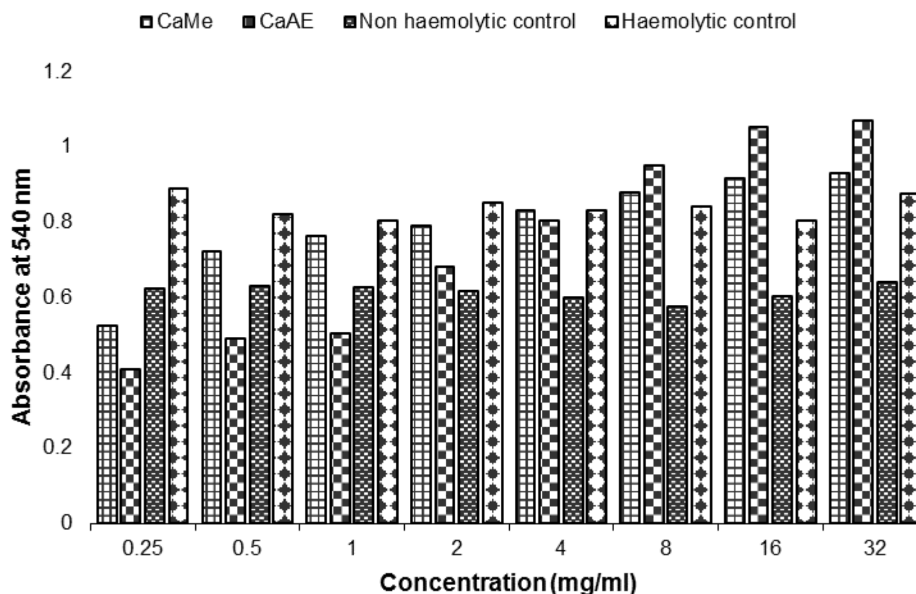

Figure 5: Haemolysis effect of *Cassia arereh* extracts using haemoglobin (Hb) release (absorbance at 540 nm) in the absence of lysis solution. Methanol extract of *Cassia arereh* (CaMe), Ethyl Acetate extract of *Cassia arereh* (CaAE), Non haemolysis control (10mM PBS, pH 7.4), Hemolytic control (PBS + 1% Triton X-100).

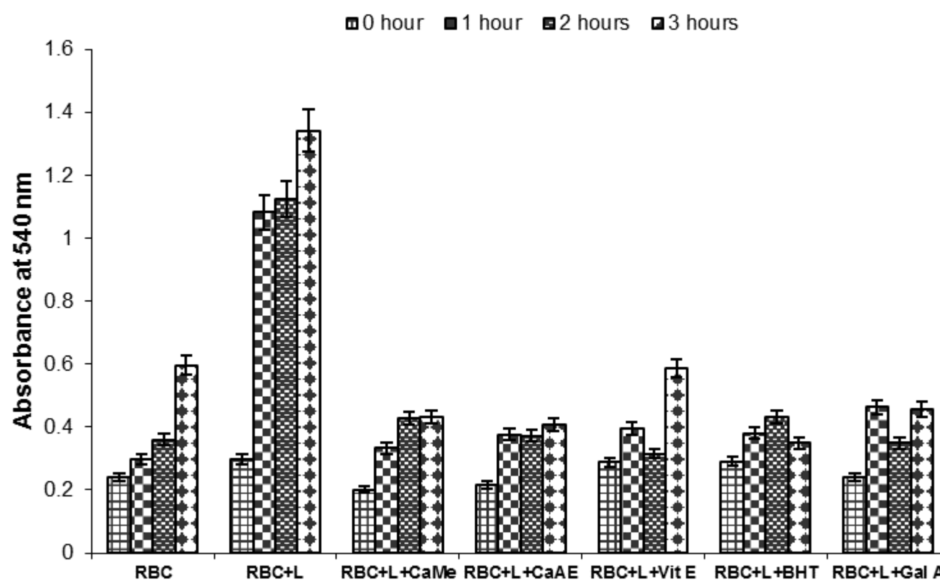

Figure 6: Effect of various treatments on haemolysis (optical density at 540 nm) for different times (0, 1, 2 and 3 h). Methanol extract of *Cassia arereh* (CaMe), Ethyl Acetate extract of *Cassia arereh* (CaAE), Vitamin E (Vit E), butylated hydroxytoluene (BHT), gallic acid (Gal A), lysis solution ($CuSO_4/H_2O_2$ 100 µM/25 µM in 100 mM PBS), red blood cells (RBCs).

Table 1: Major classes of phytocompounds of *C. arereh* extracts

Plant extract	Alc	Flav	Phe	Sap	Tan	Trit	Ster
C. arereh **(Methanol)**	+	+	+	+	+	+	-
C. arereh **(ethyl Acetate)**	+	+	+	+	+	-	-

Alc : Alcaloïdes, Flav : Flavonoïdes, Phe : Phénols, Sap : Saponines, Tan : Tanins, Trit : Triterpènes, Ster : Stérol.

Table 2: Extraction yields, total phenolic and flavonoids content of *C. arereh* extracts

Plant-extract	Yield of extraction (%)	Total phenolic GE (mg/g of dry mass)	Total Flavonoïds RE(mg/g of dry mass)
C. arereh **(ethyl Acetate)**	3	12,039 ± 1,709[a]	2,574 ± ,211[a]
C. arereh **(Methanol)**	4.5	17,456 ± 0,157[b]	6,268 ± ,447[ab]

Data are means ± standard deviation (n = 3 tests). Values with dissimilar letter (a, b and c) are significantly ($p < 0.05$) different from each other with respect to the parameter investigated. GE: gallic acid equivalent; RE: rutin equivalent.

Table 3: Antioxidant activity of *C. arereh* extracts and standards.

Plant-extracts / standards	DPPH IC_{50} (µg/ml) ± SD	Hydroxyl radical IC_{50} (µg/ml) ± SD	Reducing power EC_{50} (µg/ml) ± SD	Nitric oxide IC_{50} (µg/ml) ± SD
C. arereh **(ethyl Acetate)**	3,21 ± ,55 a	76,34 ± 3,4 c	238.89 ± 26,56 b	828,57 ± 119,04 b
C. arereh **(Methanol)**	4,27 ± ,9 a	17,06 ± ,82 b	41.597 ± 7,46 a	217,88 ± 22,25 a
Asc Ac	12,5 □ 2,99 a		12,27 ± ,11 a	74,37 ± 1,13 a
Gall Ac		1,23 □ ,1 a	3,42 ± ,25 a	53,85 □ 1,42 a

Data are means ± standard deviation (n = 3 tests). Values with dissimilar letter (a, b and c) are significantly ($p < 0.05$) different from each other with respect to the parameter investigated.

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