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Research Article

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Anti-hemolytic Activity and Antioxidant Studies of Caralluma quadrangula: Potential for Nutraceutical Development in Cancers and Blood Disorders

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ABSTRACT

Background: *Since ancient times, plants and its derivatives have been used in traditional medicine to cure human diseases. In the past few decades, the research on medicinal plants has gained significant attention due to the medicinal potential of certain phytochemicals against cancer and metabolic disorders. The present study has examined the alcoholic extract of Caralluma quadrangula (Ca qu) for its quantitative and qualitative composition and its anti-oxidant as well as anti-hemolytic properties. The findings have potential implications for plausible intervention in reactive oxygen species (ROS) mediated pathologies.* ***Materials and Methods:*** *An 80 % aqueous-methanol extract of areal parts of Ca qu was prepared. It was subjected to qualitative and quantitative phytochemical analysis. Anti-oxidant potential was determined by inhibition of 2,2-Diphenyl-1-pycrylhydrazyl (DPPH) and 2,2' -Azino-bis-3- ethylbenzthiazoline-6-sulfonic acid (ABTS) radicals; while, anti-hemolytic activity was determined by the ability of the extract to protect human RBCs from oxidative insult.* ***Results****: The extract showed abundance of polyphenolic and flavonoid compounds at concentrations of 8.6 GAE % w/w and 0.90 mg QE % w/w, respectively. Tannins, alkaloids and saponins were present at the concentration of 8.50 mg TAE % w/w, 2.8 mg % w/w and 20.07 mg % w/w, respectively. Qualitative HPLC column chromatography indicated the presence of rutin in the extract. In an increasing concentration range from 31.25 to 2000 µg/ml the extract provided significant protection to RBCs from membrane damage induced by ROS. In the DPPH and ABTS inhibition assays, the extract showed a dose-dependent inhibition of the radicals in the concentration range of 50 -1000 µg/ml and 10-250 µg/ml, respectively.* ***Conclusion:*** *The hydro-alcoholic extract of Ca qu contains several classes of important phytochemicals with known therapeutic significance. The extract possesses significant anti-oxidant and anti-hemolytic potential as demonstrated in standard assays. The findings can be exploited for advanced studies on pharmacological premises for intervention in different diseases that are associated with an imbalanced production of ROS/free radicals in cells including certain anemic disorders and cancers. The formulations derived from the plant are expected to possess therapeutic advantage as nutraceuticals or as adjuvants with standard treatment regimen.*

Key words: *Caralluma quadrangula, anti-oxidant, anti-hemolytic, nutraceutical.*

INTRODUCTION

Throughout human civilizations, plants and their derivatives have been used in traditional medicine for the therapeutic management of several diseases and thus have affected human health for centuries. The medicinal

plant *Ca qu* is found in Saudi Arabia and the neighboring countries in Arabian Gulf peninsula [1, 2]. In these regions, it is used as a component of traditional medicine in treatment of metabolic disorders including diabetes [3]. The genus caramulla is particularly known to be rich in pregnane glycosides with medicinal properties particularly for the control of obesity and hyperglycemia. In a more recent study, the rats fed with the HF diet and russelioside B (25 mg/kg; pregnane glycoside from caralluma) resulted in diminishing body weight gain as compared to the control group fed with HF diet alone. Enriching the diet with doubled russelioside B dose led to further reduction in body weight gain as compared to control group. The anti-obesity affect was partly attributed to its anti-inflammatory and adipokine modulating activities, in addition to its favorable effect on energy expenditure [4]. When administered at a dose of 50 mg/kg in streptozotocin induced diabetic rats, russelioside B was shown to regulate few key carbohydrate metabolizing enzymes, thereby improving the fasting serum glucose level, glycated hemoglobin, serum insulin level and lipid profile [5]. In a biologically guided fractionation approach, few isolated pregnane glycosides from *Ca qu* have also been shown to be cytotoxic against MFC7 breast cancer cell lines [6]. Moreover fractionated organic and aqueous extracts form *Ca qu* have also been demonstrated to possess antimicrobial properties [7].

Extensive studies have implicated the role of ROS in the pathogenesis of human diseases and disorders including cancers and few blood disorders like hemolytic anemia [8]. It important to note that the phytochemicals in herbal extracts, especially polyphenols including flavonoids, tannins, proanthocyanids, catechins have been shown to scavenge and neutralize ROS in deranged cells preventing the damage or at least partly restoring the normal cellular functions [9]. Studies have shown that nutraceuticals derived from plants are highly beneficial in human health [10] and may serve to reverse certain pathologies. Interestingly, from the last decade the genus caralluma has been studied for developing nutraceuticals given its medicinal properties. For example, in a double blind placebo controlled clinical trial, the extracts from C*aramulla fimbriata* have been shown to play a favorable role in curbing central obesity (significant weight loss), the key component of metabolic syndrome [11]. Furthermore, *C. fimbriata* extract has received Generally Recognized as a Safe (GRAS) status (FDA) for use as a nutraceutical to combat obesity, a well-known and serious public health concern across many developed and developing nations. In fact, an extract of *C. fimbriata* (Slimaluna(®), Gencor Nutrients, Anaheim, CA, USA) is used as an anti-obesity agent and appetite suppressor [12].

The aim of the current investigation was to study the basic phytochemical content and to evaluate antioxidant and anti-hemolytic potential of the extract obtained from the areal parts of *Ca qu*. As depicted in Figure 1, we hypothesize that the extract has the potential to be developed as a nutraceutical product that can be used to intervene in certain human diseases for which ROS have been associated as a causative mechanism; for example cancers and hemolytic anemia.

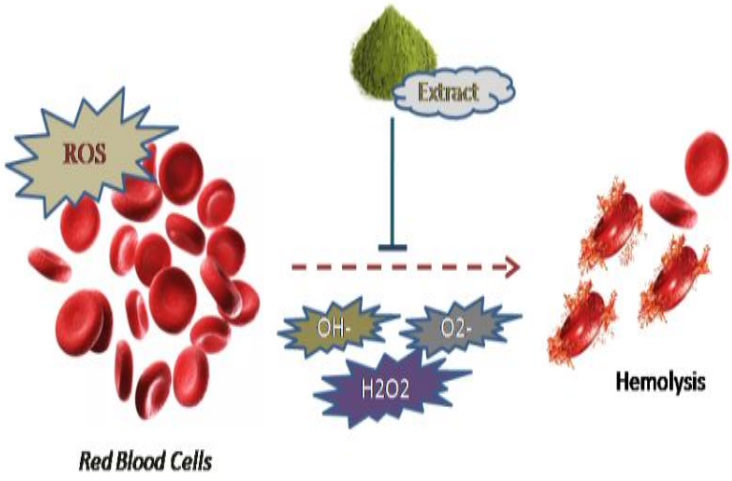

Figure 1. The scheme to hypothesize that phyto-constituents of Caralluma quadrangula extract are bio-effective antioxidants. It can be developed into a nutraceutical product to impede certain reactive oxygen species associated disorders.

MATERIALS AND METHODS

DPPH, ABTS, anhydrous potassium dihydrogen orthophosphate, orthophosphoric acid, kampferol, gallic acid, rutin, lutiolin and quercitin were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All solvents used were analytical and HPLC grade.

Plant collection and extraction: *Ca qu* was collected from its natural desert habitats from the Northwestern region of Saudi Arabia around the city of Tabuk. The plant was authenticated by an expert taxonomist by comparing the collected sample with the *Ca qu* specimen (RH 3254) preserved in the herbarium at the Department of Biology, College of Science, University of Tabuk. The Areal parts were dried under shade for few days. The dried plant material was crushed into powdered form with a wooden mortar and pestle and further ground to fine dry powder using a blender. 100 g of dry powder was then soaked in 1 L of 80% methanol in a conical flask placed in a shaking water bath at 40°C for 24 hr. The mixture was then filtered through double-layered, clean cheese cloth and then again through double-layered Whatman paper (Sigma-Aldrich Co., St. Louis, MO, USA). The procedure was repeated twice and the collective filtrate was then concentrated under reduced pressure at 35°C using a Buchi Rotavapor R-210 (Flawil, Switzerland). The extract was dried in a vacuum freeze dryer. The residual material was found to weigh 12.25 g (representing a yield of 12.25 %) and stored at -35 C for further use.

Phytochemical determination (quantitative): Several major classes of phytochemicals were determined by authenticated procedures which included phenolics, flavonoids, tannins, alkaloids, and saponins. The quantitative determination of the total phenolic and flavonoid contents were determined using gallic acid and quercitin as reference standards respectively [13,14]. Tannins were determined using tannic acid as a reference standard [15]. Alkaloids and saponins were determined by gravimetric estimation as described by Harborne et al. [16].

Phytochemical determination (qualitative): HPLC was used for the qualitative determination of flavonoids for which a standard flavonoid mixture containing kampferol, rutin, lutiolin and quercitin was used. Shimadzu LC 2010 CHT HPLC was used for the chromatographic separation equipped with autosampler and a UV detector detector (370 nm). The chromatographic separation was achieved on a Purosphere, C18, 5 µ (250 x 4.6 mm) reverse phase analytical column. Mobile phase A was HPLC grade water: buffer (0.136 g of anhydrous potassium dihydrogen orthophosphate/L, pH adjusted to 2.8 with orthophosphoric acid). It was filtered by passing through 0.45 µm filter and further degassed by using bath sonicator. The mobile phase B was HPLC grade acetonitrile. Injection volume was 10 µL and oven temp was set at 30ºC. The mobile phase was pumped at 1.5 ml/min at room temperature. The gradient program was as follows: 0-6 min B was 5%, 6-10 min B was 25%, 10-25 min B was 65%, 25-30 min B was 90%; 30-35 min B was ramped to 5% and followed by equilibration of column till another 5 minutes.

Antioxidant activity by scavenging of DPPH radicals: The DPPH radical-scavenging activity was carried out according to the Blois method [17]. DPPH (0.3 mM) was added to each sample. After incubation for 30 min in the dark at room temperature, the absorbance was measured at 518 nm using a microplate reader. Vitamin C was used as a positive control. The free radical-scavenging capacity was expressed by IC_{50}.

Antioxidant activity by scavenging of ABTS radicals: The ABTS assay was adopted to determine the ability of the *Ca qu* extract to neutralize the ABTS radical cation and compared to that of vitamin C [18]. The radical cation was prepared by mixing 7 mM ABTS with 2.45 mM potassium persulfate (1:1 v/v). The mixture was incubated for 24 h until completion of the reaction to attain a stable absorbance. PBS was mixed with ABTS radical solution to achieve an absorbance of 0.7 (±0.02) at 732 nm. The experiment was executed with diluted ABTS radical solution mixed with samples, and the measurements were taken at 734 nm after 30 min. The decrease in absorbance reflected the antioxidant capacity of the samples and was expressed by IC_{50}.

Anti-hemolytic assay (*Isolation of RBCs and protection again H_2O_2 induced hemolysis):* Human Blood was obtained by venipuncture in EDTA tubes and RBCs were isolated and stored according to Nabavi et al. [19]. Different concentrations of the each extracts (0.5 mL) were added to erythrocyte suspension (4%, 2 mL) and the volume was made up to 5 mL with saline buffer. Reaction mixtures were incubated for 5 min at room temperature and then 0.5 mL of H_2O_2 solution in saline buffer was added to induce hemolysis. After incubation (240 min) at room temperature, the reaction mixture was centrifuged at 250*g* for 10 min and the extent of hemolysis was determined by measuring the absorbance at 540 nm corresponding to hemoglobin liberation [20].

Statistical analysis: The experiments were performed in three different sets, with each set in triplicate. The data are expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed using an

analysis of variance (ANOVA), followed by an F-test using SPSS version 11.5 (SPSS, Inc., Chicago, IL). Values of P that were ≤0.05 were considered significant. For HPLC analysis LC solutions software was used.

RESULTS

Phytochemical composition (quantitative) of the extract show the presence of varied phytochemical classes: The results as tabulated in Table 1, showed that the extract contained total phenolics at a concentration of 8.60 ± 0.76 mg GAE % w/w, total flavonoids were 0.90 ± 0.01 mg QE % w/w, total tannin content at 8.50 ±0.66 mg TAE % w/w, alkaloids at 2.80 ±0.15 mg % w/w and saponins 20.07 mg %w/w. The phytochemical contents of extract displayed an array of bioactive metabolites which have been well implicated to benefit and intervene in certain metabolic diseases.

Table 1. The phytochemicals determined in the hydro-methanolic extract of *Caralluma quadrangular*. Most determined phytochemical classes are effective antioxidants and are capable of limiting reactive oxygen species associated pathologies.

Phytochemical Class	Quantity
TP (mg GAE % w/w)	08.60 ±0.76
TF (mg QE % w/w)	00.90 ±0.01
Saponins (mg %w/w)	20.07 ±1.85
Tannins mg TAE % w/w	08.50 ±0.66
Alkaloids (mg %w/w)	02.80 ±0.15

TP = Total polyphenols; TF = Total flavonoids; GAE = Gallic acid equivalent; QE = Quercitin; equivalent; TAE = Tannic acid equivalent; Values are mean ± SD of three replicates.

Qualitative phytochemical analysis display the presence of flavonoid rutin: As shown in Figure 2, the extract chromatogram displayed a peak at RT 9.000, strongly indicative of the presence of rutin in the extract. Rutin has effective pharmacological properties that include anti-carcinogenic, antioxidant, cardio-protective, vaso-protective, neuro-protective and cyto-protective activities [21].

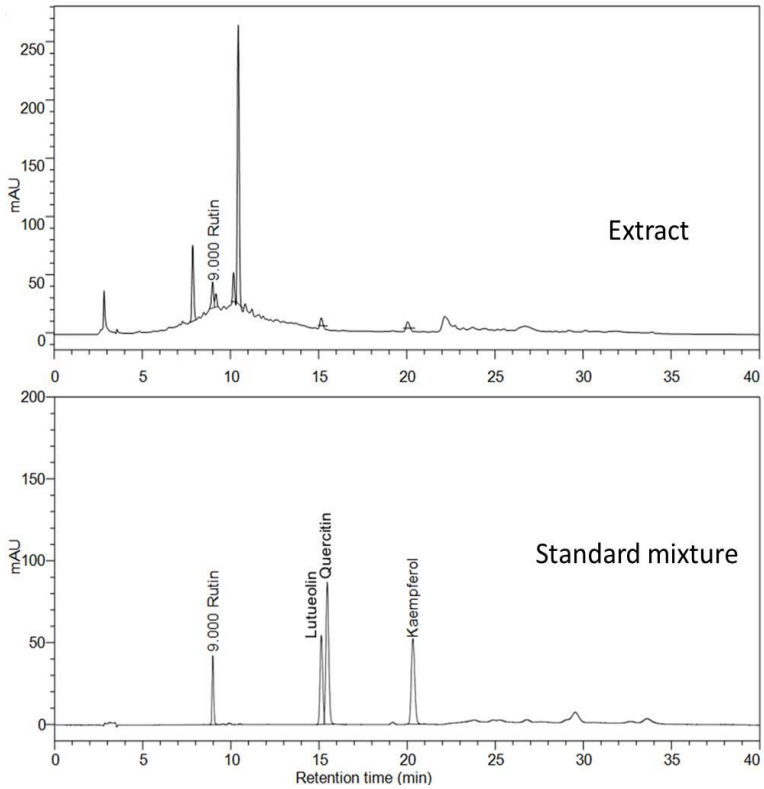

Figure 2. HPLC chromatogram of the hydro-alcoholic extract of *Caralluma quadrangula*. The peak corresponding to the retention time of 9.00 min is highly indicative of the presence of rutin. Rutin is a strong anti-oxidant and possess varied pharmacological properties as mentioned.

DPPH radical scavenging: As shown in Figure 3, the inhibition of DDPH radicals was dose- dependent in relation to concentration of the extract, and more than 85% inhibition could be seen at a concentration of 1000 µg/ml extract in the reaction mixture. The unpaired electron on DPPH confers a strong absorbance at 517 nm, giving the radical a purple color. With the exposure to antioxidants, it is reduced resulting in decreased absorbance due to the formation of yellow colored anti-radical diphenylpicryl hydrazine.

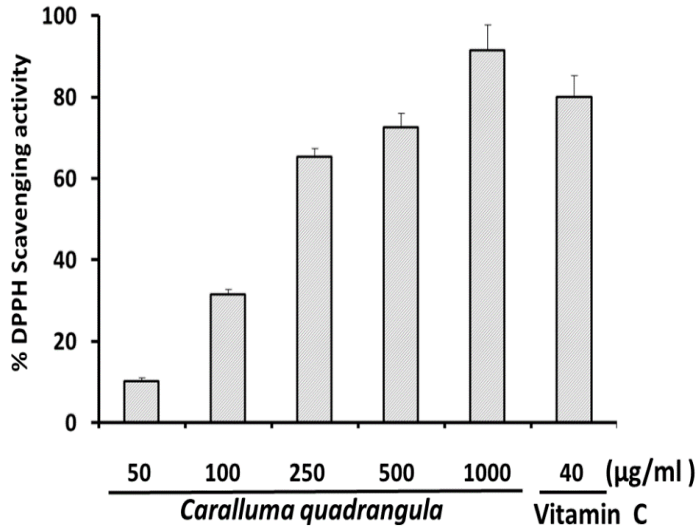

Figure 3. DPPH inhibition (%) by the hydro-alcoholic extract of *Caralluma quadrangular*.The values reported are the mean ± SEM of three independent experiments. A dose-dependent inhibition of DPPH radicals can be seen as the concentration of the extract was increased in the given range.

Inhibition of ABTS radicals: As can be observed from Figure 4, the final extract concentration in the reaction mixture in the range of 10-250 µg/ml had a dose dependent effect on the scavenging of ABTS radicals. ABTS is a well-studied substrate for peroxidases and is often utilized to examine the antioxidant properties of plant derived compounds.

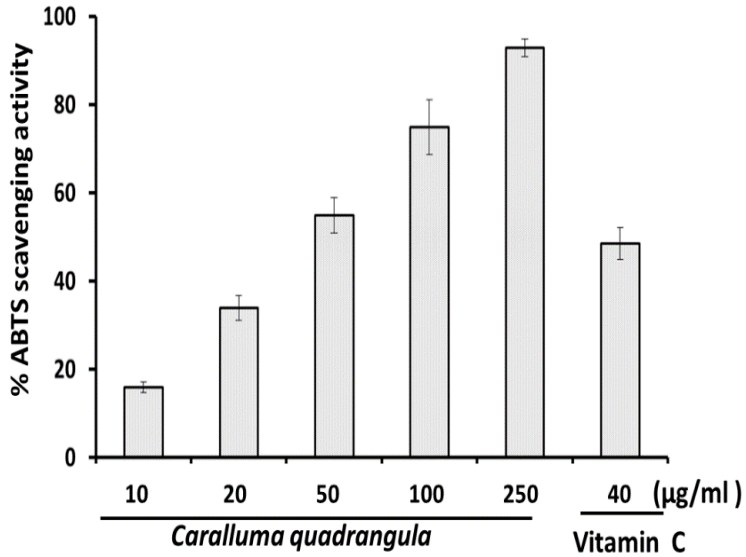

Figure 4. The radical scavenging capacity of the aqueous-alcoholic *Caralluma quadrangula* extract expressed by its ability to inhibit ABTS•+ radicals. The values reported are the mean ±SEM of three independent experiments. A dose-dependent inhibition of ABTS radicals can be seen as the concentration of the extract was increased in the given range.

The extract alleviates the hydrogen peroxide-induced RBC membrane damage (Anti-hemolytic): The results of the anti-hemolytic assay are shown in Figure 5. A clear dose- dependent relation among the extract dose and potential to inhibit RBC lysis can be observed. An inhibition of near 90% RBC lysis was observed at the highest dose of 2000 µg/ml of the extract in the reaction mixture. The phytconstuitents of the extract scavenge hydrogen peroxide and peroxide radicals generated, protecting the RBC from membrane damage induced by oxidative insult, which otherwise will lead to hemoglobin leakage into the reaction mixture.

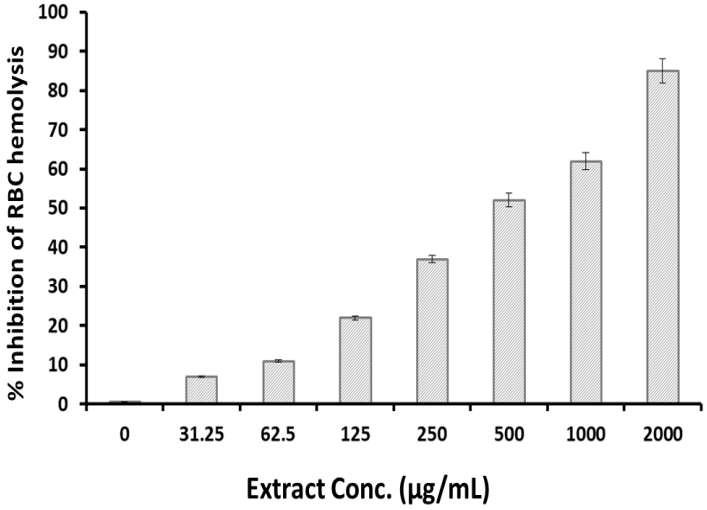

Figure 5. Anti-hemolytic assay showing the effect of increasing concentrations of the *Caralluma quadrangula* extract on inhibition of RBC lysis induced by hydrogen peroxide. As the concentration of the extract was increased, the lysis of RBCs was decreased in the given range. This can be attributed to scavenging of hydrogen peroxide which results in decreasing the oxidation of polyunsaturated fatty acids in RBC membrane, and therefore of cell lysis.

DISCUSSION

Aqueous-alcoholic solvents are excellent combinations to extract polyphenols including flavonoids such as flavonols [22, 23]. The aqueous methanolic extract of *Ca qu* showed rich contents of different phytochemicals as presented in Table 1. These plant-derived secondary metabolites possess spectra of pharmacological properties attributable to their ability to interfere with multiple signaling cascades that are critical to heterogeneous metabolic diseases [24]. Few previous studies have also demonstrated the antioxidant potential of genus *Caralluma* and richness in bioactive metabolites [25-27]. As observed in the HPLC chromatogram (Figure 2), the peak eluting at 9.000 min is highly indicative of the presence of rutin in the extract. Rutin which is mainly present in glycosidic form in plants is a potent antioxidant and has potential therapeutic implications in several chronic diseases such as different cancers and neurodegenerative disorders [28, 29]. The low toxicity associated with rutin and its derivatives in mammalian cells along with potent attenuation of ROS in human cells makes it a potential candidate for use as nutraceutical as well [30]. The two anti-oxidant assays resulted in an IC_{50} of 323.19 µg/ml and 69.68 µg/ml for DPPH and ABTS radicals, respectively. As in Figure 3, our results demonstrate significant scavenging activities of DPPH radicals. DPPH is a stable free radical that can donate hydrogen when reacts with antioxidant compounds and is reduced to diphenyl picrylhydrazine. It is therefore presumable that in our experimental system at least rutin and other unidentified anti-oxidative polyphenols contribute to attenuate the DPPH radicals as well as the peroxide radicals generated in RBC hemolysis model. Our study has specifically used the experimental model of RBC hemolysis to evaluate the scavenging capability of *Ca qu*. The phenols and tannins in the extract could easily donate electrons to H_2O_2, thereby neutralizing it into water. As shown in Figure 5, with increasing dose of the extract the hemoglobin leakage that reflects RBC membrane damage is reduced significantly. A similar reflection of a dose dependent scavenging effect of ABTS radicals can be seen in the antioxidant assay as shown in Figure 4. The enolic groups on the aromatic ring of polyphenols/flavonoids are excellent electron donors and thereby easily get oxidized. The presence of alkaloids in the extract (Table 1) possibly has an additive effect on this scavenging activity. In fact the imine group(s) in

plant alkaloids like capsaicin, protoberberines, jatrorrhizine and piperine is/are also excellent nucleophile(s) and readily donate electron to highly unstable ROS thereby neutralizing their effects. Such scavenging mechanisms have been well studied and reviewed by others [31]. Peroxidation of cellular membranes induced by ROS is a major event in oxidative damage in living systems [32, 33]. It is believed that due the presence of high content of polyunsaturated fatty acids in erythrocyte cell membrane, these serve as an acceptable model to study the lipid peroxidation. ROS generated by hydrogen peroxide/or radicals generated directly in cellular metabolism leads to the peroxidation of cell membrane lipids causing membrane damage and the consequent hemoglobin leakage. In our study, the inhibition of hemolysis reached 90% after treatment at 2000 µg/mL extract, likely reflecting a cumulative scavenging antioxidant effect of constituent polyphenols/flavonoids and alkaloids and is in agreement with other studies [34]. Several diseases and disorders are a result of excessive production of ROS including cancers and can aggravate others like hemolytic anemia. Antioxidant molecules derived from plants are effective in neutralizing ROS, thereby compromising their deleterious effects in the cells. Given *Ca qu* plant is edible and has been shown to be non-toxic towards mammalian cells, it would be of interest to investigate its potential as nutraceutical to counter ROS related pathologies. Interestingly, one nutraceutical product from genus *Caralluma* is already FDA approved, and used for appetite and weight loss to control obesity [12]. The scavenging ability of ROS by the *Ca qu* extract and its anti-hemolytic activity, as demonstrated in our study are reflective of its potential to attenuate ROS burden in diseased cells such as cancers and certain blood disorders. Several such studies in literature provide evidence for the protective effects of plant extracts and their derivatives against ROS mediated damage of red blood cells [35, 36]. It is recognized that the natural products reflect the richest source for new drugs and/or pharmaceutical leads due to their enormous structural diversity and pleiotropic action mechanism.

CONCLUSION

The hydro-alcoholic extract of *Ca qu* is rich in antioxidant phytochemicals and have possible therapeutic implication in ROS associated impairments that include chronic diseases such as cancer and certain blood disorders. We provide experimental evidences that the extract has the potential to be exploited as nutraceutical or as an adjuvant with standard therapies. Further studies are worthwhile to examine the extract for its capacity towards the development of nutraceutical formulations to contest such pathologies.

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