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In vitro assessment of *Alpinia officinarum* rhizome on red blood cells of anaemic goats

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Abstract
The objective of this study was to find out how *Alpinia officinarum* rhizome extract affected the anemic goats' red blood cells in an *in vitro* setting. A phytochemical screening of *Alpinia officinarum* rhizome extract identified the following compounds: Alkaloids, flavonoids, phenols, tannins, terpenoids, glycosides, and carbohydrates. The total phenolic and flavonoid content of AO rhizomes were 29.92±0.19mg QE /g and 12.64±0.25mg GAE/g, respectively. Normal goat red blood cells do not exhibit hemotoxicity from *Alpinia officinarum* rhizome. *In vitro* treatment of anaemic goats' red blood cells with AO rhizomes restored the generation of free radicals caused by anemia. According to our findings, the extract protects the erythrocytes of anemic goats *in vitro*.

Keywords: *Alpinia officinarum*, goat, red blood cells, antioxidant, *in vitro*

Introduction
Plants are used in the preparation of many traditional medical systems, including Ayurveda, Siddha, Unani, homeopathy, and folklore from different nations. There is a lot of research being done on the possible health benefits of medicinal plant extracts. The main components of plants that are thought to have therapeutic effects are flavonoids, coumarins, phenolic acids, and antioxidant micronutrients like copper, zinc, and magnesium (Seth & Sharma, 2004) [17].
Oxidative stress, which is brought on by reactive oxygen species (ROS) and other free radicals, is assumed to be the fundamental mechanism underlying degenerative diseases like diabetes, viral infections, autoimmune pathologies, and most likely aging. According to research, scavenging reactive oxygen species (ROS) with antioxidant compounds from food and medicinal plants can be used as a chemoprevention strategy (Finkel & Holbrook, 2000) [6].
Numerous earlier studies demonstrate how plant extracts can prevent oxidative damage, including to erythrocytes (Luqman S *et al*., 2009) [11]. *A. officinarum* is a perennial herb with showy white flowers in racemes, thick, creeping reddish-brown rhizomes, and lineolate acuminate ornamental leaves (Daniel M, 2006) [4]. Because of their anti-inflammatory, analgesic, antioxidant, antidiabetic, anti-ulcer, antidiarrhea, and anticoagulant properties, the dry root and rhizome have been used medicinally (Lee *et al*., 2009; Xie *et al*., 2013) [10, 24]. To the best of our knowledge, no research has been done on the impact of *A. officinarum* rhizome extract on anemic goat red blood cells (RBCs) *in vitro*.

Materials and Methods
Plant materials
Alpinia officinarum was procured from the Ethnoveterinary Herbal Products Research and Development Centre, Veterinary College and Research Institute, Orathanadu. The Siddha Central Research Institute, Central Council for Research in Siddha, Ministry of AYUSH, Government of India, Arumbakkam, Chennai, verified the authenticity of the plant material used in this study. The botanical identity and quality of the plant samples were guaranteed by this authentication procedure, which enhanced the study's scientific validity. The reference herbarium specimen was placed under voucher number 25/ EVHPR&D Center/2023 in the herbarium of the Ethno Veterinary Herbal Product Research and Development Centre, VCRI, Orathanadu, Thanjavur.

Preparation of extract

The extraction process relied on carefully selected solvents, referred to as menstruum, to ensure efficient extraction of active constituents from the chosen medicinal plants. In the present study, ethanol was used as solvent for the separation of active principle. To enable effective Soxhlet extraction, the plant materials were ground into a coarse powder after being shade-dried. To maximize the extraction yield and avoid the formation of fine powder, this step was essential. After that, the coarse powder was carefully placed inside a thimble and placed above a flask with a circular bottom. To condense the evaporating vapor back into the thimble, a condenser was positioned above it. Using a rotary film evaporator, the resultant extractive liquid-which contained the active ingredients-was processed once more. With the help of this procedure, the alcoholic solvent was successfully eliminated, yielding a pure extract that contained no traces of leftover solvent. For later use, the finished extract was kept in storage at 4 °C.

Qualitative phytochemical analysis of extract

The prepared extracts were subjected to phytochemical screening as per Shaikh and Patil, (2020) [18] to know the presence of secondary metabolites which includes alkaloids, carbohydrates, glycosides, flavonoids, tannins, saponins, proteins, amino acids, sterols and triterpenoids.

Estimation of total phenolic content (TPC)

The phenolic compound concentration in the alcoholic extract of *Alpinia officinarum* was assessed through the Folin - Ciocalteu method according to method of Madaan *et al*., (2011) [12].

Estimation of Total Flavonoid Content (TFC)

Total flavonoid contents in the ethanolic extract of *Alpinia officinarum* was determined by aluminium chloride colorimetric assay as described by Phuyal *et al*., (2020) [14].

Determination of *in vitro* antioxidant activity

2, 2 Diphenyl-1-picrylhydrazyl radical (DPPH) Scavenging Activity

The DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay is widely employed in antioxidant studies involving natural products due to its simplicity and sensitivity. This method is based on the principle that a substance acting as a hydrogen donor qualifies as an antioxidant (Akar *et al*., 2017) [2].

***In vitro* study of goat red blood cells**

The *in vitro* study was carried out in red blood cells of anaemic goats brought to Veterinary Clinical Complex, Orathanadu, and Thanjavur. Goats with clinical signs of pale mucous membrane and haematology showing haemoglobin below 8 g/dl were selected for the study. The study was approved by Institutional Animal Ethics Committee, Veterinary College and Research Institute, Orathanadu, Thanjavur-614625.

Collection of blood samples

3 ml of peripheral blood was collected from jugular vein puncture of apparently normal and anaemic goats using EDTA-sodium salt as the anticoagulant. Blood was centrifuged at 2000 rpm for 10 minutes. Plasma and buffy coat were removed. Subsequently the cells were washed three times with phosphate buffered saline (PBS), pH 7.2. The resultant RBC was incubated with *Alpinia officinarum* extract (0.2mg, 0.4mg, 0.5mg, 0.8mg and 1mg/ml), standard antioxidant quercetin (1mg/ml) and without extract as negative control at 37 °C for 30 minutes as per the method described by Luqman S *et al*. 2009 [11]. The antihemolytic activity was examined by the method of Naim *et al*., 1997 [13]. As per Rehman, S. (1984) [15], lipid peroxidation (LPO) was assessed in terms of malondialdehyde (MDA) production using the level of Thiobarbituric acid-reactive substances (TBARS). Reduced Glutathione (GSH) level was determined according to the method of Sedlak and Lindsay, (1968) [16]. By estimating free-SH groups using 5-5' dithiobis 2-nitrobenzoic acid (DTNB) to produce a compound that absorbs light at 412 nm, reduced glutathione (GSH) was calculated.

Statistical analysis

Data were analyzed using Graph Pad Prism version 4.00 (San Diego, California, USA). Results are expressed as Mean ± SEM. Differences in values were considered statistically significant at $p<0.05$ or $p<0.01$ (Snedecor GW and Cochran WG, 1989) [20].

Results and Discussion

In the present study, yield of *Alpinia officinarum* was 8.75% paralleling with the results of Abdul S *et al.* 2019 [1]. Results of the phytochemical screening of the *Alpinia officinarum* leaves extracts were obtained using various chemical tests to identify the presence of specific phytochemical constituents. The phytochemical screening showed that extract contained carbohydrates, phenols, tannins, terpenoids, glycosides, and alkaloids (Table 1). Proteins were notably absent in *Alpinia officinarum in* ninhydrin test.

The total phenolic content was measured by Folin-ciocalteu reagent in terms of gallic acid equivalent (GAE). The value obtained for the concentration of total phenols in the alcoholic extracts of *Alpinia officinarum*, was found to be 29.92±0.19 mg/g (Table 2). In a study by Srividya *et al*. 2010 [21] where they found total phenol content of 30.6 (mg GAE/g) in methanolic extract of *Alpinia officinarum*. Studies have also demonstrated that the phenolic composition of *AO* extract is closely related to and dependent upon its antioxidant activity.

The flavonoid content was expressed in terms of quercetin equivalent. The concentration of flavonoid in the alcoholic extracts of *Alpinia officinarum, was* found to be 12.64±0.25 mg/g (Table 2). In a study by Srividya *et al*. 2010 [21], where they observed total flavonoid content of 27.64mg QE/g in methanolic extract of *Alpinia officinarum*.

The *In-vitro* antioxidant assay conducted on the alcoholic extract of *Alpinia officinarum* demonstrated the presence of antioxidant potential. The results indicated a concentration-dependent scavenging of free radicals by the test compounds. At 500 µg /ml the maximum percentage of inhibition (86.56%), The IC50 value was found to be 75.31µg/ml suggested that the *Alpinia officinarum* effectively neutralized free radicals, highlighting its antioxidant activity in a concentration-dependent manner that is depicted in the table 3.

The present study indicated that *in vitro* anti-hemolytic activity of ethanolic extracts of *Alpinia officinarum* rhizome shown in Table 4. In the present study, H_2O_2 caused the *in vitro* destruction of RBC membranes. The maximum protection of RBC membrane lysis (46.33±0.33) was observed in ethanolic extract of *Alpinia officinarum* rhizome. These results were compared with standard drug quercetin (63±1.52 at 1mg/ml). The minimum RBC protection

(19.17±0.6) was observed at 0.2mg of *Alpinia officinarum* rhizome extract. Anti hemolytic activity was increased with increasing concentration of plant extracts.

Oxidative stress, defined as an imbalance between the production of reactive oxygen species (ROS) and the antioxidant defense mechanisms, has a major impact on the pathophysiology of red cell diseases. Due to their unique combination of methemoglobin reductase, glutathione/ glutathione peroxidase, superoxide dismutase, catalase, and membrane-bound alpha-tocopherol, erythrocytes are well suited to degrade reactive oxygen species (Halliwell & Gutteridge, 1986) [7].

In the present study, erythrocytic GSH were significantly decreased in anemic goats as compared to standard antioxidant quercetin. Quercetin administration protects sickle cell anemia patients' erythrocytes from hemoglobin oxidation and peroxide-induced cellular changes, according to in vitro research. (Henneberg R *et al*., 2013) [8]. Incubation of Red blood cells *in vitro* with *Alpinia officinarum* rhizomes (0.8mg and 1mg) for 30 minutes significantly increased GSH level (Table 5). Lower extract concentrations did not significantly shield erythrocyte GSH levels from oxidative stress brought on by anemia in goats.

Reduced glutathione is one of the main intracellular nonprotein sulfhydryl compounds and has several biological functions. One of the functions is to maintain membrane protein -SH groups in their reduced form, since their oxidation can change the structure and function of cells (Di Simplicio *et al*., 1996) [5]. Under physiological and pathological oxidative stress, membrane-SH group oxidative damage may be a significant molecular mechanism causing changes in the membrane micro elasticity or whole cell deformability of erythrocytes (Wang *et al*., 1999) [23].

During oxidative stress, the RBC membrane is vulnerable to lipid peroxidation, which results in the production of MDA, a biomarker that is used to investigate the oxidation of lipids in several situations. In the present study, anaemia caused an increase in MDA concentration above the basal level in erythrocytes. MDA concentration significantly decreased in quercetin (1mg/mL) treated anaemic erythrocytes and thereby limiting MDA formation in erythrocytes (Table 6). This implies that quercetin may prevent the production of peroxynitrite radicals.

The plant extracts, when present in the incubation medium at a concentration of 0.8mg and 1mg/mL, were found to protect the erythrocytes from the anaemia induced oxidative stress. When compared to normal erythrocytes, stressed erythrocytes exhibit altered levels of MDA and GSH concentration, which are markers of an increased pro-oxidant/antioxidant ratio. Elevated erythrocyte MDA concentration, according to Bryszewska *et al*. (1995) [3], decreases the membrane fluidity of the lipid bilayer, which can result in long-term complications in diseases like diabetes, hypertension, atherosclerosis, cardiovascular disease, cancer, and neurological disorders. (Halliwell & Gutteridge, 1990; Krouf *et al*., 2003; Siemianowicz *et al*., 2003) [7, 9, 19].

The presence of polyphenols, tannins, anthocyanins, and glycosides in *Alpinia officinarum* extracts may have the ability to scavenge free radicals, activate antioxidant enzymes, or inhibit oxidases. These compounds may also be responsible for the protective effects of the extracts on erythrocyte GSH and MDA concentration in anemic goats. Additionally, *Alpinia officinarum's* ethanolic extract has strong antioxidant properties. Our findings agree findings of Vijayabharathi *et al*. 2017 [22] who reported protective effect of *Alpinia officinarum* extract against dextran sulphate sodium induced anaemia in rat model.

Conclusion

Alpinia officinarum alcoholic extracts significantly protect the erythrocytes in anemic goats. Because the extracts shield erythrocytes from oxidative stress, baseline levels of MDA and GSH concentrations can be maintained. Medicinal plant extracts have been demonstrated to provide protection against erythrocyte GSH and MDA oxidation because of their high concentration of antioxidant-rich compounds, including polyphenolics, anthocyanins, tannins, and glycosides. Consequently, *Alpinia officinarum's* ethanolic extract has therapeutic potential for treating anaemia in goats. To isolate, characterize, and identify the active phytoceuticals causing the antioxidant activity in animal models, more research is required.

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