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Effect of *Centella asiatica* leaf extract on red blood cells of anaemic goats: An *in-vitro* study

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Abstract
The objective of the present study was to investigate the effect of *Centella asiatica* leaf extract on red blood cells of anaemic goats *in vitro*. Phytochemical screening of alcoholic extract of *Centella asiatica* revealed the presence of carbohydrates, phenols, tannins, terpenoids, glycosides, flavonoids and alkaloids. Total phenolic and total flavonoids of CA leaves were 5.50 ± 0.29 mg GAE /g and 23.00 ± 0.58 mg mg QE /g. *Centella asiatica* leaves is not hemotoxic in normal goat red blood cells. Free radical generation due to anaemia induced oxidative stress was restored with treatment with CA leaves in red blood cells of anaemic goats. Our data revealed that the extract exhibits an *in-vitro* protective effect on erythrocytes in anaemic goats.

Keywords: *Centella asiatica*, goat, red blood cells, antioxidant, *in vitro*

Introduction
The potential benefits of medicinal plant extracts on human health are being investigated extensively. Many traditional medical systems, such as Ayurveda, Siddha, Unani, homeopathy, and folklore from various countries, are prepared using plants. The primary plant constituents believed to possess medicinal properties include flavonoids, coumarins, phenolic acids, and antioxidant micronutrients such as magnesium, zinc, and copper (Seth & Sharma, 2004) [18].

Free radicals such as reactive oxygen species (ROS) induce oxidative stress, which appears to be the fundamental mechanism underlying degenerative diseases like diabetes, viral infections, autoimmune pathologies, and most likely aging. According to research, antioxidant compounds found in food and medicinal plants can be used to scavenge ROS as a means of chemoprevention. (Finkel & Holbrook, 2000) [5].

Several previous reports highlight the protective effect of plant extracts against oxidative damage, including on erythrocytes (Luqman S *et al*. 2009) [11]. *C. asiatica* extracts have been shown to have a wide range of beneficial effects in Ayurvedic and Chinese traditional medicine, including the ability to heal wounds, preserve nerves, hearts, and livers, as well as have anti-inflammatory, antioxidant, anti-allergic, anti-depressant, anxiolytic, antifibrotic, antibacterial, anti-arthritic, anti-tumor, and immunomodulatory properties. (Bandopadhyay *et al*., 2023) [2]. However, to the best of our knowledge, no studies have been performed so far on the effect of *Centella asiatica* leaf extract on anaemic goat red blood cells (RBCs) *in vitro*.

Materials and Methods
Plant materials
Centella asiatica was sourced from the Ethnoveterinary Herbal Products Research and Development Centre, Veterinary College and Research Institute, Orathanadu. The plant material in this study were authenticated by the Siddha Central Research Institute, Central Council for Research in Siddha, Ministry of AYUSH, Government of India, Arumbakkam, Chennai. This authentication process ensured the botanical identity and quality of the plant samples, contributing to the scientific validity of the study. The reference herbarium specimen was deposited in the Herbarium, Ethno Veterinary Herbal Product Research and Development Centre, VCRI, Orathanadu, Thanjavur under the voucher number 24/ EVHPR&D Centre/2023.

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, methanol was used as solvent for the separation of active principle. The plant materials, after being shade-dried, were ground into coarse powder to facilitate efficient Soxhlet extraction. This step was crucial to prevent the formation of fine powder and maximize the extraction yield. The coarse powder was then carefully packed into a thimble and positioned above a round-bottom flask. A condenser was placed above the thimble to condense the evaporating vapor back into the thimble. The resulting extractive liquid, containing the active constituents, was subjected to further processing using a rotary film evaporator. This process effectively removed the alcoholic solvent, resulting in the collection of a pure extract that was completely free from any residual solvent. The final extract was stored at 4 °C for further usage.

Qualitative phytochemical screening

The prepared extracts were subjected to phytochemical screening as per Shaikh and Patil, (2020) [19] 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 *Centellas asiatica* 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 alcoholic extract of *Centellas asiatica* was determined by aluminium chloride colorimetric assay as described by Phuyal *et al*., (2020) [14].

Determination of *Invitro* 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) [1].

***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, 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

About 3 ml of peripheral blood was obtained from jugular vein puncture of apparently normal and anaemic goats by 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 *Centella asiatica* extract (0.2 mg, 0.4 mg, 0.5 mg, 0.8 mg and 1 mg/ml), standard antioxidant quercetin (1mg/ml) and without extract as negative control at 37 ^{0}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]. Lipid peroxidation (LPO) was evaluated in terms of malondialdehyde (MDA) production by using thiobarbituric acid-reactive substances (TBARS) level according to the method of Rehman, S (1984) [16]. Reduced Glutathione (GSH) level was determined according to the method of Sedlak and Lindsay, (1968) [17]. Reduced glutathione (GSH) was determined by estimating free-SH groups, using 5-5' dithiobis 2- nitrobenzoic acid (DTNB) to give a compound that absorbs light at 412nm.

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) [21].

Results and Discussion

In the present study, yield of *Centella asiatica* was 43.86% paralleling with the results of Idris *et al.* (2021) [9]. Results of the phytochemical screening of the *Centella asiatica* 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 *C.asiatica 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 *Centella asiatica,* was found to be 5.50 ± 0.29 mg/g (Table 2). In a study by Quyen *et al*. 2020 [15] where they found total phenol content of 2.14 ± 0.29 (mg GAE/g) in ethanolic extract of *Centella asiatica*. Studies have also demonstrated that the phenolic composition of *CA* 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 *Centella asiatica, was* found to be 23.00 ± 0.58 mg/g (Table 2). Our findings are in accordance with the findings of Quyen *et al*. 2020 [15], where they observed total flavonoid content of 23.03 ± 2.89 mg QE/g in ethanolic extract of *Centella asiatica* leaves.

The *In-vitro* antioxidant assay conducted on the alcoholic extract of *Centella asiatica* 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 (88.80%), The IC50 value was found to be 72.31 µg /ml suggested that the *Centella asiatica* 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 *Centella asiatica* leaf shown in Table 4. *In vitro* RBC membrane destruction was induced by H_2O_2. The maximum protection of RBC membrane lysis (52 ±1.15) was observed in ethanolic extract of *Centella*

asiatica leaf. These results were compared with standard drug quercetin (63 ± 1.52 at 1mg/ml). The minimum RBC protection (21 ±0.57) was observed at 0.2 mg of *Centella asiatica* leaf extract. Anti hemolytic activity was increased with increasing concentration of plant extracts.

The pathophysiology of red cell diseases is significantly influenced by oxidative stress, which is defined as an imbalance between the generation of reactive oxygen species (ROS) and the antioxidant defense mechanisms. Because they contain methemoglobin reductase, glutathione/glutathione peroxidase, superoxide dismutase, catalase, and membrane-bound alpha-tocopherol, erythrocytes are special cells that 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. Research conducted *in vitro* has demonstrated that administering quercetin to sickle cell anemia patients' erythrocytes can shield them from peroxide-induced cellular modifications and hemoglobin oxidation (Henneberg R *et al*., 2013) [8]. Incubation of Red blood cells *in vitro* with *Centella asiatica* leaves (0.8 mg and 1 mg) for 30 minutes significantly increased GSH level (Table 5). Lower concentration of extracts did not show significant protection of GSH concentration in erythrocytes against the anaemia induced oxidative stress in goats.

One of the primary intracellular nonprotein sulfhydryl compounds, reduced glutathione serves a variety of biological purposes. One of these functions is the preservation of membrane protein -SH groups in their reduced form, as their oxidation can result in changes to cellular structure and function (Di Simplicio *et al*., 1996) [4]. Under circumstances of physiological and pathological oxidative stress, membrane-SH group oxidative damage may be a significant molecular mechanism causing changes in the membrane microelasticity or whole cell deformability of erythrocytes (Wang *et al*., 1999) [22].

Under oxidative stress, the erythrocyte membrane is vulnerable to lipid peroxidation, which results in the production of MDA, a biomarker used to investigate the oxidation of lipids under various circumstances. In the present study, anaemia caused an increase in MDA concentration above the basal level in erythrocytes. MDA concentration significantly decreased in quercetin (1 mg/mL) treated anaemic erythrocytes and thereby limiting MDA formation in erythrocytes (Table 6). This suggests that quercetin may inhibit peroxynitrite radical formation.

The plant extracts, when present in the incubation medium at a concentration of 0.8 mg and 1 mg/mL, were found to protect the erythrocytes from the anaemia induced oxidative stress. Alteration in the normal level of MDA and GSH concentration in stressed erythrocytes are indicators of an increased pro-oxidant/antioxidant ratio compared with normal erythrocytes. According to Bryszewska *et al*., 1995 [3] elevated erythrocyte MDA concentration reduces the lipid bilayer's membrane fluidity, which can lead to long-term complications in conditions like diabetes, hypertension, atherosclerosis, cardiovascular disease, cancer, and neurological disorders (Halliwell & Gutteridge, 1990; Krouf *et al*., 2003; Siemianowicz *et al*., 2003) [7, 10, 20].

The protective effects of *Centella asiatica* extracts on erythrocyte GSH and MDA concentration in anaemic goats may be attributed to the presence of polyphenols, tannins, anthocyanin, and glycosides which either have the capacity to scavenge free radicals or activate antioxidant enzymes or inhibit oxidases. Further, the methanolic extract of *Centella asiatica* possess good antioxidant activity. Our findings agree findings of Ghosh *et al*. 2017 [6] who reported protective effect of *Centella asiatica* extract against isoniazid induced oxidative stress in rat erythrocytes.

Table 1: Phytochemical screening of methanolic extract of leaves of *C. asiatica*

Herbal plant	Carbohydrate	Proteins and Amino acids	Alkaloids	Glycosides	Flavonoids	Phenolic compounds and tannins	Sterols and Triterpenoids
Methanolic extract of *Centella asiatica*	+	-	+	+	+	+	+

Table 2: TPC and TFC of methanolic extract of leaves of *C. asiatica*

Herbal plant	Total polyphenol content (mg GAE/g)	Total flavonoid content (mg QE/g)
Methanolic extract of *Centella asiatica*	5.50 ± 0.29 mg/g	23.00 ± 0.58 mg/g

Table 3: DPPH Scavenging Activity and Percentage of Inhibition

Tested sample concentration (µg/ml)	Percentage of inhibition
Ascorbic acid (Standard)	92.87
500 µg/ml (CAE)	88.80

Table 4: Anti-haemolytic activity of methanolic extract of leaves of *C. asiatica*

CA extract	0.2 mg/ml	0.4 mg/ml	0.6 mg/ml	0.8 mg/ml	1 mg/ml	Quercetin (1 mg/mL)
% Anti haemolytic activity	21±0.57	34±0.58	40.67±0.67	46±0.57	52±1.15	63±1.52

Table 5: Effect of *Centella asiatica* leaf extract on erythrocytic GSH in anaemic goats

Parameter	0.2 mg/ml	0.4 mg/ml	0.6 mg/ml	0.8 mg/ml	1 mg/ml	Negative control	Quercetin (1 mg/mL)
GSH	2.97±0.09	2.94±0.03	2.78±0.03	2.45±0.10[a]	2.08±0.08 [a]	3.12±0.07	1.03±0.12 [a]

Table 6: Effect of *Centella asiatica* leaf extract on erythrocytic LPO in anaemic goats

Parameter	0.2 mg/ml	0.4 mg/ml	0.6 mg/ml	0.8 mg/ml	1 mg/ml	Negative control	Quercetin (1 mg/mL)
LPO	6.23±0.04	6.21±0.03	6.12±0.04[a]	6.02±0.04 [a]	5.49±0.03 [a]	6.44±0.07	2.57±0.12 [a]

[a] significant difference ($p<0.01$) compared to negative control

Conclusion

In anemic goats, the erythrocytes are considerably protected by the alcoholic extracts of *Centella asiatica*. By shielding erythrocytes from oxidative stress, the extracts can keep the concentrations of MDA and GSH at baseline levels. The protective effect of medicinal plant extracts against erythrocyte GSH and MDA oxidation may be attributed to their high content of polyphenolics, anthocyanins, tannins, and glycosides, which are known for their antioxidant properties. Therefore, the methanolic extract of *Centella asiatica* can be used as a drug for the treatment of anaemia in goats. Future research is necessary to isolate, characterize, and identify the active phytoceuticals that are responsible for the antioxidant activity in animal models.

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