Original Article

# *In vitro* Antihemolytic Activity of Gymnema Sylvestre Extracts Against Hydrogen Peroxide ($H_2O_2$) Induced Haemolysis in Human Erythrocytes

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## ABSTRACT

Gymnema sylvestre (*Asclepiadaceae)* is a good source of large number of bioactive substances. It has deep roots in history, being one of the major botanicals used in traditional medicine to treat conditions ranging from diabetes, malaria, to snakebites. This study was conducted to quantitatively evaluate anti-haemolytic activity of aqueous extract of stem, leaf and flower parts of this plant, against hydrogen peroxide induced haemolysis using human erythrocyte in an *in vitro* assay. Prior to the addition of $H_2O_2$ to induce haemolysis, different concentrations (50-500 mg/ml) of the extract was added to 2ml of 4% erythrocyte suspension and allowed to incubate for 5 minutes at room temperature. The mixture was centrifuged and the colour density of the supernatant was measured spectrophotometrically. Quercetin was used as standard. The percentage haemolysis and $IC_{50}$ values were calculated. The extracts were potent against haemolysis of the erythrocyte in concentration dependent manner. The leaf extract exhibited the highest anti-haemolytic effect with $IC_{50}$ = 29.83 mg/ml followed by the flower with $IC_{50}$ = 34.96 mg/ml and the least was the stem with $IC_{50}$ = 37.75 mg/ml. the $IC_{50}$ value of Quercetin was 386.72 mg/ml. The lower the $IC_{50}$ the more protection offered against haemolysis by the extracts. These results suggest that the plant extracts are better anti-haemolytic agents and offered significant biological action compared with standard compound used.

**Keywords**: Quercetin, *Gymnema sylvestre*, Anti-haemolytic activity, Erythrocytes hydrogen peroxide.

## INTRODUCTION

A medicinal plant is any plant which in which one or more of its organs, contain substance that can be used for therapeutic purposes or which are precursors for chemo-

pharmaceutical semi-synthesis. Healing with medicinal plants is as old as mankind. The use of medicinal plants as fundamental component of the African traditional health care system is perhaps the oldest and most assorted of all the therapeutic systems. In any part of rural Africa, traditional healers prescribing medicinal plants are the most easily accessible and affordable health resources available to the local community and at times, the only therapy that subsists[1].

Many plants have been known to produce biologically active substances, some of which are related to special flavour or taste and others are found to be useful as antioxidants and or antimicrobial agents. Antioxidants decrease oxidative damage to cells and bio-molecules caused by reactive oxygen/nitrogen species (ROS/RNS)[2].

During the past years, ROS and RNS have been implicated in the oxidative deterioration of food products as well as in the pathogenesis of several human diseases such as atherosclerosis, diabetes mellitus, chronic inflammation, neurodegenerative disorders, including Alzheimer's disease, and certain types of cancer[3].

The putative protective effects of antioxidants against these deleterious oxidative stress-induced diseases have received increasing attention in recent times, especially within biological, medical, nutritional and agrochemical areas. Among the dietary antioxidants, phenolic compounds, secondary metabolites from plants, are the most abundant natural antioxidants [2]. Phenolics act as antioxidants in a number of ways such as reducing agents, hydrogen donors, free radical scavengers and singlet oxygen quenchers and therefore act as cell saviours[4].

Erythrocytes, which are the most abundant cells in human body, possessing desirable physiological and morphological characteristics, are exploited extensively in drug delivery[5]. Oxidative damage to the erythrocyte membrane (lipid/protein) may be implicated in haemolysis associated with some hemoglobinopathies, oxidative drugs, transition metal excess, radiation, and deficiencies in some erythrocyte antioxidant systems[6].

This assay is useful either for screening studies on various molecules and their metabolites, especially on one hand, molecule having an oxidizing or anti-oxidizing activity or on the other hand, molecule having a long term action[7].

*Gymnema sylvestre* R.Br (*G. Sylvestre*) is a perennial, woody climber belonging to the class dicotyledonous of the family *Asclepiadaceae* or the "milk weed" family[8]. The plant is a good source of a large number of bioactive substances[9]. Traditionally, the leaves of *G. sylvestre* were used for the treatment of diabetes, and other disorders, while the flowers and bark are given in diseases related to phlegun[10]. Reports in the ancient literature suggested that the plant has multiple medicinal applications, namely, antihemolytic, antipyretic, astringent, an alexipharmic, anodyne, cardiotonic, liver tonic, digestive, diuretic, cough dyspepsia, laxative, stimulant, etc. The root bark is useful as an emetic, expectorant and analgesic for body ache and root juice in the treatment of snakebite[11]. The plant also exhibits medicinal importance in the treatment of constipation, cardiopathy, asthma, bronchitis, amenorrhoea, conjunctivitis, renal and vesical calculi etc [11].

The leaves of *G. sylvestre* contain triterpene saponins belonging to oleane and damarane classes. The major constituents like gymnemic acids and Gymnema saponins are members of oleane type of saponins while gymnema sides are dammarane saponins[12,13].

Lipid peroxidation processes in living organisms lead to many pathological events, including changes in physicochemical properties of cell membrane[14]. Hence, membrane protection against peroxidation is a very important task and much time has

recently been devoted to find and apply natural and synthetic compounds of anti oxidation properties[15].

In this study, an attempt has been made to determine anti-haemolytic activity of the aqueous extract of stem, leaf and flower of *G. sylvestre* plant against hydrogen peroxide ($H_2O_2$) induced - haemolysis in human erythrocyte in an in vitro assay and also compare the level of inhibition of haemolysis among the various extracts.

## MATERIALS AND METHODS

### Chemicals and reagents

Quercetin, $H_2O_2$ (Sigma Chemicals Co. St. Louis, MO, USA). Normal Saline (0.9% NaCl) Phosphate buffered – PBS (0.2M/pH 7.4) Distilled water ($H_2O$).

### Collection of plant material

Stem, leaf and flower of *G. sylvestre* were collected green and fresh from a farm land at Ajaokuta, Ajaokuta Local Government Area in the Central Senatorial District of Kogi State, Nigeria. The plant had already been authenticated by Dr. William D. Hawthrone, a James Martin Research fellow, plants for the 21st Century, Department of Plant Science, University of Oxford.

The collected plant parts were rinsed with clean water to remove dirt particles. The leaves and flowers were plucked from the stem and were separately spread and air-dried under shade at room temperature for three weeks.

### Preparation of plant extracts

The air-dried, leaves and flowers were pulverized into powder using an electric blender while the stem was first pounded into semi-powder with mortar and pestle, which was further pulverized into fine powder utilizing mechanical grinder. Cold extraction method was used to obtain aqueous extracts of the samples. Portions (197.7g) of leaf, 271.15g of powdered stem and 103.89g of powdered flower were soaked in different containers with 2000ml of distilled water each.

They were properly stirred and left for four (4) days with continuous stirring each day. The mixtures were then filtered using the high pressure vacuum pump machine. The filtrates in beakers were concentrated by evaporation at $60^0$C to dryness in a water bath.

### Preparation of Erythrocyte Suspension and Determination of Anti-haemolytic Activity

Erythrocyte suspension was prepared as described by Naim[16]. Human blood sample was purchased from the blood bank of Grimad Hospital, Anyigba, Nigeria, and was centrifuged at 1000 x g for ten minutes and erythrocytes were separated from the plasma and were washed three times. The erythrocytes separated were then diluted with phosphate buffered saline (0.2M, pH 7.4) to give 4% suspension. To 2ml of the erythrocyte suspension, 50-500 mg/ml of extract of buffered saline was added and the volume was made up to 5ml with buffered saline. The mixture was incubated for 5 minutes at room temperature and then 0.5 ml of $H_2O_2$ solution in buffered saline was added to induce oxidative degradation of the membrane lipid (haemolysis).

In another set, quercetin (50-500mg/ml) was taken as a reference compound and treated in the similar manner. Therefore, the tubes were centrifuged at 1000 x g for 10 min and the colour density of the supernatant was measured spectrophoto-metrically at 580nm.

To obtain 100% haemolysis (control), 2 ml of distilled water was added to 2 ml of RBC suspension. The relative haemolysis in the control, which was taken as 100%.

Inhibitory activity of the extracts on haemolysis was calculated and expressed as percent haemolysis.

% Hemolysis = Absorbance of control – absorbance of extract / Absorbance of control X 100.

## RESULTS

Table 1: shows the anti-haemolytic activity of the extracts of *G. sylvestre* in the presence of toxicant – $H_2O_2$ where quercetin was taken as reference standard. From the table, RBC treated with $H_2O_2$ along with the stem, leaf and flower extracts, a marked reduction in haemolysis was observed. Haemolysis of the RBC decreased with increase in extract and quercetin concentrations. The leaf extract showed the highest anti-haemolytic activity.

## DISCUSSION

Medicinal herbs are in use for thousands of years and are renowned for their effectiveness in many diseases. These natural herbs are very effective in boosting the immune system, increasing the body's resistance to infections, healing the allergies, raising and renewing the body's vitality[17].

Phytochemicals present in commonly consumed plant foods are normally non-toxic and have the potential of preventing chronic diseases. The plant extracts encompasses high concentration of flavonoids and phenolic compounds. The multiple properties of these phytochemicals have made them more attractive, as they can modulate various aspect of disease like lipid peroxidation involved in atherogenesis, thrombosis, carcinogenesis, hepatotoxicity and variety of disease conditions[18].

Plant ingredient is an important source of natural products which differ broadly in their structures, biological properties and mechanism of action.

Different phytochemical components especially polyphenols, flavonoids, phenolic acid etc are responsible for the free radical scavenging and antioxidant activity of the plants. Polyphenols possess many biological effects, mainly attributed to their antioxidant activities in scavenging free radicals, inhibition of peroxidation and chelation of transition metals[19].

Erythrocytes are considered as major target for the free radicals owing to the presence of both high membrane concentration of polyunsaturated fatty acids (PUFA) and the oxygen transport associated with redox active hemoglobin molecules, which are potent promoters of activated oxygen species[20].

The extent of haemolysis was found to be much greater, when red blood cells were treated with hydrogen peroxide (toxicant). This could be attributed to the oxidizing nature of hydrogen peroxide with respect to the destruction of cell membrane and subsequent liberation of haemoglobin from the cells. Mobilization of $Fe^{2+}$ by $Ca^{2+}$ via fenton reaction is also caused due to hydrogen peroxide which further leads to the production of OH radicals[21].

All these factors, in unison, cause deterioration of cell membrane, which way, perhaps, be the key episode of the lyric of cell[22]. Nevertheless, the anti-haemolytic activity is the expression of collaborative action of the various antioxidant mechanisms which function in nature.

The application of the extract of *G. sylvestre* plant parts showed generally a remarkable effect in inhibiting haemolysis. When red blood cells were treated with the extracts along with $H_2O_2$, marked reduction in haemolysis was found (Table 1). This may be because of radical scavenging activity of the bioactive components of the extracts showing potent anti-haemolytic nature of the extracts. The result shows that the protective effect of all the extracts against haemolysis increased with increase in concentration. Each extract exhibited a correspondingly increasing anti-haemolytic activity with increase in their concentration.

Scavenging of $H_2O_2$ by *G. sylvestre* extract may be attributed to their phenolics and other active components, which can donate electrons to $H_2O_2$, thus neutralizing it to water molecule[23]. The extracts were capable of scavenging $H_2O_2$ in a concentration dependent manner. The leaf extract exhibited the highest anti-haemolytic activity with $IC_{50}$ value of 29.83 mg/ml.

Although quercetin being the standard had lower haemolytic activity with $IC_{50}$ = 386.72 mg/ml compared with those of the extracts it also showed a good anti-haemolytic activity. Anti-haemolytic activity of quercetin[24] and the relation between iron chelating activity against oxidative damage to erythrocyte membrane by flavonoids was previously reported[25]. The wide gap in potential between all the plant extracts (leaf, flower and stem) and the standard (quercetin) shows that the plant has a very high anti-haemolytic activity.

Order of increasing anti-hemolytic activity of test sample is as follows: Quercetin ($IC_{50}$ = 386.72 mg/ml) ⟶ Stem ($IC_{50}$ = 37.75 mg/ml) ⟶ Flower ($IC_{50}$ = 34.96 mg/ml) ⟶ Leaf ($IC_{50}$ = 29.83 mg/ml). This implies that the leaf extract has more phenolic compounds and flavonoids above the stem and flower extracts. In addition, the extracts of *G. sylvestre* leaf, flower and stem displayed potent anti-haemolytic activity due to the presence of flavonoids and glycosides[26] which can guard RBC from free radical mediated haemolysis[27]. Also, binding of flavonoids to the red blood cell membrane significantly inhibits lipid peroxidation and at the same time, enhances their integrity against lysis[28]. As expected, the compounds present in the various parts of *G. sylvestre* quenched $H_2O_2$ before it attacked the biomolecules of the erythrocyte membrane to cause oxidative haemolysis as reported by[29] on the effects of green tea.

Although hydrogen peroxide itself is not very reactive, it can sometimes cause cytotoxicity by giving rise to hydroxyl radical in the cell. Thus removing $H_2O_2$ is very important throughout food systems. The exact mechanism by which the *G. sylvestre* extracts reduced $H_2O_2$-induced haemolysis remains to be elucidated.

## CONCLUSION

The present study showed that the extracts have anti-haemolytic activity comparable to the reference standard – quercetin. All the extracts exhibited concentration – dependent inhibitory activity towards hydrogen peroxide-induced haemolysis of erythrocytes attributed to the bioactive constituents present in *G. sylvestre* parts which exert protective effects against oxidative injury to biological macromolecules like lipids and proteins in the erythrocyte membrane. Further work is needed to confirm the results obtained in an *in vivo* environment.

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### Conflict of interest statement

We all declare that we have no conflict of interest in this work.

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**Table 1.** Antihemolytic activity of aqueous stem, leaf and flower extracts of G. Sylvestre

| Plant parts | Extract conc. (mg/ml) | Log cong. | % Hemolysis | $IC_{50}$ |
|---|---|---|---|---|
| **Stem** | 50 | 1.6990 | 40.22 | 37.75[a] |
| | 100 | 2.0000 | 32.20 | |
| | 200 | 2.3010 | 29.99 | |
| | 300 | 2.4771 | 16.85 | |
| | 400 | 2.6021 | 10.02 | |
| | 500 | 2.6990 | -7.19 | |
| **Leaf** | 50 | 1.6990 | 25.89 | 29.83[b] |
| | 100 | 2.0000 | 21.83 | |
| | 200 | 2.3010 | 5.65 | |
| | 300 | 2.4771 | -15.41 | |
| | 400 | 2.6021 | -24.86 | |
| | 500 | 2.6990 | -41.03 | |
| **Flower** | 50 | 1.6990 | 43.30 | 34.96[c] |
| | 100 | 2.0000 | 32.77 | |
| | 200 | 2.3010 | 30.15 | |
| | 300 | 2.4771 | 24.71 | |
| | 400 | 2.6021 | 16.95 | |
| | 500 | 2.6990 | 4.37 | |
| **Quercetin** | 50 | 1.6990 | 78.53 | 386.72[d] |
| | 100 | 2.0000 | 66.36 | |
| | 200 | 2.3010 | 58.86 | |
| | 300 | 2.4771 | 53.98 | |
| | 400 | 2.6021 | 49.77 | |
| | 500 | 2.6990 | 46.43 | |

a Linear equation: $y = 41.227 x + 115.02$
b Linear equation: $y = 66.599 x + 148.28$
c Linear equation: $y = 32.79 x + 100.67$
d Linear equation: $y = 30.888 x + 129.92$

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**Figure 1.** *Gymnema sylvestre*