Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 5, Suppl 3, 2013

Research Article

A STUDY ON INVITRO ANTIINFLAMMATORY ACTIVITY OF A POLYHERBAL FORMULATION USING HRBC MODEL

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Received: 25 May 2013, Revised and Accepted: 18 Jun 2013

ABSTRACT

Objective: The aim of this research was to investigate the role of the methanolic extract of polyherbal formulation has powerful retardation effect on phenyl hydrazine induced hemolysis.

Methods: RBC has got the simplest structure and can be used as a very good model to detect the direct effect of a toxin on the cell membrane as well as protective effects by antidotes. Destabilization of the cell membrane in RBC can lead to lysis of the cell and release of haemoglobin in the medium. The extent of hemolysis can help us to reveal the extent of toxicity.

Results: The anti-hemolytic activity may be because of the presence of Phytochemicals such as flavoniods and tannins, which are believed to be potent antioxidants. The results of the present investigation indicate that the possibility of employing the polyherbal formulation extract as an antioxidant substance to ameliorate the oxidative damage of cells.

Conclusion: The extent of hemolysis is amelioriatiated by our polyherbal formulation by 61.52%.at 500μg/ml.

Keywords: Polyherbal formulation, Hemolysis, Phenylhydrazine, HRBC, Flavonoids.

INTRODUCTION

The imbalance of oxidants and antioxidants of the body leads to an oxidative stress resulting in destruction of unsaturated lipids, DNA, proteins and other essential molecules. Increasing evidence suggests that oxidative damage to cell components has a relevant pathophysiological role in several types of human diseases**[1]**. Free radicals have been reported to cause red blood cell lysis in patients with blood pathologies such as thalassemia **[22]**. The erythrocytes are highly susceptible to oxidative damage due to the high polyunsaturated fatty acid content of their membrane and the high cellular concentration of oxygen and hemoglobin, all of which are powerful promoters of oxidative processes **[6]**. Exposure of erythrocytes to free radicals leads to a number of membrane changes including lipid peroxidation**,** reduction in deformability **[15],** changes in cell morphology**,** protein cross-linking and fragmentation **[22]**. These are the most common configuration damage leading to lysis of red blood cells.

Focusing our attention on natural sources of antioxidants for the protection of the body from oxidative stress, we investigated the protective effect of the methanolic extract of a polyherbal formulation against free radical-induced hemolysis. It has numerous uses in popular folk medicine. Its leaves and roots serve as anti-rheumatic, anti-inflammatory, antiseptic and anti-diabetic remedies in Brazil **[1 ,16**]. In Guatemala, Caribe, Japan and India it has been used in inflammation, diabetes and stomach problems**.** Phytochemical studies revealed that polyherbal formulation contained alkaloids, saponins, glycosides, phenolic constituents, reducing sugars and free acids. The presence of 5-hydroxytryptamine in bitter melon has also been reported. The extract polyherbal formulation was reported to exhibit hypoglycemic activity comparable to that of tolbutamide**.** Treatment with bitter melon was found to lower blood glucose levels in animal and human studies **[11].**

The extract also demonstrated potent purgative effect and produced contractions of the guinea ileum **[21].** Other effects of bitter melon include dose- related analgesic activity in rats and mice**[4],**anti-inflammatory actions**,** and treatment for GI ailments, such as gas, ulcer, digestion, constipation, dysentery**[5,12],**or hemorrhoids**.** The plant has also been used for skin diseases (eg, boils, burns, infections, scabies, and psoriasis) and for its lipid effects and hypotensive actions. Bitter melon has also been used as an insecticide **[10, 12].** It exhibits genotoxic effects in Aspergillusnidulans **[20].**

MATERIALS AND METHODS

Chemicals

Sodium chloride, disodium hydrogen phosphate and potassium di hydrogen phosphate were obtained from S.D Fine- Chem. Ltd; Mumbai. Phenylhydrazine was obtained from Loba chemic PVT. LTD. Mumbai Methanol was obtained from S.D Fine- Chem LTD, Mumbai.

Plant material collection and Identification

Fresh plants were collected from karipatti herbal garden salem in dec 2012.It was identified botanically using a handbook of Indian Medicinal plant-Volume 4 by S.RaghunethaIyer – orient Longman PVT. LTD publication. Identification was authenticated by the Faculty of Biotechnology, M.G.R. College, Hosur.

Preparation of plant extract

About 200g of shade dried powdered materials of polyherbal formulation were exhaustively extracted with methanol. The residue was filtered and concentrated to a syrupy consistency. The extract was then stored in a dessicator until further use.

Table 1: Showing the polyherbal formulation combination of 10 medicinal plants

S. No.	Name of the plant	Common name	Family	Part of the plant used
1	*Azadiriachtaindica*	Neem	Myrtaceae	Leaves
2	*Curcuma longa*	Turmeric	Zingiberaceae	Rhizome
3	*Ocimumtenuiflorum*	Tulsi	Lamiaceae	Leaves
4	*Cassia auriculata*	Cassia	Ceasalpinaceae	Flowers
5	*Ficusbengalensis*	Aalam	Moraceae	Seed
6	*Trigonellafoenagraceum*	Fenugreek	Leguminasae	Seed
7	*Phyllanthusniruri*	Keelanelli	Euphorbiaceae	Leaves
8	*Phyllanthusemblica*	Nelli	Euphorbiaceae	Seed
9	*Tinosporacardifolia*	Seendal	Convolvulaceae	Root
10	*Abitulonindicum*	Tuthi	Malvaceae	Leaves

Preliminary Phytochemical Screening

Known amounts of the lyophilized powder were extracted with 95% methanol. All the solvents used were of analytical grade. The solvents were evaporated in a rotary evaporator at 40- 50°C under reduced pressure.

Known amounts of individual solvent – free extracts were suspended in water to obtain the desired concentration and subjected to qualitative phytochemical screening for the detection of alkaloids, phenols, glycosides, flavonoids, tannins, proteins, aminoacids, carbohydrates, anthraquinones, saponins, sterols and triterpenoids (Harborne, 1984). The phytochemical analysis of individual solvent free extracts revealed the presence of relatively more number of active ingredients in methanolic extract and hence methanolic extract was used in this study. However, solvents, which showed appreciable results alone, were not presented.

Investigation of Anti-Hemolytic Activity

Blood Sample

The normal; anticoagulated blood was collected from the students of vysya college, salem.

Preparation of RBC cell suspension

The collected anti-coagulated blood was washed several times with phosphate buffered saline to remove (protein) Buffy coat.3 ml of anticoargulated blood was mixed with 10 ml of phosphate buffered saline and then centrifuged at 1500-1800 rpm for 5 minutes. The supernatant was discarded. To the pellet, 10 ml of phosphate buffered saline was added centrifuged and discarded the supernatant. This washing was repeated for 3-4 times. Total volume of RBC was found by the formula,

Total volume = packed cell volume/designed cell concentration*100

Using the total volume, RBC suspension preparation was made

RBC suspension = Total volume-volume of packed cell

The suspension was prepared by using phosphate buffered saline, at a concentration of 5%.

Amelioration of phenyl hydrazine induced hemolysis:

To assess the efficacy of extracts in amelioration of phenyl hydrazine induced toxicity on human RBC, 4 sets of tubes containing 0.1ml of RBC suspension were prepared as mentioned below:

Control tubes containing only RBC suspension.

Tubes containing RBC suspension and phenyl hydrazine (1 to 500μg).

Control tubes containing RBC suspension and test compound (100-500μg)

Tubes containing RBS suspension and phenyl hydrazine (500μg) with varying concentration of test compound (100 to 500μg).

The volume of each tube is made up to 2ml with phosphate buffered saline in ordered to have the equal volume in all the tubes. The tubes were shaken gently and incubated at 37ºc for 4 hours with intermittent shaking. After that the tubes were centrifuged at 1000g for10 minutes and the colour density of the supernatant was measured spectrophotometrically at 540nm.The percent hemolysis was calculated using the formula below:

Percent hemolysis = Absorbance of the individual tube x 100
Absorbance with 100% haemolysis

To achieve 100 percent hemolysis, 1.9ml of distilled water was added to 0.1ml of RBC suspension.The percent retardation of test compound was calculated using the formula:

Percent retardation = A-B x 100
A

Where, A=phenyl hydrazine induced haemolysis.

B = haemolysis caused by concurrent addition of phenyl hydrazine and test compound

Statistical analysis

Test was carried out in triplicate. All results are expressed as mean + S.E.M. Statistical analysis was performed using Student's t test. P-values less than 0.05 were considered statistical significant.Linear regression analysis was used to calculate the IC50 values.

RESULTS

Results shown indicate that addition of phenyl hydrazine [1-500 μg/ml] to the RBC suspension caused significant ($P<0.05$) rise in hemolysis. The cell pellet in the bottom of the tubes reduced to reddish colored supernatant indicating hemolysis. The effect was concentration dependent. The present investigation clearly indicates that phenyl hydrazine causes hemolysis and toxicity to RBC.

The concurrent addition of phenyl hydrazine along with methanolic extract of polyherbal formulation (100-500 μg/ml) to the RBC suspension significantly ($P<0.05$) reduced phenyl hydrazine induced hemolysis. The protective effects of polyherbal formulation extract and reference standard ascorbic acid on the hemolysis induced by phenyl hydrazine are shown in figure presenting the percentage of hemolysis inhibition at various concentrations. IC50 of thepolyherbal formulation extract and ascorbic acid were 426.66 and 15.18μg/ml respectively. The polyherbal formulation extract showed maximum inhibitory effect 61.52%.at 500μg/ml.

Phytochemical analysis

Total yield of exrtract was 6.53% (w/w). The whole plant yielded triterpenoids, saponins, alkaloids, flavonoids, tannins and amino acids as major constitutents. (Table1)

Table 2: Phytochemical screening of extract

S. No.	Chemical Constituents	Test	Result
1	Test for alkaloids	Mayers Test	+
		Wagners Test	+
		Hagers Test	-
		Dragendroffs test	+
2	Test for saponins	Foam test	+
		Haemolytic test	-
		Bromine water test	+
		Legal's test	-
3	Test for triterpenoids	Salkowski test	+
		Liebermann Burdchard	+
4	Test for /carbohydrates	Molisch's test	+
		Barfoed's test	-
		Benedict's test	-
5	Test for Flavonoids	Ferric Chloride test	+
		Shinoda test	-
		Alkaline reagent test	+

		Lead Acetate test	+
6	Test for tannins	Ferric chloride test	-
		Gelatin test	-
		Lead acetate test	-
		Potassium dichromate	-
7	Test for amino acids	Ninhydrintes	+

+ Present , - Absent

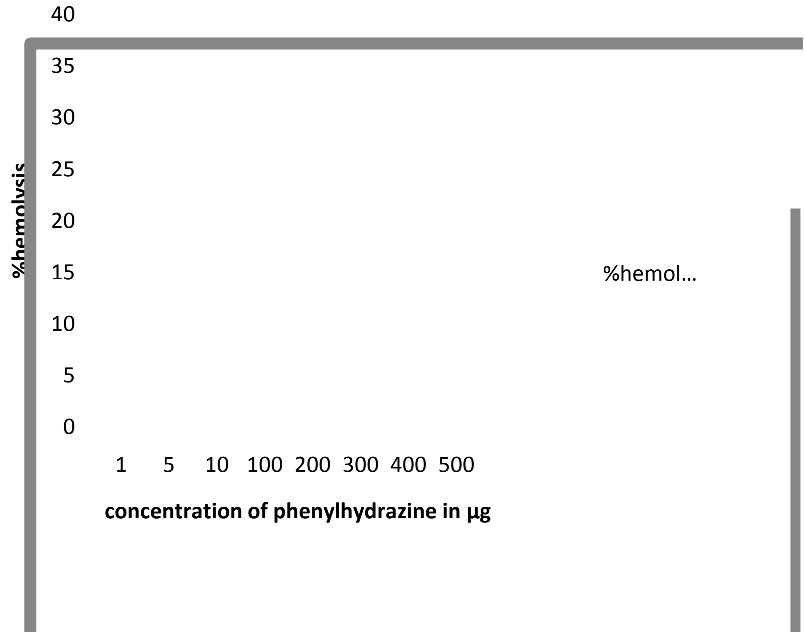

Fig. 1: Phenlyhydrazine induced hemolysis

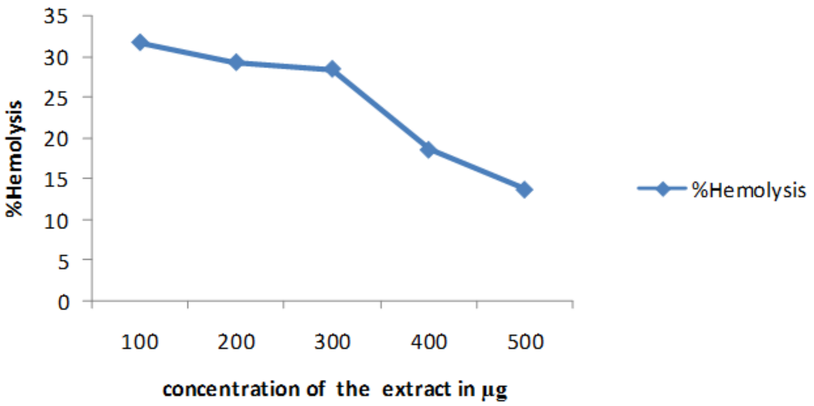

Fig. 2: Effect of methanolic extract of polyherbal formulation on phenylhydrazine induced hemolysis.

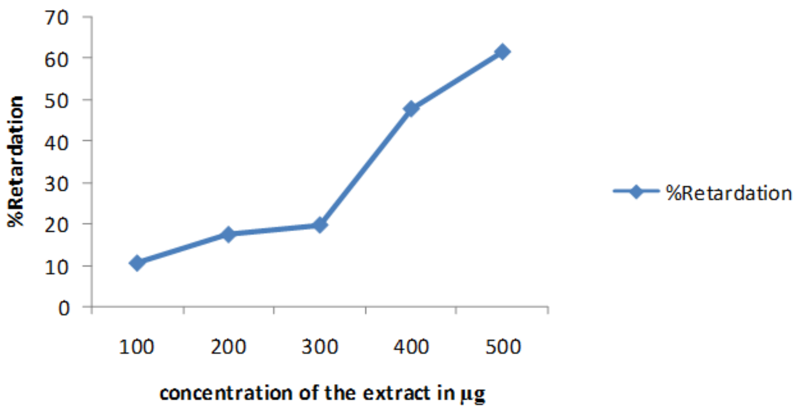

Fig. 3: Effect of methanolic extract of polyherbal formulation on phenylhydrazine induced Retardation

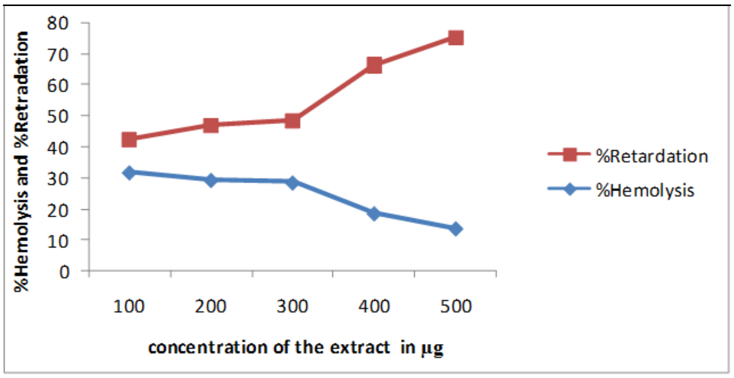

Fig. 4: Comparison on effect of methanolic extract of polyherbal formulation on phenylhydrazine induced hemolysis

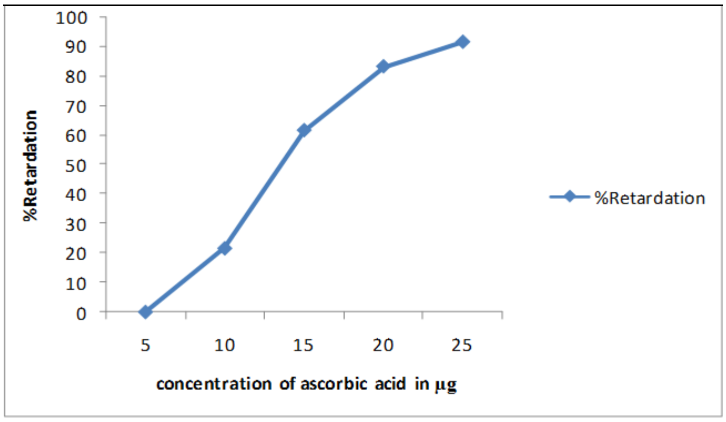

Fig. 5: Percentage retardation of ascorbic acid standard.

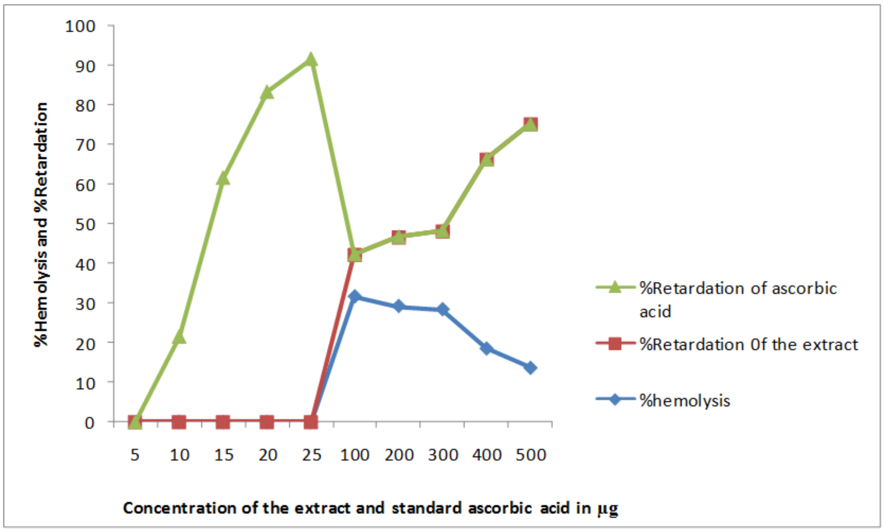

Fig. 6: Comparison between %Hemolysis, %Retardation of Polyherbal formulation extract and Standard Ascorbic acid

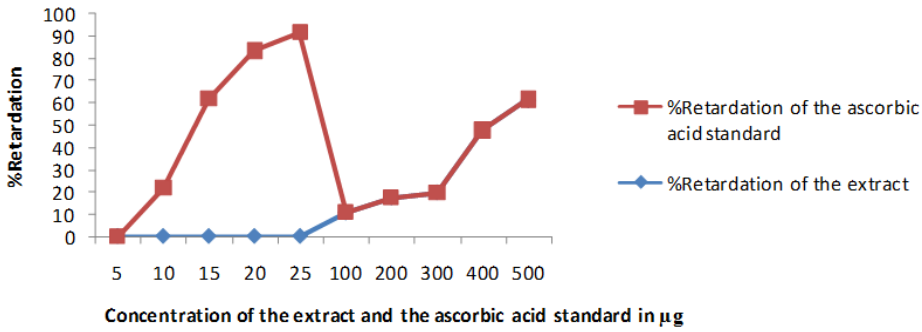

Fig. 7: Comparison between % retardation of the Polyherbal formulation extract and Standard ascorbic acid

DISCUSSION

The autoxidation of phenylhydrazine

Misra and Fridovich**[17]** showed phenylhydrazine to be stable in acid solutions but autoxidised in neutral and alkaline buffers. The oxidation was catalysed by traces of metal ion complexes, of which oxyhaemoglobin was the most effective. The scheme they proposed to account for the observations is shown below. Phenyl-diazene rapidly decays **[13]** giving traces of benzene and biphenyl. A kinetic analysis emphasized **[17]** the role of superoxide. Following initiation, these are true possible routes to phenyl diazene:

a> A superoxide-dependent propagation producing hydrogen peroxide.

b> A Super-independent autocatalysis via a benzadiazonium cation intermediate.

Therefore, after an initial lag phase, the autoxidation of phenylhydrazine to phenyldiazene may not be effectively inhibited by superoxide dismutase (SOD), and may indeed be stimulated by (SOD) **[17].**

Mechanism of phenylhydrazine induced hemolysis

ROS production was associated with extensive binding of oxidized and denatured hemoglobin to the membrane cytoskeleton. Phenyl hydrazine-induced hemolytic injury seems to be derived from oxidative alternations to red blood cell proteins rather than to membrane lipids.

Phenyl hydrazine increases reactive oxygen species (ROS) and lipid peroxidation and decreases glutathione (GSH), these effects are reversed by N-acetyl cysteine, a known ROS scavenger **[6,14].**

Phenyl hydrazine induces Heinz body formation and oxidative degradation of spectrin without any cross-linking of membrane protein; both these findings impair erythrocyte deformability**.** Formation of Phenyl radicals and the replacement of haeme with phenyl-substituted protoprophyrins cause the destabilization of haemoglobins to induce Heinz bodies and haemolytic anemia with phenylhydrazine**[19].**

Phenylhydrazine treatment increases the transport rates in Na-K pump, Na-H exchange, Na-Li exchange, and K-Cl co-transport in vivo, while a decreases in Na-K pump, Na-H exchange, Na-Li exchange and increases K-Cl co-transport were found in rabbit red cells**[3].**Phenylhydrazine modulate immune reactions. It was found to be mitogen and activator of lymphoid cells**.**This study has demonstrated the antioxidant activity and the protective effect against oxidative-induced hemolysis of the polyherbal formulation extract. Polyphenols, tannins and flavonoids are very valuable plant constituents possessing scavenging action due to their several phenolic hydroxyl group **[13].** It is well known that the vitality of cells depend on the integrity of their membranes**.**

Exposure of red blood cells to injurious substances such as hypotonic medium and phenylhydrazine results in lysis of its membrane accompanied by haemolysis and oxidation of haemoglobin**[20].**The haemolytic effect of hypotonic solution is related to excessive accumulation of fluid within the cell resulting in the rupturing of its membrane. Such injury to RBC membrane will further render the cell more susceptible to secondary damage through free radical-induced lipid peroxidation **[1].** This notion is consistent with the observation that the breakdown of bio-membrane leads to the formation of free radicals which in turn enhance cellular damage **[3].** It is expected that compounds with membrane-stabilizing properties, should offer significant protection of cell membrane against injurious substances **[8].** Compounds with membrane-stabilizing properties are well known for their ability to interfere with the early phase of inflammatory reaction, namely the prevention of the release of phospholipases that trigger the formation of inflammatory mediators [**9**]. In similarity with above compounds our study showed the protective effect of polyherbal formulation against the drug induced inflammation and osmosis indused inflammation. Our polyherbal formulation methanol extract showed 90% protection**.**

Summary

The Plant material was collected locally, leaves were detached, washed and shade dried. The dried leaves were powdered and extracted using methanol. To evaluate the anti-hemolytic activity, RBC suspension was used as a model system with phenyl hydrazine as hemolysin As a result of the present study; polyherbal formulation was found to haveanti-hemolytic activity with the maximum percentage of inhibition of hemolysis 61.52%.and IC50 value also was found to be 426.66µg.

CONCLUSION

The methanolic extract of polyherbal formulation has powerful retardation effect on phenyl hydrazine induced hemolysis.This may be because of the presence of Phytochemicals such as flavoniods and tannins, which are believed to be potent antioxidants.RBC has got the simplest structure and can be used as a very good model to detect the direct effect of a toxin on the cell membrane as well as protective effects by antidotes. Destabilization of the cell membrane in RBC can lead to lysis of the cell and release of haemoglobin in the medium. The extent of hemolysis can help us to reveal the extent of toxicity. The results of the present investigation indicate that the possibility of employing the polyherbal formulation extract as an antioxidant substance to ameliorate the oxidative damage of cells However, further attempts shall be made to investigate the possible protective effect of this extract against phenylhydrazine induced cytotoxicity *in vivo* condition.

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