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RESEARCH ARTICLE

PROTECTION AGAINST OXIDATIVE DAMAGE USING MOMORDICA CHARANTIA EXTRACT INCASE OF PHENYL HYDRAZINE INDUCED HEMOLYSIS

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ABSTRACT

Increasing evidence suggests that oxidative damage to cell components has a relevant pathophysiological role in several types of human diseases. Free radicals have been reported to cause red blood cell lysis in patients with blood pathologies such as thalassemia. 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 haemoglobin, all of which are powerful promoters of oxidative processes. 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 *Momordica charantia* (cucurbitaceae) against free radical-induced hemolysis. *Momordica charantia* L. (cucurbitaceae) is a creeping plant native of Asia and found throughout the world. 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. Phytochemical studies revealed that *Momordica charantia* contained alkaloids, saponins, glycosides, phenolic constituents, reducing sugars and free acids. The extract also demonstrated potent purgative effect and produced contractions of the guinea ileum and other effects of bitter melon include dose- related analgesic activity in rats and mice**,** anti-inflammatory actions and treatment for GI ailments, such as gas, ulcer, digestion, constipation, dysentery or hemorrhoids**.** 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; *Momordica charantia* was found to have anti-hemolytic activity with the maximum percentage of inhibition of hemolysis 61.52%.and IC50 value also was found to be 426.66µg.

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 [Ames *et al*., 1993]. Free radicals have been reported to cause red blood cell lysis in patients with blood pathologies such as thalassemia [Vives-Corrons *et al*.,1995]. 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 haemoglobin, all of which are powerful promoters of oxidative processes [Clemens *et al*., 1987]. Exposure of erythrocytes to free radicals leads to a number of membrane changes including lipid peroxidation [Koster and Slee, 1983;Lal *et al*., 1980], reduction in deformability [Kurata *et al*., 1994], changes in cell morphology [Shinar *et al*., 1989], protein cross-linking and fragmentation [Vissers *et al*., 1994]. 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 *Momordica charantia* (cucurbitaceae) against free radical-induced hemolysis. *Momordica charantia* L.(cucurbitaceae) is a creeping plant native of Asia and found throughout the world. It has numerous uses in popular folk medicine. Its leaves and roots serve as anti-rheumatic, anti-inflammatory, antiseptic and

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anti-diabetic remedies in Brazil [Leatherdale *et al*, 1981; Anila and Vijayalakshmi, 2000]. In Guatemala, Caribe, Japan and India it has been used in inflammation, diabetes and stomach problems [Giron *et al*., 1991].Phytochemical studies revealed that *Momordica charantia* contained alkaloids, saponins, glycosides, phenolic constituents, reducing sugars and free acids. The presence of 5-hydroxytryptamine in bitter melon has also been reported [Dhalla *et al*., 1981].The extract from the leaves of *Momordica charantia* was reported to exhibit hypoglycemic activity comparable to that of tolbutamide [Platel and Srinivasan, 1997; Lotlikar and Rajarama, 1966].Treatment with bitter melon was found to lower blood glucose levels in animal and human studies [Lotlikar and Rajarama, 1966].

The extract also demonstrated potent purgative effect and produced contractions of the guinea ileum [Sofowora, 1979]. Other effects of bitter melon include dose- related analgesic activity in rats and mice [Biswas *et al*.,1991], anti-inflammatory actions [Raman *et al*.,1996], and treatment for GI ailments, such as gas, ulcer, digestion, constipation, dysentery[Chevallier,1996;Duke,1989],orhemorrhoids[Hockin g,1997]. The plant has also been used for skin diseases (eg, boils, burns, infections, scabies, and psoriasis) [Duke, 1989] and for its lipid effects [Raman *et al*., 1996] and hypotensive actions [Duke, 1989; Raman *et al*., 1996]. Bitter melon has also been used as an insecticide. [Cunnick *et al*., 1993; Duke, 1989] It exhibits genotoxic effects in Aspergillus nidulans [Ramos *et al*., 1996]. The n-hexane extract of seeds of *Momordica charantia* has been reported to contain conjugated octadecatrienoic fatty acids and α-eleostearic acid. These acids have been studied for their anti-oxidant activities and are proven to be successful in an *in vitro* study. Thus it may help to reduce the risk of coronary heart diseases in non-diabetic as well as diabetic patients (Dhar, P. *et al*., 2007).

Phenyl hydrazine derivatives were used firstly as antipyretics but the toxic action on red blood cells made their use dangerous [Ranvers, 1891]. For many years phenylhydrazine was used for experimental induction of anemia in animals until Morawitz and Pratt suggested it as a drug for polycythemia Vera (Falconer, 1933), a colan disorder (Spivak, 2002) which is known by a net increase in the total number of erythrocytes in the body.

Phenyl hydrazine decreases hemoglobin level, red blood cell concentration, and packed cell volume, and impairs erythrocyte deformability. It induces reticulocytosis, increased osmotic resistance, free plasma haemoglobin means corpuscular hemoglobin (MCH), means corpuscular hemoglobin concentration (MCHC), and erythropoietin levels, and extramedular haematopoiesis in the spleen and liver (cf, Hara and Ogawa, 1975, Berger, 1985a, Stern, 1989).It has long been suspected that [Nicholson and Cohen, 1966] phenyl radical are produced in the oxidation of phenyl hydrazine. The autoxidation of phenylhydrazine is catalysed by oxyhemoglobin within the erythrocytes, producing high level of hydrogen peroxide [Cohen and Hochstein, 1964] and phenyl radical [Hill and Thornalley, 1981]. Induced anemia in rats following a single phenyl hydrazine intraperitoneal administration at a dose of 20mg/kg body weight (aqueous solution): erythrocyte concentration lowered to about 50% and haemoglobin level to about 60% of normal values in the course of 4 days (Yeshoda (1942).

It is well known that phenylhydrazine causes formation of methaemologbin and Heinz bodies as well as haemolysis both in vivo and in vitro. Oxidative stress is involved in phenylhydrazine induced erythrocyte damage [Stern, 1989].Phenyl hydrazine can penetrate to the O2 binding site of the haemoglobin molecule and react with it. Phenyl hydrazine oxidation results in the formation of superoxide and hydrogen peroxide [Goldberg & Stern, 1975; Goldberg *et al*, 1976; Goldberg & Stern, 1977; Jain & Hochstein, 1979]. Several reactive intermediates of Phenylhydrazine [Maples, Jordon, & Mason, 1988; Saito & Itano, 1981; Hill & Thronalley, 1981] are formed as well as complexes of these reactive products with haemoglobin. In the erythrocyte membrane, lipid peroxidation can occur [Goldstein, *et al*., 1980] as well as formation of a new antigen which is recognized by autologus IgG [Law, *et al*., 1985]. It is possible that the cytotoxic activity, (Goldberg and Stern, 1977), of phenyl hydrazine is due to the attack of phenyl radicals on the membrane thus initiating the oxidation of unsaturated fatty acid residues in phospholipids, (Gardner and Agric, 1979) and aggregation of membrane proteins, (Hochstein and Jain, 1981). Phenyldiazene presumably produced via the 2-electron oxidation of phenylhydrazine by oxyhaemoglobin, was found to hemolyse red cells rapidly and convert oxyhaemoglobin into methemoglobin, hemichromes, and other haemoglobin products. The hemolysis produced in the presence of phenyldiazene and oxygen is related to lipid peroxidation in the red cell membrane (Goldberg and Stern, 1997). The present investigation was aimed to assess the anti-hemolytic activity of the *Momordica charantia*. The study was carried out with the Collection and identification of the herbs and Soxhlet extraction of *Momordica charantia* using methanol. Anti-hemolytic activity of methanolic extract of *Momordica charantia* was evaluated using RBC as model system.

MATERIALS AND METHODS

Chemicals: Sodium chloride, disodium hydrogen phosphate and potassium dihydrogen 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: *Momordica charantia* leaves were collected from Bagalur in December 2008.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 Mrs. D. Lakshmi Faculty of Biotechnology, M.G.R. College, Hosur.

Preparation of plant extract: About 200g of shade dried powdered leaves of *Momordica charantia* were exhaustively extracted with methanol using Soxhlet apparatus. The residue

was filtered and concentrated in vacuo to a syrupy consistency. The extract was then stored in a dessicator until further use.

Investigation Of Anti-Hemolytic Activity

Blood Sample: The normal; anticoargulated blood was collected from the Meenakshi Hospital, Hosur.

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. 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:

1. Control tubes containing only RBC suspension.
2. Tubes containing RBC suspension and phenyl hydrazine (1 to 500µg).
3. Control tubes containing RBC suspension and test compound(100-500µg)
4. 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: % hemolysis= (Absorbance of the individual tube/Absorbance with 100% haemolysis) *100. 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)/A x100. 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 $\pm$ 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 in table 1 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. [Table 1]

The concurrent addition of phenyl hydrazine along with methanolic extract of *Momordica charantia* (100-500 µg/ml) to the RBC suspension significantly ($P<0.05$) reduced phenyl hydrazine induced hemolysis. As shown in table 2 the effect was concentration dependent. The protective effects of *Momordica charantia* 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. IC 50 of the *Momordica charantia* extract and ascorbic acid were 426.66 and 15.18 µg/ml respectively. The *Momordica charantia* extract showed maximum inhibitory effect 61.52% at 500µg/ml.

Table 1 Phenyl hydrazine induced hemolysis. Each value represents the mean± SEM; n=3; $p<0.05$.

S.No	Concentration (µg/ml)	OD	% hemolysis
1	1	0.03± 0.009	7.41 ± 3.17
2	5	0.026± 0.012	8.38± 4.39
3	10	0.030± 0.139	9.67± 4.56
4	100	0.033± 0.014	10.64± 3.83
5	200	0.036± 0.014	11.61± 4.65
6	300	0.040±0.0163	12.90± 5.27
7	400	0.050±0.0163	16.12± 5.27
8	500	0.110±0.039	35.48± 12.44
LC 50 = 832.35 µg			

Table-2 Effect of methanolic extract of Momordica charantia on phenyl hydrazine induced hemolysis. Each value represents the mean± SEM; n=3; $p<0.05$.

S.No	Concentration in (µg/ml)	OD	% Hemolysis	% Retardation
Control	-	0.057±0.0027	13.82±0.664	-
1	100	0.13±0.00472	31.71±1.150	1063±3.24
2	200	0.12±0.00471	29.27±1.150	17.50±3.24
3	300	0.116±0.00981	28.45±2.39	19.81±6.74
4	400	0.076±0.00273	18.53±0.66	47.77±1.87
5	500	0.056±0.00272	13.65±0.66	61.52±1.87
IC 50 = 426.66				

Table-3 Effect of Standard Ascorbic acid on phenyl hydrazine induced hemolysis.
Each value represents the mean± SEM; n=3; $p<0.05$.

S.No	Concentration (µg/ml)	OD	% inhibition
control	-	0.06	-
1	5	0.06±0.00	0±0.00
2	10	0.047±0.0054	21.6±9.07
3	15	0.023±0.0027	61.66±4.53
4	20	0.010±0.00	83.33±0.00
5	25	0.005±0.0017	91.66±2.98
IC 50 = 51.18 µg			

Table-4 RBC Suspension and Methanolic extract of Momordica charantia. Each value represents the mean± SEM; n=3; $p<0.05$.

S.No	Concentration (µg)	OD	% hemolysis	% Retardation
1	100	0.35	33.65±0.45	5.16±1.28
2	200	0.14	13.46±0.00	62.06±0.00
3	300	0.10	9.62±0.45	72.89±1.28
4	400	0.09	8.65±0.91	75.62±2.56
5	500	0.12	11.54±0.78	67.47±2.1

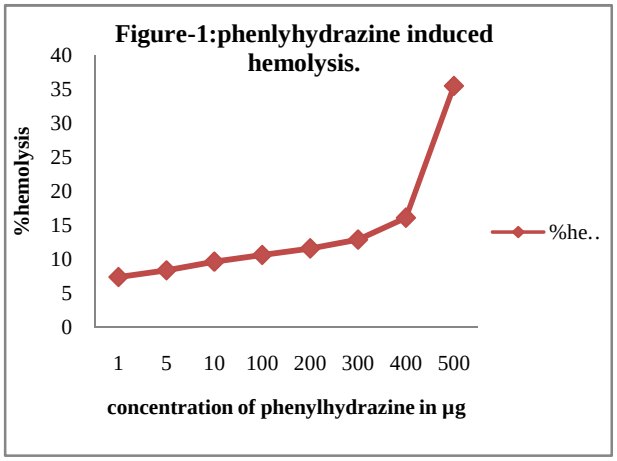

Figure 1 Graphs shows the percentage of hemolysis induced by phenyl hydrazine (µg). Each value represents the mean± SEM; n=3; p<0.05.

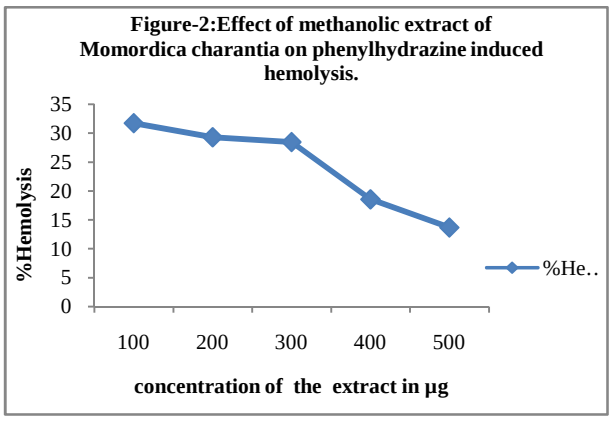

Figure 2 This graph shows the decrease in percentage hemolysis of methanolic extract treated RBC cells at an increasing concentration (µg) of the methanolic extract. Each value represents the mean± SEM; n=3; p<0.05.

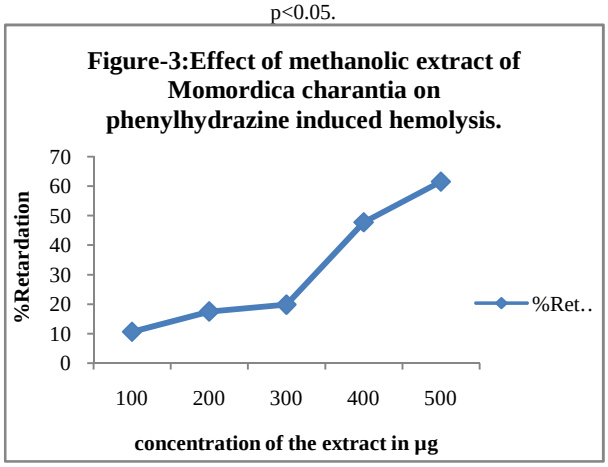

Figure 3 This graph shows the increase in percentage retardation of methanolic extract treated RBC cells at an increasing concentration (µg) of the methanolic extract. Each value represents the mean± SEM; n=3; p<0.05.

DISCUSSION

Misra and Fridovich [Misra and Fridovich, 1976] 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 [Huang and Kosower, 1968] giving traces of benzene and biphenyl. A kinetic analysis emphasized [Mira and Fridovich (1976)] the role of superoxide. 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 *Momordica charantia* to the RBC suspension significantly reduced phenyl hydrazine induced hemolysis. The effect was concentration dependent. The protective effects of *Momordica charantia* extract and reference standard ascorbic acid on the hemolysis induced by phenyl hydrazine were clearly seen by there increase in percentage retardation and decrease in percentage hemolysis.

CONCLUSION

The methanolic extract of *Momordica charantia* 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 *Momordica charantia* 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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