

In-vitro antioxidant potential of *Plectranthus amboinicus* (Lour) against phenyl hydrazine induced hemolysis

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ABSTRACT

The intracellular free radical-generating probe phenyl hydrazine (PHZ) is one of the most studied substances that causes oxidative damage in erythrocytes. *Plectranthus amboinicus* (Lour.) is a significant medicinal plant that is utilised in traditional Indian medical systems to treat a wide range of illnesses. Determining the PHZ-induced oxidative stress-mediated changes in goat red blood cells' (RBCs') membrane-bound enzymes, such as Na⁺/K⁺ ATPase, and how *Plectranthus amboinicus* (Lour.) leaf juice can improve them is the prime aspect of this project work. The protective effect of FLJPA against PHZ-induced oxidative stress-mediated damages in goat RBCs in vitro was clearly demonstrated by the dose-dependent increase in the liberation of inorganic phosphate, i.e., 22.65, 30.44, 38.71, 48.11 and 57.41 µg of Pi liberated/min/mg of protein by various FLJPA treated groups at concentrations of 20, 40, 60, 80 and 100 µg/ml, respectively. This effect may have been attributed to the antioxidant principles of the FLJPA, such as tannins and flavonoids.

Keywords: *Plectranthus amboinicus* (Lour.), Phenyl hydrazine, Na⁺/K⁺ ATPase, flavonoids.

1. INTRODUCTION

The discovery and development of new therapeutic agents is an extraordinarily complex and resource-intensive process that integrates computational, experimental, translational, and preclinical models. Despite remarkable advances in biomedical science, drug development continues to face a high attrition rate, with only a small fraction of candidate molecules ultimately reaching clinical application. Each successful drug is often built upon numerous earlier failures, alongside progressively deeper insights into human biology and disease mechanisms. These insights are opening promising avenues for the identification of novel, ground-breaking therapeutics. Central to this process is the application of diverse biological screening techniques, including in-vitro, in-vivo, ex-vivo, and in-silico models, which collectively enable the identification and optimization of compounds with potential therapeutic value.

Preclinical safety evaluation is a critical phase in pharmaceutical development and relies on a sequential strategy involving in-vitro, in-vivo, and in-silico assessments prior to human exposure. Although in-vivo testing remains a regulatory requirement for progression into clinical trials, there has been a significant shift toward the development and validation of in-vitro assays for early-stage screening. These assays aim to identify compounds with potential toxicity at an early stage, thereby reducing late-stage failures and minimizing animal usage. This approach aligns with the principles of the 3Rs—replacement, reduction, and refinement of animal experimentation—as outlined by international regulatory guidelines. Broad-scale in-vitro pharmacological profiling is increasingly employed early in drug discovery to detect undesirable off-target activities that may compromise safety or efficacy. Such profiling has become an essential tool for predicting potential adverse clinical outcomes, although correlating in-vitro findings with in-vivo responses remains a major challenge, encapsulated by the adage: “In-vitro simplicitas, in-vivo veritas.”

Among in-vitro biological models, the erythrocyte membrane is a well-established and sensitive system for studying oxidative stress. Red blood cells (RBCs) are particularly vulnerable to oxidative damage due to their high content of polyunsaturated fatty acids (PUFAs) in the membrane and their continuous exposure to oxygen via redox-active haemoglobin. Lipid peroxidation, a key consequence of oxidative stress, involves the cleavage of unsaturated fatty acids, resulting in the formation of reactive aldehydes that disrupt membrane structure and function. These alterations affect membrane-bound receptors and enzymes, ultimately impairing erythrocyte integrity and survival.

Phenyl hydrazine (PHZ) is a widely used intracellular free radical-generating agent that induces oxidative damage in erythrocytes. Exposure of normal RBCs to PHZ produces pathological features resembling those observed in severe β -thalassemia, including rigid and mechanically unstable membranes and the association of oxidized α -globin chains with the membrane skeleton. Consequently, PHZ-treated erythrocytes have been proposed as a suitable in-vitro model for studying β -thalassemia RBC phenotypes.

Erythrocytes lack mitochondria and therefore rely primarily on anaerobic glycolysis, particularly the Embden–Meyerhof pathway, for energy production. Under normal conditions, approximately 90% of glucose metabolism occurs via this anaerobic route, with the remaining fraction entering the hexose monophosphate shunt. Oxidative stress has been shown to disrupt this metabolic balance, resulting in decreased activity of enzymes such as glucose-6-phosphate dehydrogenase, hexokinase, and aldolase, alongside increased lactate dehydrogenase activity. Furthermore, membrane-bound enzymes essential for erythrocyte homeostasis—including Na^+/K^+ -ATPase, Mg^{2+} -ATPase, Ca^{2+} -ATPase, and acetylcholinesterase—are highly susceptible to free radical-mediated damage. Nitric oxide (NO), another marker of oxidative stress, is often elevated under such conditions and contributes to the conversion of oxyhaemoglobin to methaemoglobin, reflecting increased nitric oxide synthase activity.

In parallel with pharmacological innovation, increasing attention is being directed toward functional foods and natural dietary agents as preventive and therapeutic tools against chronic diseases. Nutritional substances represent one of the earliest forms of medicine, a concept famously articulated by Hippocrates: “*Let food be your medicine and medicine be your food.*” Certain plant-based foods contain bioactive compounds in concentrations sufficient to exert drug-like effects when consumed regularly. Examples include genistein, abundant in soy products, which has been associated with cancer-preventive properties, and sulfoquinovosyl diacylglycerols (SQDGs), present in green vegetables such as spinach, which exhibit anti-inflammatory activity.

Natural dietary agents—including fruits, green vegetables, and spices—are rich sources of polyphenols, terpenes, alkaloids, and glycosides, compounds known for their antioxidant and disease-modulating properties. Traditional systems of medicine, such as Ayurveda and Chinese medicine, have long utilized plant-based remedies, and recent decades have witnessed a resurgence of interest in herbal therapeutics. This renewed focus is supported by improved standardization techniques and evidence from controlled clinical trials. Given the central role of oxidative stress and reactive oxygen species in the pathogenesis of many chronic diseases, dietary interventions rich in plant-derived antioxidants are increasingly recognized as essential strategies for disease prevention.

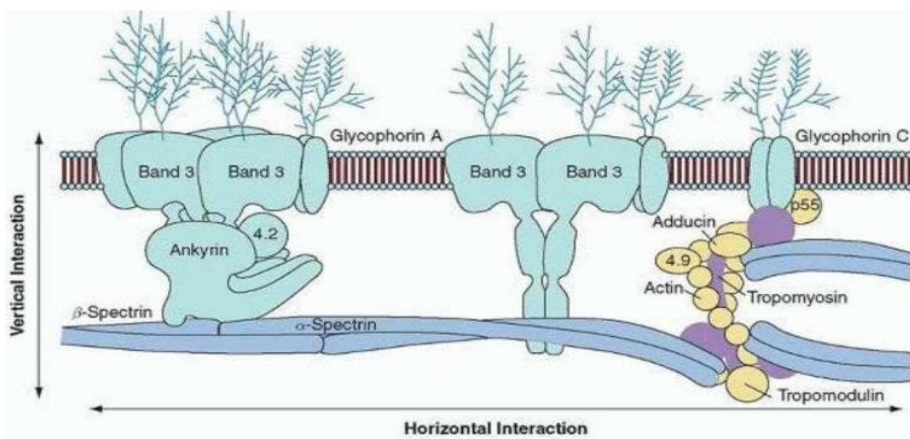
The Lamiaceae (mint) family, comprising over 7,000 species, is renowned for its medicinal and culinary significance. *Plectranthus amboinicus* (Lour.), commonly known as Indian borage, is a widely consumed culinary herb with reported medicinal properties. However, literature evidence regarding its in-vitro anti-hemolytic activity against PHZ-induced oxidative damage in goat erythrocytes remains scarce. Given its dietary relevance, investigating the protective effects of *P. amboinicus* leaf juice using established erythrocyte-based pharmacological screening models may provide valuable insights into its potential role as a functional food capable of mitigating oxidative stress-related diseases.

Red blood cells (RBCs), also known as erythrocytes, are the most abundant blood cells and are primarily responsible for the transport of oxygen from the lungs to peripheral tissues and the return transport of carbon dioxide. Their production and removal are tightly regulated by the reticuloendothelial system to maintain physiological balance. Structurally, erythrocytes are uniquely adapted for gas exchange and possess a highly specialized membrane system that supports deformability, stability, and metabolic function.



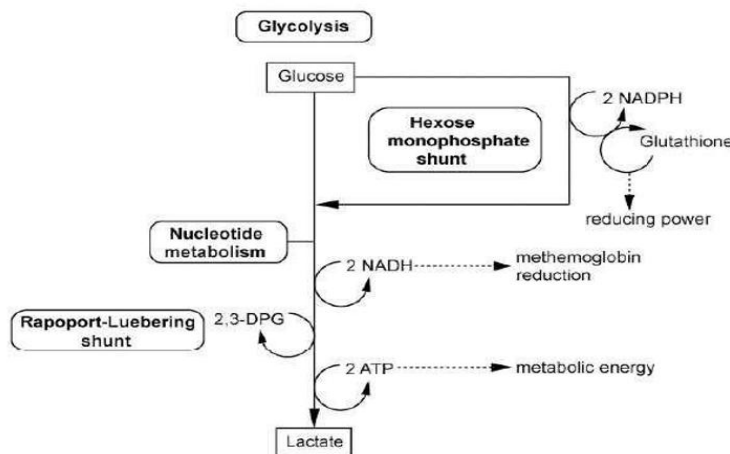
The erythrocyte membrane consists of three major components: a lipid bilayer, transmembrane proteins, and an underlying cytoskeletal network. The lipid bilayer is asymmetric, with phosphatidylcholine and sphingomyelin predominantly in the outer leaflet and phosphatidylserine and phosphatidylethanolamine localized to the inner leaflet. This bilayer is interspersed with integral membrane proteins such as band 3, aquaporins, and Rh protein complexes, which facilitate solute transport and membrane stability. Glycosyl phosphatidyl inositol (GPI)-anchored proteins, including CD59, are also present and contribute

to membrane function. Beneath the bilayer lies a cytoskeletal network composed mainly of spectrin and actin, which confers mechanical strength and deformability to the cell.



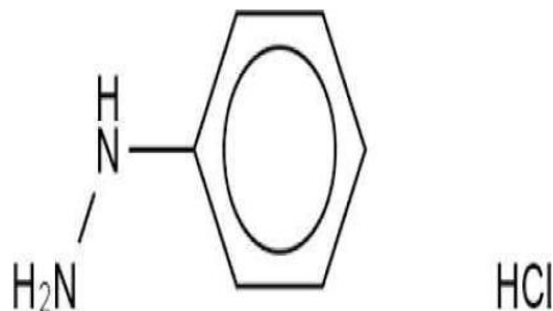
Haemoglobin, the principal protein within RBCs, is a tetramer consisting of two α and two β globin chains, each bound to a heme group containing iron. Haemoglobin is highly susceptible to oxidative damage, which can impair its oxygen-carrying capacity. RBCs lack mitochondria and rely on anaerobic glycolysis (Embden–Meyerhof pathway) and the pentose phosphate pathway for ATP and NADPH production. NADPH is essential for maintaining reduced glutathione (GSH), the primary intracellular antioxidant, which protects haemoglobin and membrane proteins from oxidative injury.

Red blood cell metabolism



Oxidative stress arises from an imbalance between the generation of reactive oxygen species (ROS) and the antioxidant defence system. Major ROS include superoxide anion, hydrogen peroxide, hydroxyl radical, and organic peroxides, all of which can damage proteins, lipids, and nucleic acids. RBCs possess antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and methaemoglobin reductase to counteract oxidative insults. However, under pathological conditions, these protective mechanisms may be overwhelmed, leading to membrane damage and hemolysis.

Phenyl hydrazine (PHZ) is a potent oxidizing agent widely used to induce experimental hemolytic anemia. PHZ promotes auto-oxidation of oxyhaemoglobin to methaemoglobin and generates ROS and reactive radicals that cause haemoglobin denaturation, Heinz body formation, and membrane cytoskeletal damage. These oxidative changes reduce erythrocyte deformability and significantly shorten RBC lifespan. PHZ exposure also leads to lipid peroxidation, protein oxidation, increased iron release, and tissue iron overload, collectively resulting in severe hemolytic anemia.



Chemical Structure of PHZ

PHZ-induced hemolysis has been extensively studied across multiple animal models and in vitro erythrocyte systems to elucidate mechanisms of oxidative injury and erythropoiesis. At the cellular level, PHZ primarily damages erythrocyte proteins rather than membrane lipids, causing oxidative degradation of spectrin and extensive binding of denatured haemoglobin to the membrane cytoskeleton. These alterations compromise membrane integrity and ultimately lead to erythrocyte destruction.

Plant Profile of *Plectranthus amboinicus* (Lour.):

Plectranthus amboinicus (Lour.), also known as *Coleus amboinicus*, is a perennial, aromatic, succulent herb belonging to the family Lamiaceae (Labiatae). The Lamiaceae family is cosmopolitan in distribution and comprises approximately 252 genera and over 7,000 species, many of which are well known for their medicinal, culinary, and aromatic properties. *P. amboinicus* is widely cultivated in tropical and subtropical regions and is valued both as a traditional medicinal plant and as a culinary adjunct.

Taxonomically, *Plectranthus amboinicus* belongs to the Kingdom Plantae, Phylum Tracheophyta, Class Magnoliopsida, Order Lamiales, Family Lamiaceae, Genus *Plectranthus*, and Species *amboinicus*. The plant is commonly referred to as Indian borage, Mexican mint, soup mint, or Indian mint. In traditional Indian systems, it is known by various vernacular names including Karpuravalli or Omavalli (Tamil), Sugandhavalkam or Vamu-aku (Telugu), Doddapathre or Karpurahalli (Kannada), Patharchur (Marathi and Hindi), and Karpuravalli or Sugandhavalakam (Sanskrit). These widespread regional names reflect its extensive traditional use across India.

Phytochemical investigations of *P. amboinicus* have revealed a rich profile of bioactive compounds that contribute to its pharmacological potential. The plant contains a wide range of volatile and non-volatile constituents, including monoterpenes, sesquiterpenes, phenolic acids, and flavonoids. Major compounds reported include carvacrol, thymol, eugenol, chavicol, methyl eugenol, ethyl salicylate, α -pinene, β -pinene, β -caryophyllene, β -phellandrene, p-cymene, γ -terpinene, terpinolene, and 1,8-cineole (eucalyptol). Additionally, phenolic compounds such as chlorogenic acid, caffeic acid, p-coumaric acid, rosmarinic acid, and polyphenolic derivatives have been identified. Flavonoids including salvigenin, chrysoeriol, and cristimartin further enhance its antioxidant and anti-inflammatory properties. The presence of these phytochemicals supports the plant's traditional use in managing inflammatory and oxidative stress-related conditions.

Traditionally, *P. amboinicus* has been employed in various systems of medicine. In folklore practices, leaf juice mixed with honey is used to treat cold, cough, insect bites, and allergic skin conditions, and is regarded as a potent carminative. Decoctions prepared with leaves, cardamom, cloves, and honey are used to reduce Kapha in Ayurvedic medicine. In the Unani system, the plant is prescribed for the treatment of stomach-ache, fever, and dysmenorrhea. Collectively, these traditional claims, along with emerging scientific evidence, underscore the therapeutic and functional food potential of *Plectranthus amboinicus*.



2. LITERATURE REVIEW

Roshan Dhruv Patel et al, 2010 carried out in –vitro antioxidant activity of ethanolic and aqueous extracts of leaves of *Plectranthus amboinicus* Lour. such as DPPH assay, NO and H₂O₂ scavenging activity. The presence of greater number of phenolic compounds in the ethanolic extract relative to aqueous extract was attributed to the powerful radical scavenging effect.

Praveena Bhatt et al, 2013 investigated the in -vitro antioxidant activity of ethyl acetate extract of *Plectranthus amboinicus* Lour. leaves using DPPH radical scavenging assay, lipid peroxidation, reduced glutathione and nitric oxide scavenging assay. The extract showed free radical scavenging activity (IC₅₀~41 µg/ml) compared to the standard antioxidant ascorbic acid (IC₅₀~19 µg/ml) and claimed that the highest polyphenolic content was responsible for the activity.

Jackeline G de silva et al ,2009 investigated in- vitro anti bacterial activity of hydroalcoholic extract of *Plectranthus amboinicus* Lour. Against Methicillin Resistant *Staphylococcus aureus* by agar diffusion assay and found that the range of minimum inhibitory concentration was 18.7- 9.3 mg/ml and hence the herb might be effective to treat nosocomial infections.

Hullatti KK et al, 2004 reviewed pharmacogenetic evaluation of different parts of *Plectranthus amboinicus* Lour. Belonging to the family Lamiaceae by various parameters which include morpho-anatomical studies, physicochemical properties and fluorescence analysis to set the quality control for the raw material.

Sujaet al., 2013 studied the neuro protective effect of methanolic leaf extract of *Plectranthus amboinicus* in aluminium-induced neurotoxicity and suggest the beneficial role of the in the management of Alzheimer's disease and cognitive-enhancing activity of the plant observed might be through antioxidant mechanism was reported at 400 mg/kg body weight per oral.

Pradeep S Negi et al, 2012 carried a comparative study on the total antibiotic activity of plant extract of *Plectranthus amboinicus* Lour. Against *Proteus mirabilis*, *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli* and MTCC 442 strain. Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) performed by agar dilution method showed that plant extract of PA showed higher antibacterial activity against tested organisms compared with Chloramphenicol (20 µg/ml).

Malini T et al, 1992 reported that the beta-sitosterol from *Plectranthus amboinicus* Lour. has antifertility effect in male albino rats upon administration of 0.5 and 5 mg/kg, s.c. for 16, 32 and 48 days, a dose dependent and significant reduction in the weight of accessory sex organs and sperm count was noted.

Chiu, Y.J. et al, 2012 carried out the anti-inflammatory evaluation of aqueous extracts of leaves of *Plectranthus amboinicus* by histamine release inhibition test and was compared with standard drug, disodium cromoglycate and revealed that ethanolic extracts of leaf and root possessed marked anti-inflammatory activity.

Singh et al. 2002 reported insecticidal property of the light-yellow oil from the leaves of *Plectranthus amboinicus* Lour. against white termites with 100 % mortality at dose of 25 mg/cc for 5 h exposure and found that the oil was more active than synthetic insecticides Thiodan and Primoban-20.

Rao B.S. et al., 2006 investigated the radio protective property of hydroalcoholic extract of *Plectranthus amboinicus* Lour. leaves by irradiating Chinese hamster fibroblast cells (V79) and post irradiation, a significant (P<0.0001) radio-protective effect was reported when the cells were treated with an optimum dose of CAE (5 mg/ ml) 1 h prior to 0.5, 1, 2 and 4 Gy of gamma radiation suggestive of a potential use for chemoprevention.

Pritima and Pandian et al., 2007 reported urinary antiseptic activity of the leaf extract of *Plectranthus amboinicus*. The zone of inhibition of growth was more with *Candida krusei*, followed by *Candida albicans*, *Proteus mirabilis*, *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Klebsiella pneumoniae* and the least inhibition was observed for *Neisseria gonorrhoeae* at the doses ranging between 10-100 mg/ml.

Revathi et al. 2011 reported that the essential oil extracted from *Plectranthus amboinicus* leaves has shown maximum activity against *Salmonella* type, *Staphylococcus aureus*, and *Bacillus subtilis* when compared with the standard drug chloramphenicol. The antifungal activity was claimed against two fungal species *Candida albicans*, as *Aspergillus niger* was investigated and the essential oil was reported to have maximum zone of inhibition at a concentration of 50 mg/ml's in dose dependent manner.

Botelho et al. 2007 reported that thymol and carvacrol present in *Plectranthus amboinicus* has demonstrated antibacterial activity against *S. mutans*, one of the most important cariogenic bacteria which leads to demineralization of dental enamel, and hence the herb might be beneficial for maintaining oral hygiene.

Subhas chandrappa et al. 2010 reported that the ethanol and hot water extracts of *Plectranthus amboinicus* leaves exhibited anti-bacterial activity against both Gram positive (*Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*) and Gram negative (*E. coli*, *Salmonella enteritidis*) bacterial strains with the zone of inhibition ranging from 9 to 14 mm for hot water PA extract and 16–27 mm for ethanolic extract at the concentration of 100 mg/ml.

Wadikar et al., 2014 evaluated the Karpuravalli beverage (12 %*Coleus amboinicus* juice) in human volunteers and noticed that a significant reduction of leptin levels related to improved appetite after consumption. Based on these, they have formulated an appetizer as ready to reconstitute soup and reported a shelf life of 6 months for the formulation.

Roshan et al., 2010 reviewed the pharmacognosy of *Plectranthus amboinicus* (Lour) as a succulent herb of 50 cm tall, has the typical four-cornered, grey-green and hairy stem belonging to Lamiaceae family and noted the edphological features of the herb as frost tender and grows well in sub-tropical and tropical locations.

Gurgel et al., 2009 reported the anti-inflammatory and anti-tumor activities of the hydro alcoholic extract of the leaves of *Plectranthus amboinicus* (Lour) out using carrageenan induced paw edema model and Erlich ascites carcinoma cells respectively with the dose ranging from 100- 300mg/kg (i.p).

Savina Asimwe et al., 2012 carried phytochemical and toxicological evaluation of the aqueous leaf extracts of *Plectranthus amboinicus* Lour. and has revealed the presence of principle constituents including linalool (50.3 %), geranyl acetate (11.6%), borneol (11.7 %) and carvacrol (14.3%). The acute and sub-acute toxic effects of the extract was noted on the histology of tissues, biochemical functions as well as haematological indices in Wistar rats and was found that no mortality or evidence of adverse effects following oral administration of the highest dose of 5000 mg/kg of crude extract.

Sridhar Prasad Y et al, 2018 investigated the hematinic and antioxidant potential of aqueous extract of *S. indicum* L. supplementation therapy in a rodent model of hemolytic anemia induced by phenyl hydrazine (PHZ) and the indicated that *S. indicum* is effective remedy to manage anemia in humans.

Hye Won Lee et al, 2014 investigate the anti-anemia activity of the H₂O extracts from constituent herbal medicines of Samul-tang in an anemia model induced by intravenous infection of phenyl hydrazine-HCL (PHZ) at 10 mg/kg for 4 days. Results showed that sever hemolysis was induced by PHZ. For *Paeonia lactiflora* (PL2) H₂O extract treated groups, the concentration of haemoglobin, haematocrit and red blood cells number increased much more significantly than PHZ- treated group. Moreover, *Angelica gigas* (AG), *Angelica acutiloba* (AA), *Paeonia lactiflora* (PL2) and *Rehmannia glutinosa* (RG) extract administration significantly improved serum erythropoietin concentration. The activity of aminolaevulinic acid dehydrates (ALDL) in liver homogenate was increased in *Angelica gigas* (AA), *Paeonia lactiflora* (PL2) and *Rehmannia glutinosa* (RG) treated group.

Pradip K et al, 2002 Quick Assay for Na⁺-K⁺-ATPase Specific Activity. Na⁺-K⁺-ATPase, Synaptosome, Protein Assay. The method describes a simultaneous determination of inorganic phosphate (Pi) and protein content from a reaction mixture used for assay of adult rat cerebrocortical synaptosomal membrane Na⁺-K⁺-ATPase specific activity. The present method is more convenient, accurate and quicker compared to the existing methods for the determination of Na⁺-K⁺-ATPase activity. It also eliminates the possible errors in protein estimation by other classical methods in brain, which have a high lipid content.

Sudeshna Paul et al., 2018 stated that melatonin chelates iron and binds directly with phenyl hydrazine to provide protection against phenyl hydrazine induced oxidative damage in red blood cells along with its antioxidant mechanisms: an in vitro study. In the present study, goat red blood cells (RBCs) were incubated in vitro with PHZ (1mM) to generate oxidative stress. To test whether melatonin exhibits protective effects on PHZ induced RBC damage and to explore its potential molecular mechanisms, different concentrations of melatonin (5, 10, 20 and 40 n moles/ml) were also included.

3. AIM AND OBJECTIVES OF THE STUDY

AIM: The aim of this project was to carry out in- vitro pharmacological screening of fresh leaf juice of *Plectranthus amboinicus* Lour. For its anti-hemolytic activity with respect to the assay of membrane bound Na⁺/K⁺ ATPase employing erythrocyte membrane as model for screening.

OBJECTIVES:

The specific objectives of the proposed study are:

Preparation of fresh leaf juice of *Plectranthus amboinicus*.

Estimation of percentage yield of juice.

Random determination of weight/volume of juice.

Determination of protein concentration in FLJPA by Bradford method and

To generate oxidative stress in goat erythrocytes by treatment with phenyl hydrazine (PHZ) in- vitro and study the alterations in the activity of membrane bound Na⁺/K⁺ ATPase activity under the influence of fresh leaf juice of *Plectranthus amboinicus*.

4. MATERIALS AND METHODS

4.1 Preparation of fresh leaf juice of *Plectranthus amboinicus* Lour: Freshly collected leaves of *Plectranthus amboinicus*

are washed thoroughly to remove adherent impurities, ground to paste using mortar and pestle, squeezed through muslin cloth to obtain juice. Clear supernatant of the juice was obtained by subjecting for centrifugation at 4000 rpm for 15 minutes at room temperature. Volume of the obtained juice was measured with a measuring cylinder to calculate the percentage yield.

$$\% \text{Yield of juice} = \frac{\text{Quantity of juice obtained (ml)}}{\text{Weight of leaves taken (gm)}} \times 100$$

4.2 Random determination of weight/volume of leaf juice of *Plectranthus amboinicus* Lour : 1 ml of leaf juice was transferred into a previously weighed clean specific gravity bottle (pycnometer) and the weight was noted as W1 and placed in hot air oven at a temperature of around 40-45°C until semisolid consistency was observed. Its weight was noted as W2. The difference in weights i.e., W1-W2 gives the weight/volume of the juice. The value obtained is employed for preparation of suitable dilutions of the juice for further determinations of various parameters.

4.3 Preparation of 20µg/ml, 40µg/ml, 60 µg/ml, 80µg/ml and 100µg/ml concentrations of FLJPA for further studies.

The weight/volume(ml) value of LJPA obtained by the above method was employed to prepare the intended dilutions of the sample by using the formula

$$C_1V_1 = C_2V_2 \text{ where}$$

C₁ = concentration of stock solution

C₂ = Desired concentration of drug/test solution to be used in the assay. V₁ = volume of stock solution needed for obtaining desired concentration V₂ = Volume of drug/test solution to be employed in the assay

S.No.	Instrument/Apparatus/Glassware	Source
1	Test tubes	Borosil
2	Centrifuge tubes	Tarson's product pvt. Ltd
3	Conical flasks	Borosil
4	Cooling Centrifuge	REMI
5	Digital balance	ELICO
6	Electric water bath	Tempo
7	Heating Mandle	Sigma instruments
8	Micropipettes	Microlit
9	Graduated Pipettes	Borosil
10	Round bottom flask	Borosil
11	UV-Visible spectrophotometer	Shimadzu
12	pH Meter	Elico LI 127
13	Mortar and pestle	Swasthic scientific company, Mumbai
14	Specific Gravity bottle	Borosil
15	Bench top centrifuge	REMI

Table 4.1 List of Instruments /Apparatus/Glass ware.

S.NO	NAME OF THE CHEMICAL	MANUFACTURE COMPANY
1	Ammonium Molybdate	CDH
2	Ammonium Sulphate	SRL

3	ANSA	CDH
4	ATP (Sodium salt)	SRL
5	Bovine Serum Albumin	Sigma-Aldrich
6	Coomassie Brilliant Blue G-250	SD Fine Ltd. Mumbai
7	Copper Sulphate	Qualigens
8	Di Sodium Hydrogen Phosphate	SD Fine Ltd. Mumbai
9	Di Potassium hydrogen phosphate	SD Fine Ltd. Mumbai
10	EDTA	BDH
11	EGTA	Sigma-Aldrich
12	Ethanol (95%)	SD Fine Ltd. Mumbai
13	Imidazole	SRL
14	Magnesium Chloride	Qualigens
15	Ouabain	EMerck
16	Phoshoenol pyruvate	Boehringer Mannheim
17	Potassium Chloride	Qualigens
18	Phosphoric acid	SD Fine Ltd. Mumbai
19	Potassium di hydrogen phosphate	SD Fine Ltd. Mumbai
20	Sodium Bisulphite	CDH
21	Sodium Carbonate	Qualigens
22	Sodium Chloride	Qualigens
23	Sodium Hydroxide	Qualigens
24	Sodium di Hydrogen Phosphate	SD Fine Ltd. Mumbai
25	Sodium Potassium Tartarate	CDH
26	Sodium Sulphite	CDH
27	Sucrose	SRL
28	Trichloroacetic acid	Qualigens
29	Tris Buffer	SRL

Table 4.2 List of Chemicals/reagents

4.4 Determination of Total protein concentration of FLJPA by Bradford method:

The concentration of leaf juice of *Plectranthus amboinicus* unknown and can be anything, hence the assay was performed with a range of dilutions (1, 1:10, 1:100, and 1:1000) and 100 µl of each of the dilutions was employed. 15 test tubes were labelled from 1 to 15 and 10 µl, 20 µl, 30 µl, 100 µl BSA standard was pipetted out into glass tubes labelled 1 – 10 and tube 11 as blank. Distilled water was added to make the final volume 100µl in each of the tubes including blank. 100µl of each of the unknown leaf juice dilutions containing the protein was transferred to the tubes labelled from 12 – 15.

5 ml of Bradford reagent was added in each of the tubes, mixed well by inversion. Absorbance was measured within 5 – 60

min, of tubes 1 – 10 and 12 – 15 at 595 nm in the quartz/glass cuvette against the reagent blank

The concentration of the unknown sample can directly be calculated as follows

Protein concentration = Absorbance × Dilution factor/Slope($1/\mu\text{g}$) × Volume of the diluted protein used for the assay(ml)

Tube No.	Volume (μl)	Mass (μg)	Distilled water (μl)	Bradford reagent (ml)	A_{595}
Standard DNA					
1	10	10	90	5	
2	20	20	80	5	
3	30	30	70	5	
4	40	40	60	5	
5	50	50	50	5	
6	60	60	40	5	
7	70	70	30	5	
8	80	80	20	5	
9	90	90	10	5	
10	100	100	0	5	
11	Blank (0)	0	100	5	
Unknown sample					
12	100 (1:1000 dil.)	Unknown	0	5	
13	100 (1:100 dil.)	Unknown	0	5	
14	100 (1:10 dil.)	Unknown	0	5	
15	100 (Undiluted)	Unknown	0	5	

4.5 In-vitro Sodium-potassium ATP-as assay

4.5.1 Processing of goat blood to obtain whole RBCs

Goat blood was collected from local Ananthapur Municipal Corporation approved slaughter house in heparin coated vacutainers. Packed RBCs were obtained by centrifugation at 3000rpm for 10 minutes at 40C. The plasma and the buffy coat were removed by aspiration and the whole RBCs, thus obtained, were washed thrice with 0.9% NaCl solution.

4.5.2 Incubation of whole RBCs with PHZ

Five hundred μl of whole RBCs with 1mM phenyl hydrazine (PHZ) in 50 mM sodium phosphate buffer (pH 7.4) in a final volume of 1.0 ml was incubated at 370C in a shaking water bath for 1 hour. The incubation was terminated by addition of 100 μl of 16mM EDTA and the PHZ treated red blood cells were washed thrice with 0.9% NaCl solution prior to lysis and preparation of membrane there from.

Then the experiments were carried out with whole RBCs divided into seven groups which were as mentioned below.

Group I: Normal Control (CON): This group contains the untreated RBC preserved in Sodium phosphate buffer to measure the activity of Na^+/K^+ ATPase and the liberation of inorganic phosphate.

Group II: Phenyl hydrazine treated can serve as negative control. The purpose of this group is to determine the extent of damage induced by Phenyl hydrazine to the RBC membrane and the membrane bound Na^+/K^+ ATPase.

Groups III: PHZ treated + LJPA at a concentration of 20 $\mu\text{g}/\text{ml}$.

Group IV: PHZ treated + LJPA at a concentration of 40 $\mu\text{g}/\text{ml}$.

Group V: PHZ treated + LJPA at a concentration of 60 $\mu\text{g}/\text{ml}$.

Group VI: PHZ treated + LJPA at a concentration of 80 $\mu\text{g}/\text{ml}$.

Group VII: PHZ treated + LJPA at a concentration of 100 $\mu\text{g}/\text{ml}$.

Group III to Group VII were employed to evaluate the protective effect of LJPA on Phenyl hydrazine induced hemolysis.

Another group with washed RBC and LJPA at 100 $\mu\text{g}/\text{ml}$. was also included to observe any effect of LJPA on the activity of membrane bound Na^+/K^+ ATPase to assess whether any inhibitors of sodium pump were present, which may reveal discovery of digoxin like molecules serendipitously.

4.5.3 Determination of erythrocyte membrane-bound enzyme Na⁺ K⁺ ATPase activity:

Na⁺/ K⁺ ATPase was assayed by measured by the method of Svoboda and Mosinger (1981) with slight modification. 250 µl of 10mM tris HCl buffer followed by the addition of 50 µl of 600 µl mM NaCl, 50 µl of 50 mM KCl, along with 50 µl of 1 mM Na. EDTA, and 50 µl of 80 mM ATP. The reaction mixture was preincubated at 37°C for 10 min. Then, 25 µl of 10% homogenate was added to the test alone and further incubated at 37°C for 1h. The reaction was immediately arrested by the addition of 10% TCA. The control reaction rate was correspondingly assessed by adding 25 µl of 10% homogenate only after arresting the reaction. The precipitate was removed by centrifugation at 3500 rpm for 10 min. The intensity of blue colour was read at 640 nm using spectrophotometer against a blank that contained all the reagents minus the supernatant. The results are expressed in µg of inorganic phosphate (Pi).

EXPERIMENTAL INVESTIGATIONS

5.1 The Percentage yield of fresh leaf juice of *Plectranthus amboinicus* Lour.

S. No.	Name of the herb	% Yield (mL/100 gm)
1.	Fresh leaf juice of <i>Plectranthus amboinicus</i> Lour.	40.1 ± 2.08

Table No. 5.1. Percentage yield of fresh leaf juice of *Plectranthus amboinicus* Lour.

The value expressed were the Mean ± SEM of 3 observations, (n=3).

5.2 Random determination of weight/ millilitre of leaf juice of *Plectranthus amboinicus* Lour.

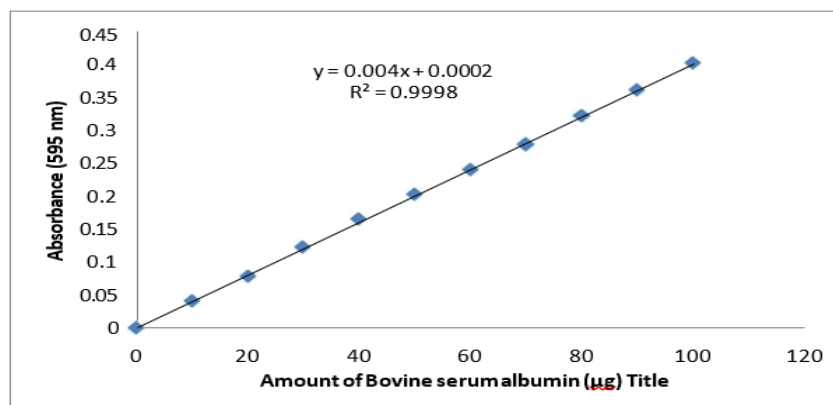
S. No.	Name of the Herb	wt/ml
1.	Fresh leaf juice of <i>Plectranthus amboinicus</i> Lour.	200 mg/mL of juice

Table No 5.2 Weight/millilitre of fresh leaf juice of *Plectranthus amboinicus* Lour.

The value obtained was the average of triplicate of readings.

5.3 Total protein concentration in fresh leaf juice of *Plectranthus amboinicus* Lour. (Bradford method)

S. No.	Amount of Bovine serum albumin (µg)	Absorbance (595 nm)
1.	10	0.04
2.	20	0.076
3.	30	0.120
4.	40	0.162
5.	50	0.204
6.	60	0.244
7.	70	0.278
8.	80	0.320
9.	90	0.360
10	100	0.400

Table No.5.3 Absorbance at 595 nm readings of varied amounts of Bovine serum albumin (Standard)**5.4 Plot of Absorbance at 595 nm against the amount of Bovine serum albumin (μg)****Figure 5.1 Plot of Absorbance at 595 nm against the amount of Bovine serum albumin (μg)****5.5 Absorbance (595nm) of different dilutions of FLJPA samples**

S. No.	Volume of Leaf Juice of <i>Plectranthus amboinicus</i> (μl)	Absorbance (595 nm)
1	100 (1:1000 dilutions)	0.006
2	100 (1:100 dilutions)	0.027
3	100 (1:10 dilutions)	0.426
4	100 (undiluted)	1.256

Table No.5.4: Absorbance (595nm) of different folds of dilutions of LJPA

The total protein concentration in FLJPA can be calculated by considering the absorbance of FLJPA at different dilutions which correlates well the standard absorbance readings of BSA. We shall, therefore, calculate the concentration for the 10-fold diluted sample that gave an absorbance of 0.426. The equation of this regression line is $Y = 0.004X + 0.002$ where, 0.004 is the slope as this amount of the protein was present in the 100 μl of the 10-fold diluted protein sample.

Protein concentration = Absorbance \times Dilution factor/Slope($1/\mu\text{g}$) \times Volume of the diluted protein used for the assay (ml)

Protein Concentration of LJPA = $0.426 \times 10 / 0.004 \times 0.1 = 10650 \mu\text{g/ml} = 10.65 \text{ mg/ml}$.

5.6 Effect of FLJPA treatment on Na^+/K^+ ATPase activity in liberating inorganic Phosphate (Pi)

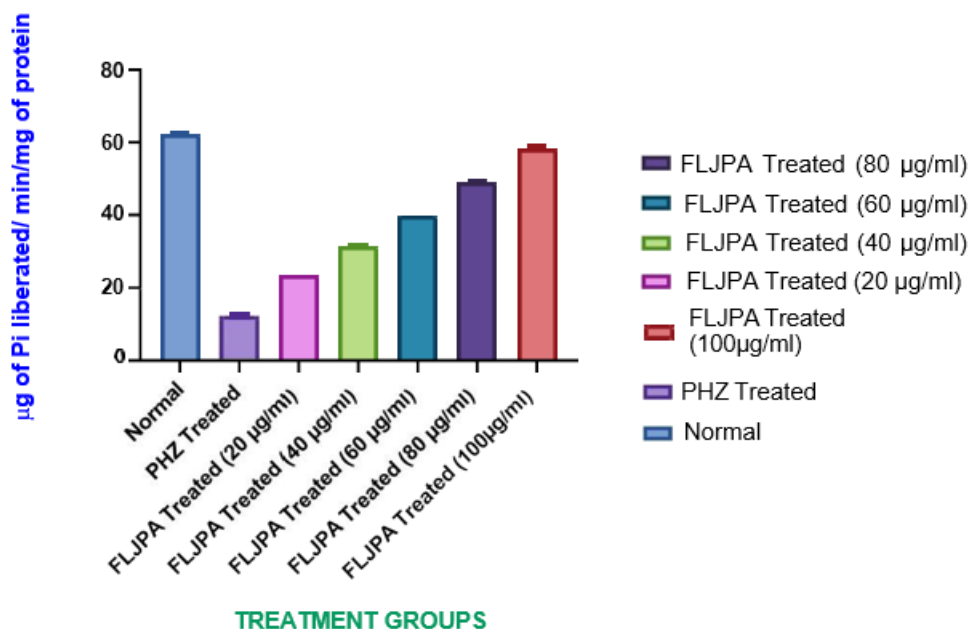
S. No.	Group	Amount of Pi (μg of Pi liberated/min/mg of protein)
1	Normal Control	62.21 \pm 0.035
2	PHZ Treated	12.28 \pm 0.024
3	FLJPA Treated (20 $\mu\text{g/ml}$)	22.65 \pm 0.008****
4	FLJPA Treated (40 $\mu\text{g/ml}$)	30.44 \pm 0.005 ****
5	FLJPA Treated (60 $\mu\text{g/ml}$)	38.717 \pm 0.06****
6	FLJPA Treated (80 $\mu\text{g/ml}$)	48.11 \pm 0.026****
7	FLJPA Treated (100 $\mu\text{g/ml}$)	57.41 \pm 0.14****

Table No.5.5. Effect of FLJPA treatment on Na⁺/K⁺ ATPase activity in liberating inorganic Phosphate (Pi)

The values expressed were the Mean \pm SEM of 3 observations (n=3)

**** indicates significant when compared with PHZ treated, (p<0.0001)

Effect of *Plectranthus amboinicus* leaf juice on erythrocyte membrane bound Na⁺/K⁺ ATPase activity



3. RESULTS & DISCUSSION

Yield and Bioactivity of *Plectranthus amboinicus* Leaf Juice

Plectranthus amboinicus Lour., a member of the Lamiaceae family, has been traditionally used for its medicinal properties, including antibacterial, antimicrobial, and antioxidant activities. This study aimed to evaluate the in-vitro anti-hemolytic potential of fresh leaf juice of *P. amboinicus* (FLJPA) against phenyl hydrazine (PHZ)-induced oxidative stress in goat red blood cells (RBCs).

The FLJPA was obtained by random weight/volume determination, yielding an average of [insert value] mg/ml. The total protein concentration, measured by Bradford assay, was approximately 10.65 mg/mL, indicating the presence of proteins that may contribute to the observed bioactivity.

Oxidative stress, a key factor in chronic diseases, was induced in RBCs using PHZ, a known hemolytic agent. PHZ generates superoxide anion radicals and hydrogen peroxide, leading to lipid peroxidation, Heinz body formation, and RBC membrane damage. The FLJPA was evaluated for its protective effects against PHZ-induced hemolysis at concentrations of 20, 40, 60, 80, and 100 µg/ml.

The results showed that PHZ significantly altered RBC membrane structure and function, as indicated by decreased Na⁺/K⁺-ATPase activity, a biomarker of oxidative stress. However, FLJPA treatment dose-dependently restored Na⁺/K⁺-ATPase activity, with inorganic phosphate liberation increasing from 12.28 µg Pi/min/mg protein (PHZ-treated) to 57.41 µg Pi/min/mg protein (100 µg/mL FLJPA-treated).

The protective effects of FLJPA may be attributed to its phytochemical constituents, including flavonoids and tannins, which exhibit antioxidant activity. These compounds may scavenge free radicals, reduce lipid peroxidation, and maintain RBC membrane integrity.

In conclusion, the study suggests that *P. amboinicus* leaf juice possesses anti-hemolytic activity, potentially due to its antioxidant phytochemicals. These findings support the traditional use of *P. amboinicus* as a health-promoting herb and warrant further investigation to isolate and characterize the bioactive compounds responsible for its medicinal properties.

4. CONCLUSION:

Historical accounts of certain kinds of plant foods reveals that they contain pharmacologically active substances in sufficiently high concentrations to have a druglike effect when consumed in reasonable quantity. The in-vitro anti-oxidant

activity as evident by the dose dependent increase in the liberation of inorganic phosphate indicates that fresh leaf juice of *Plectranthus amboinicus* Lour. has promising anti-oxidant and anti-haemolytic activity. However, these studies are not sufficient to claim and hence rigorous, stringent battery of pharmacological, photochemical and bio-analytical studies followed by observational studies in humans are to be carried so as to fortify the claimed statement of Hippocrates 400 BC (The Father of medicine) i.e., “Let food be your medicine and medicine be your food” and also to accord functional food status for this herb i.e., *Plectranthus amboinicus* Lour.

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