RESEARCH PROPOSAL

Anti-anemic properties of *Pterocarpus marsupium* latex and its cytotoxic and embryotoxic effects

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ABSTRACT

Anemia is a hematological disorder, and a large number in the developing world are affected with significant morbidity and mortality. Among them, most depend on traditional medicine to strengthen the hematopoietic action. The availability, low cost, and minor side effects are thought to be associated with this high selection rate of traditional medicine. The *Pterocarpus marsupium* is a medicinal plant native to Sri Lanka, India, and Nepal. The different components are used by traditional medical practitioners in treating different disease conditions, specifically latex, to enhance hemoglobin production in anemic patients. However, there is minimal scientific evidence for using the *P. marsupium* latex as an anti-anemic agent. Therefore, this study is planned to evaluate its anti-anemic effect, erythropoietin (EPO)-like effect, toxicity, phytochemical composition, and bioactive ingredients. Consequently, we aim to apply *in-vitro* and *in-vivo* assays to investigate the toxicity and favorable anti-anemic and other hematopoietic potentials of the plant latex using normal human cell cultures, rat-derived hematopoietic stem cell cultures, zebrafish embryos, and phenyl hydrazine-induced anemic rats with identification of bioactive ingredients that might mediate such potentials. If we discover a similar action of EPO in this plant latex, it would be beneficial in the way of treating anemia-associated disease conditions such as chronic renal failure, myelodysplasia, rheumatoid arthritis, human immunodeficiency virus (HIV), and cancer as a replacement for recombinant human EPO (rhEPO). This will be beneficial in the way of reducing the high cost of rhEPO treatment as well. Finally, an attempt to discover a therapeutic agent for anemia from a safe, natural source will considerably contribute to traditional medicine/herbalism due to the crucial role of traditional medicine in the prophylaxis or therapy of hematological diseases in Sri Lanka.

Keywords: *Pterocarpus marsupium*, Indian kino, Anemia, Toxicity, Latex, Erythropoietin.

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Introduction

The practice of traditional medicine is increasing throughout the world, specifically in low and middle-income countries. A major part of the health care is provided in such countries, and up to 80% of the population may depend on traditional medicine for their primary health care needs, irrespective of their cultural affiliation.[1] The use of medicinal plants for the above purpose has a long history and is described as herbalism or botanical medicine.[2] The substances found in medicinal plants with therapeutic activity are known as bioactive ingredients. In traditional practice, these bioactive ingredients are mostly unidentified by the traditional healers, even if they differ from plant to plant. Anthraquinones, flavonoids, glycosides, saponins, tannins, morphine, atropine, codeine, steroids, lactones, and volatile oils are some components present in medicinal plants that possess therapeutic value for the treatment of different diseases.[3,4] More recently, these bioactive ingredients in medicinal plants have been extracted and used to treat different disease conditions, such as infusions, syrups, concoctions, decoctions, infused oils, essential oils, ointments, and creams.[2] Also, some medicinal plants are used in the treatment of hematological disorders as a source of iron, other minerals, and hematopoietic vitamins such as riboflavin (vitamin B_2), pyridoxine (vitamin B_6), cyanocobalamin (vitamin B_{12}), and folic acid (vitamin B_9). These types of plants are classified as Rasayana in Ayurveda and are believed to be useful in strengthening an individual's hematopoietic and immune systems.[5-7]

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Pterocarpus marsupium is one such plant that has been used for a long period of time to treat different diseases with multiple pharmacological activities. The different components of the plant, such as heartwood, leaves, flowers, and latex, have been scientifically proven for hypolipidemic, hepatoprotective, antiulcer, anti-inflammatory, and anti-diabetic activities. It has also been reported as a curing agent of dysentery, diarrhea, fever, toothache, obesity, arthritis, gout, asthma, elephantiasis, leukoderma, and the greying of hair.

Anaemia is a hematological disorder characterized by a reduction of circulating hemoglobin concentration of less than 13 g/dL for males and less than 12 g/dL for female adults.[8,9] It has been estimated that 24.8% of the world's population is affected, and it has become a moderate public health problem in Sri Lanka. It was reported that 33% of preschool, 39% of nonpregnant, and 34% of pregnant women are affected.[10-12] Among the types of anemia, hemolytic anemia is common and may be inherited due to glucose-6-phosphate dehydrogenase (G6PD) deficiency or, acquired due to exposure to hemolytic agents resulting in intra or extra-vascular destruction of erythrocytes. Also, chronic diseases such as chronic renal disease and chronic respiratory diseases are considered causes affecting hematopoiesis, resulting in the shortening of red blood cell lifespan, dysregulation of iron homeostasis, and impaired bone marrow erythropoietic responses.[13,14] Exposure to some chemicals may be associated with hemolysis along the course of therapy and oxidative stress predisposes to peroxidation of erythrocyte membrane. Since traditional medical practitioners use the *P. marsupium* to enhance hemoglobin production in anemic patients, evaluating its efficacy and toxicity risks is of utmost importance.[15] Therefore, an attempt to discover a therapeutic agent for hemolytic anemia from a safe, natural source will considerably contribute to traditional medicine/herbalism due to the crucial role of traditional medicine in the prophylaxis or therapy of hematological diseases in Sri Lanka. Consequently, in this study, we aim to apply *in-vitro* and *in-vivo* assays to investigate the toxicity and favorable anti-anemic and other hematopoietic potentials of the latex of *P. marsupium* using normal human cell cultures, rat bone-marrow-derived hematopoietic stem cell cultures, zebrafish embryos and phenylhydrazine-induced anemic rats with identification of bioactive ingredients that might mediate such potentials.

Literature Review

Anemia

As defined by the World Health Organization (WHO), anemia is a condition in which the number of red blood cells or the hemoglobin concentration within them is lower than normal.[16] Further, the values indicated as in a criterion for anemia in men is less than 13 g/dL, whereas in women it is less than 12 g/dL. The concentration of hemoglobin required to meet normal physiologic needs depends on age, sex, living at high altitudes, smoking habits, and pregnancy status.[8,17] Based on the statistics, anemia affects about 1.62 billion people globally, accounting for 24.8% of the population. The highest prevalence was reported among preschool-age children (47.4%) and the lowest prevalence was reported among men (12.7%). However, 468.4 million non-pregnant women are affected the greatest number, with prevailing significant morbidity and mortality, particularly in the developing world.[8,18] When the prevalence of anemia is about 20 to 39.9% of the general population, it is usually considered a moderate public health problem. In Sri Lanka, anemia has become a moderate public health problem contributing significantly to maternal and child morbidity and mortality. According to epidemiological studies conducted in Sri Lanka, 39% of females are affected, and 34.6% of women of reproductive age are most affected by anemia.[10,12] There are common causes of anemia apart from nutritional deficiencies, including iron deficiency, deficiencies in folate, vitamins B_{12}, and vitamin A. Haemoglobinopathies and infectious diseases, such as malaria, tuberculosis, HIV, and some parasitic infections, are also considered important.[16,19] An early study done in Sri Lanka to address the contribution of hemoglobinopathies in assessing anemia burden in the community found that iron deficiency anemia (48%) and β thalassemia (12%) are common in children aged between 6 to 59 months.[20] Another survey carried out in an adolescent population of Sri Lanka reports that iron deficiency anemia occurred in 34.9% of the population, and 3.9% of them had β thalassemia.[21]

- *Hemolytic anemia*

Hemolytic anemia is defined as the destruction of red blood cells (RBCs) before their normal 120-day lifespan and is classified as normocytic anemia with an MCV of 80 to 100 fL. Because of the early destruction of red blood cells, it presents with low hemoglobin, increased hemoglobin catabolism, and an increase in the efforts of bone marrow to regenerate products.[17] It is a form of inherited or acquired anemia resulting from either intravascular or extravascular red blood cell destruction.[22] There are numerous etiologies of hemolysis. The hemoglobinopathies, which include sickle cell disease and thalassemia, are likely mechanisms of the destruction of red blood cells, while inherited protein deficits lead to increased destruction in membranopathies (*e.g.*, Hereditary spherocytosis and hereditary elliptocytosis). Overwhelming oxidative stress results in enzymopathies (*e.g.*, G6PD deficiency and pyruvate kinase deficiency) while antibody-mediated phagocytosis or complement activation leads to immune-mediated hemolytic anemia. Microangiopathic hemolytic anemia, infections, direct trauma, and drug-induced hemolysis are considered extrinsic nonimmune causes.[23,24] Numerous external and internal causes for hemolysis have been identified, and some are either relatively harmless or life-threatening in nature.

The association between hemolytic anemia and exposure to some chemicals has also been identified. It was found that the

hemolytic activity of aryl hydrazine, such as phenylhydrazine, dapsone, hydroxylamine, phenylamine, and divicine, may lead to acute hemolytic anemia in vertebrates.[22] A study conducted by inducing anemia in rats following a single intraperitoneal administration of phenylhydrazine hydrochloride at a dose of 20 mg/kg body weight showed that the reduction of erythrocyte concentration (about 50%) and hemoglobin level (about 60%) in the course of 4 days.[5] Another animal study carried out by Lee *et al*. in 2014 using melted phenylhydrazine (10 mg/kg body weight) revealed that the number of RBCs decreased by about 17% compared to control groups within four days.[25] An experiment was performed using phenylamine (aniline) to induce anemia in rats. They injected aniline suspension intraperitoneally (0.003 mL/g bw/day) for five days, and blood samples showed a significant reduction in RBC and hemoglobin levels compared to control groups.[26] The above hemolytic action of aniline is mediated *via* its active metabolite, phenylhydroxylamine. This active metabolite oxidizes oxyhemoglobin to methemoglobin and hydrogen peroxide, which causes lipid peroxidation on the red blood cells' membrane, leading the membrane to lysis.[27]

- *Diagnosis of hemolytic anemia*

Hemolytic anemia is often discovered through laboratory tests. However, the history and physical examination of an individual provide important clues about the presence of hemolysis and its underlying cause. The patient may complain of dark urine and back pain associated with intravascular hemolysis, and their skin may appear pale or jaundiced. If the anemia is pronounced, a resting tachycardia with a flow murmur may be present. However, hepatosplenomegaly suggests an enlarged spleen may reflect hemolysis.[23,28,29] According to the literature, about 70% of clinical diagnoses of hemolytic anemia are supported by laboratory investigations such as peripheral blood films and hematological tests. Peripheral blood films are a basic but highly informative hematological tool in the diagnosis and monitoring of disease progression as well as therapeutic response. It exposes the pathognomonic morphology of red blood cells, such as spherocytes or schistocytes, white blood cells, and platelets for coexisting hemolytic disorders.[17,30,31] Reticulocytosis is a characteristic feature of hemolysis. Hemolytic anemia is usually normocytic, although a marked reticulocytosis can lead to an elevated mean corpuscular volume (MCV). Apart from MCV, the other hematological parameters such as packed cell volume (PCV), total and differential white blood cell counts (WBC), red blood cell count (RBC), hemoglobin concentration (Hb), mean corpuscular Hb concentration (MCHC), platelet count (PLT), mean platelet volume (MPV) and red cell distribution width RDW are equally important in assessing anemia. Other than the hematological parameters, other chemical tests are also considered important in assessing the levels of bilirubin, lactate dehydrogenase, haptoglobin, and hemoglobinuria.[28,32]

- *Erythropoietin*

Erythropoietin (EPO) is a glycoprotein hematopoietic growth factor that regulates the number of erythrocytes in peripheral blood. It acts as a major regulator of erythropoiesis by promoting the survival, proliferation, and differentiation of erythroid progenitor cells in the bone marrow. The major site of EPO production in adults is the kidneys, while in fetal life, the liver is the principal site of EPO gene expression. EPO has been used clinically over the past decade for the treatment of anemia. It plays an important role in the acute renal failure repair process by rapidly correcting anemia and enhancing renal tubular regeneration in addition to its hematopoietic effect.[33,34] In response to hypoxia, kidneys produce EPO and increase the red cell mass, improving tissue oxygenation. EPO is expressed in the cortical peritubular cells within the kidney, and EPO receptors are expressed in glomerular, mesangial, and tubular epithelial cells in human, rat, and mouse kidneys.[35]

Quantification of serum erythropoietin (EPO) concentration serves as a diagnostic tool in the determination of anemia. Aplastic, hemolytic, and iron deficiency anemia results in elevated serum EPO concentration. It is a glycoprotein hormone produced primarily by the fetal liver and adult kidney. It has been identified as the humoral factor regulating red blood cell production. Hypoxia, anemia, and bleeding positively regulate the expression of the EPO gene up to 1000-fold above the normal levels.[36] The hemopoietic effect of extracts from constituent herbal medicines of Samul-tang on phenylhydrazine-induced hemolytic anemia in rats was evaluated by Lee *et al*. in 2014.[25] They measured the serum EPO using a Rat EPO ELISA kit, and results revealed the plant extract has a boosting action of serum EPO as in the treatment group increased EPO by 15%. The stimulatory effect of EPO by medicinal plants was also discovered by a study done with *Beta vulgaris* leaf and stalk extract in a phenylhydrazine model of anemia. Higher EPO concentration than the control group was observed after the 4th day of the treatment.[11]

The importance of flavonoids in medicinal plants is described elsewhere. Usually, flavonoids are free radical scavengers and possibly compete with Hb in RBC for oxygen, resulting in hypoxia, which then stimulates the synthesis of RBC by inducing erythropoietin.[36,37]

- *Testosterone and thyroxine*

Experimental evidence demonstrates that testosterone influences erythropoiesis during male puberty, and a decline in testosterone levels may negatively affect erythropoiesis, hence, anemia.[38] The mechanism that is included as testosterone stimulates the production of hematopoietic growth factors and increases iron bioavailability.[39] Thyroxine is another hormone associated with anemia. Normal synthesis and metabolism of thyroid hormones are dependent on many trace elements such as iron, iodine, selenium, and zinc. Deficiencies of these elements can impair thyroid functions, especially in the anemia of iron deficiency.[40]

Use of traditional medicine in treating anemia

The practice of traditional medicine using medicinal plants is as old as the origin of man, which falls outside the mainstream of Western medicine. This type of traditional medical practice is also known as indigenous medicine or folk medicine. The most crucial facts of this traditional medicine include the use of scientifically unproven plants with minimal side effects. However, the herbal practice is gaining increasing attention mainly in developing countries as two-thirds of the world's population entirely relies on such therapies as their primary form of health care.[2,6]

- *Use of medicinal plants in treating anemia*

A large number of medicinal plants have been traditionally employed in treating hematological disorders such as anemia. Some of these plants have been identified elsewhere as *Telfeira occidentalis* (Common name - Fluted pumpkin), *Jatropha curcas* (In Sinhala - Weta erandu), *Flacourtia flavenscens* (In Sinhala - Uguressa) and *Justicia carnea* (Common name - Flamingo-flower).[19] A study conducted in Kerala, India focusing on a tribal group and their traditional medicine found that *Ageratum conyzoides* (In Sinhala – Hulanthala), *Boerhavia diffusa* (In Sinhala – Pita sudu sarana), *Centella asiatica* (In Sinhala – Gotukola), *Hemidesmus indicus* (In Sinhala – Iramusu), *Ichnocarpus frutescens* (In Sinhala – Kiri vel), *Momordica charantia* (In Sinhala – Karawila), *Moringa oleifera* (In Sinhala – Murunga), *Phyllunthus amarus* (In Sinhala – Pitawakka), *Phyllunthus emblica* (In Sinhala – Nelli), *Punica granatum* (In Sinhala – Delum), *Ocimum tenuiflorum* (In Sinhala – Heen maduruthala), *Solanum americanum* (In Sinhala – Kalu kammeriya) are useful plants in the treatment of anemia.[41] *Adenia gummifera*, *Allophylus rubifolius*, *Albizia versicolor*, *Brackenridgea zanguebarica*, *Bridelia cathartica*, *Comniphora africana*, *Hibiscus sabdariffa* (In Sinhala – Heen napiriththa), *Sorgum bicolor* (In Sinhala – Idal iringu), *Theobroma cacao* (Common name – Cocoa), *Triumfetta rhomboidea* (In Sinhala – Epala) were also reported to be used in conditions of anemia.[5,42]

The anti-anemic effect of *Brillantaisia nitens* on phenylhydrazine-induced anemia in rats was described by Akah *et al*. in 2010.[7] They demonstrated that the oral administration of aqueous and methanol extracts of their leaves for two weeks exhibit a significant ($p < 0.05$) hematinic activity by increasing blood parameters such as Haemoglobin, PCV, WBC, and RBC. The analysis of vitamins and iron in their leaves showed that there is an adequate amount of hematopoietic vitamins such as vitamins B_6, B_{12}, C, E, folic acid, and iron. Phytochemical analysis indicated a high concentration of glycosides, alkaloids, and resins while flavonoids, terpenoids, carbohydrates, and saponins occurred in trace amounts. The oral LD_{50} value of the plant was detected at greater than 5000 mg/kg. Finally, they justified its use in the management of anemia especially in children. These findings were supported by several other studies which followed same the methods using various plants. One study indicated the use of *Justicia carnea* leaves as a treatment for anemia. They induced the anemia in Wistar albino rats using phenylhydrazine and treatment with an aqueous leaf extract revealed that there was a significant increment of PCV and RBC levels, but no significant change in WBC level after the 4th day of treatment. The study also evaluated the phytochemical composition of the plant, where they found increased amounts of phenols and flavonoids. Calcium and iron have been identified as the dominant minerals while vitamins C, B_2, and B_1 are the dominant vitamins. The acute toxicity test showed no deaths or adverse reactions, even during the high concentrations of the aqueous extract as 5000 mg/kg bw.[19] Similar results were obtained by another study carried out with *Justicia* leaves by Wood *et al*. in 2020.[43] The anti-anemic activity of *Jatropha tanjorensis* Ellis & Saroja in rabbits was investigated in a study conducted by Idu, Igbafe, and Erhabor in 2014.[32] Their findings revealed that the treatment with the plant extract significantly improved the PCV, Hb, RBC, MCV, and MCHC levels of the animals but reduced the WBC level by day 14 of the experiment. It was also found some minerals such as calcium, potassium, iron, and zinc are abundant and believed to have an anti-anemic potential. However, their sodium levels were found to be low. A study conducted elsewhere evaluating the anti-anemic potential in leaves of *Moringa oleifera* (In Sinhala – Murunga) using aniline-induced anemic rats found significantly increased levels of hematology parameters and total iron content in the blood. As observed in the study, the iron content in Moringa leaves is high, it can act as the main ion in hematopoiesis. Flavonoids, alkaloids, saponins, tannins, phenols, steroids, and glycosides are also reported in high levels while high vitamin C content in Moringa leaf extraction increases iron absorption in the body. When observing the blood films of the plant extract-treated group, there was no recovery of the shape of red blood cells.[26]

- *Toxic effects of medicinal plants*

The investigation of the toxic effects of medicinal plants/plant extracts is equally important prior to the use of them as a treatment option as their long-term use could be hazardous to body organs. A toxicological study of an aqueous extract of *Syzigium aromaticum* (Common name – Clove/In Sinhala – Karambuneti) performed on Wistar rats found that there is no significant difference in the organ weight in the treated group except that of the stomach after 90 days of treatment. The spleen, heart, and testes did not show any morphological changes on gross and histopathological examinations, but severe and irreversible toxicity was observed in the liver, brain, kidney, and stomach.[43] A similar study conducted in Sri Lanka to assess the toxic effects of the immature inflorescence of *Cocos nucifera* (Common name – King coconut) on female Wistar rats found a gradual increase in the mean body weight in treated groups during the 28-day study period. Their vital organs including the liver, kidney, spleen, heart, and uterus of test groups did not show any abnormality in color and texture while their histopathological evaluation showed a normal morphological architecture without any treatment-related pathological changes compared to the control group.[44]

- *P. marsupium (In Sinhala – Gammalu)*

P. marsupium is commonly known as Indian kino, which has a long history as a medicinal plant. This plant is a large deciduous plant native to Sri Lanka, India, and Nepal and belongs to the family Fabaceae (Leguminoceae).[45] The plant grows as a medium to large tree with a height of up to 30 meters. The various parts of this plant have unique morphological features including rough and vertically cracked outer bark, golden yellow inner heartwood, light yellow sapwood, compound leaves, yellow flowers, circular winged fruits, and blood-like latex. The bark is used in treating heartburns and diabetes mellitus, while the heartwood is specially used in diabetes mellitus. Their leaves are used on boils, sores, and various skin diseases and gum is useful in toothaches.[46,47]

P. marsupium is reported as a versatile medicinal plant because of its multiple pharmacological activities. The studies carried out on phytochemicals investigation of the plant found the presence of carbohydrates, glycosides, saponins, tannins, flavonoids, alkaloids, phenolic compounds, fixed oils, and fats and it is a rich source of polyphenolic compounds as well.[45,48,49] According to previous studies, it is extensively used as an antidiabetic agent and also effective as a hepatoprotective and antihyperlipidemic agent. It was believed that the presence of high flavonoids and phenolic contents in the plant mediate such antihyperglycemic and antihyperlipidemic actions. The plant was found equally effective in dysentery, diarrhea, fever, and inflammations as well as in cancer treatment.[45,48,50]

The aqueous infusion of the heartwood is reported to have antidiabetic potential. Studies conducted elsewhere showed the treatment with heartwood extract significantly increases the antioxidant enzyme activity and glucose uptake into cells resulting antidiabetic effect. It also revealed the downregulation of some metabolic intermediates such as cholic acid derivatives, coenzyme A, and sphingolipids. The cholic acid and sphingolipid pathways were identified as the primary targets of heartwood extract and decreased synthesis leads to hypolipidaemic activity.[45,50-53] Another study conducted by Jhoshi *et al*. in 2004 using a methanolic extract of heartwood on normal and non-insulin-dependent diabetic mellitus rats has revealed the blood sugar-lowering effect on both test groups.[54] These findings were supported by Mishra *et al*. in 2012 indicating the action of ethanol extract of heartwood in regenerating pancreatic beta cells.[47]

Pterostilbene extracted from the dried heartwood of *P. marsupium* was found to have significant antioxidant activity and the obtained SPF value could be used as an ingredient in topical sun-protective formulations.[55]

The aqueous extracts of the bark of *P. marsupium* have been evaluated for their antimicrobial action. The results showed growth inhibition against bacteria such as *S. aureus*, *B. cereus*, *K. pneuminiae,* and *P. aeruginosa*.[49,51] A study conducted by Sikdar *et al*. in 2013, using three types of *P. marsupium* leaf extracts revealed the effective peripheral analgesic actions.[56] The study confirmed marked analgesic activity in the methanol leaf extract followed by the ethyl acetate and petroleum ether extracts respectively.

The latex of the plant comes out through the bark when an incision is made up to the cambium and which is traditionally used as a powerful astringent as well as an antidiarrheal agent.[46,47] The composition of *P. marsupium* latex was found to have tannic acid (75%), kino tannic acid, kinoin and kino-red. Catechol, protocatechuic acid, resin pectin, and gallic acid were also present in small quantities.[46] The latex obtained from the plant is widely used to treat diabetes in Sri Lanka and a daily dose of one teaspoon is considered to be effective.[57] A patent was granted for a Sri Lankan ayurvedic product made with anti-diabetes plants in which one of the ingredients is the latex of *P. marsupium*.[58]

The scientific evidence for using the *P. marsupium* latex as an anti-anemic agent is minimal. Therefore, this study will be performed to evaluate the hematological efficacy of *P. marsupium* latex in the anemia-induced animal model.

Objectives

General objective

To investigate the phytochemical composition, cytotoxicity, embryotoxicity, and the hematopoietic effects of the latex of *P. marsupium* using human cell cultures, zebrafish embryos and phenylhydrazine induced hemolytic anemia in Wistar rats compared to controls.

Specific objectives

To investigate the hematopoietic efficacy of latex of P. marsupium by

- performing tetrazolium-based MTT assay in rat bone-marrow-derived hematopoietic stem cell cultures calculating percentage cell proliferation.
- determining the anti-anemic and erythropoietin-like effect in Wistar rats by
  - study of blood films in anemia-induced rats compared to controls using Leishman staining and light microscopy.
  - quantitative analysis of the hematological parameters (Reticulocytes, Hemoglobin, PCV, RBC, MCHC, MCV, MCH, WBC, and PLT, MPV, and RDW) in anemia-induced rats compared to controls using an automated hematology-analyzer.
  - quantitative analysis of serum electrolyte composition (Na^+, K^+, Ca^{2+}, Fe^{2+}, Mg^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+}, and Co^{2+}) in anemia-induced rats compared to controls using an automated analyzer.
  - quantitative analysis of serum erythropoietin, thyroxine, and testosterone in anemia-induced rats compared to controls using commercially available ELISA kits.

To determine the toxic effects of latex of P. marsupium by

- calculation of the percentage change in the body weight in anemia-induced Wistar rats compared to controls.
- calculation of relative organ weight (kidney, heart, liver, and spleen) of anemia-induced Wistar rats compared to controls.
- observation of histopathology of organs (kidney, heart, liver, and spleen) in anemia-induced Wistar rats compared to controls using Haematoxylin and Eosin stain.
- observations of their signs of behavioral toxicity and mortality in acute and sub-acute toxicity studies.
- drawing an ethogram based on the observations of animal behavior.
- performing tetrazolium-based MTT assay in rat bone-marrow-derived hematopoietic stem cell cultures calculating viable cell percentage and IC_{50}.
- performing tetrazolium-based MTT assay in normal human kidney, spleen, liver, and heart cell lines.
- conducting acute embryotoxicity tests using zebrafish embryos.

To analyze the composition of latex of P. marsupium by

- qualitative analysis of phytochemicals (alkaloids, flavonoids, tannins, saponin, carbohydrates, glycosides, and steroids) using standard chemical tests.
- analysis of functional groups of active components using Fourier Transformed Infrared spectroscopy (FTIR).
- quantitative analysis of hematopoietic vitamins using the High-Performance Liquid Chromatography (HPLC) method (vitamin B_6, vitamin B_{12}, vitamin B_9, vitamin B_2, vitamin A, and vitamin E) and titrimetric method (vitamin C).
- quantitative analysis of hemoglobin using a commercially available hemoglobin assay kit.
- quantitative analysis of metals (Na, K, Ca, Fe, Zn, Cu, Mg, and Co) using inductively coupled plasma mass spectrometry *(ICP-MS)*.

Methodology

Study design

This study will be carried out in three phases (Figure 1). A completely randomized design (CRD) will be employed in phase I where animal studies are involved. The animals will be randomly assigned to the study groups.

Statistics plan

Sample size calculation for animal study

The sample size was calculated using G*Power (version 3.1.9.4).[59]

For a fixed-effect ANCOVA model with five treatment groups (positive control, negative control, and three experimental dose regimens), one covariate (baseline parameters), power 0.8, alpha 0.05, and expected large effect (0.4), the calculated sample size per group is 16. The final sample size per group after allowing for a 10% attrition rate is 18. Therefore, 18 rats will be included in one study group.

For the acute and subacute toxicity studies, the animal study will be planned in accordance with OECD guidelines. However, it should be noted that separate protocols, specific to the main study, will be developed to ensure the comprehensive evaluation of our research objectives.

Statistical analysis

Statistical analysis will be done using statistical software (SPSS). Quantitative tests such as an analysis of covariance (ANCOVA) and analysis of variance (ANOVA) will be conducted to make decisions on quantitative data.

In phase I, an ANCOVA will be conducted to compare the effect of the treatment on sub-acute toxicity. The independent variable will be the type of treatment (positive control, negative control, and three experimental doses) and the dependent variables will be the hematological parameters, serum electrolyte, hormone levels, and body weight. Baseline hematological parameters, serum electrolytes, hormone levels, and body weight will be used as the covariates in the analyses. One-way between-group ANOVA will be used to compare the weight of organs at the end of the study in different treatment groups. Subgroup analyses will be performed to explore the associations between gender and dose-response effects of the individual treatment. Preliminary analyses will be conducted to ensure no violation of the assumptions of normality, linearity, homogeneity of variances, and homogeneity of regression slopes. Alternative non-parametric methods will be used in case of violations of the assumptions for the proposed parametric tests. All analyses will be performed at a priori alpha of 0.05.

During phase II, the IC_{50} of each plant latex concentration will be calculated through the linear regression graph. One-way ANOVA will be conducted to determine the cytotoxicity of the plant latex toward kidney, heart, liver, and spleen cell cultures. The *p-value* according to the significance level will be determined indicating whether the differences between some of the means are statistically significant or not by assessing the null hypothesis. Mean ± standard

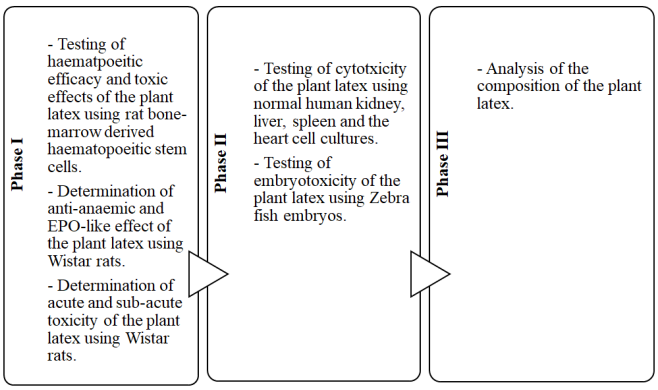


Figure 1: Three phases of the study

error of the mean (SEM) will be calculated for independent 7 test concentrations and then Student's t-test with $p < 0.05$ will be considered statistically significant in the stem cell cultures. The acute embryotoxicity will be determined using a simple linear regression. The ANOVA with $p < 0.05$ will be considered statistically significant. All the data will be interpreted as mean ± SEM with a $p < 0.05$ indicating the results are statistically significant based on the statistical hypothesis testing.

During phase III, the quantitative analysis of six vitamins will be carried out using three independent readings of each vitamin. The quantity of each vitamin will be expressed as the mean ± standard deviation (SD) value. The functional groups of active components, plant hemoglobin concentration, and electrolyte composition will be expressed as same with mean ± SD value.

The Shapiro-Wilk test will be used to assess the normality of the data for each group.

Ethical considerations

As in vivo studies are included, ethics approval needs to be obtained from a recognized Ethics Review Committee prior to conducting animal studies. Furthermore, all animals should be housed and treated in accordance with the internationally accepted guidelines for laboratory animal use and care.

Collection of plant latex

Prior permission to collect and utilize the latex samples will be obtained from the Department of Forest, Sri Lanka. Identification and authentication of the *P. marsupium* will be done from the herbarium of the botanical garden, Peradeniya, Sri Lanka. The herbarium of each plant sample will be deposited with a voucher specimen number in the Department of Physiology, Faculty of Medicine, University of Colombo for future reference.

The areas identified for latex specimen collection are Kosgama and Belihuloya areas of Sri Lanka while minimizing potential contaminations from environmental toxins, microorganisms, and agricultural contaminants. The collection will be carried out at the same time of the day from the same plant by making an incision in the trunk and allowing the latex to drain into a sterile container. They will be kept in a refrigerator at 4˚C until use.

Hematopoietic effect of *P. marsupium* latex

Effect on rat bone-marrow-derived hematopoietic stem cells (HSC)

HSC will be isolated from a 200 to 250 g weighted male rat by cutting of femur and tibia into small pieces and flushing the marrow. The harvested cells will be filtered through a $40\ \mu m$ cell strainer and centrifuged at 3000 rpm for 7 minutes. The HSC will be resuspended in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% of penicillin-streptomycin, 100 ηg/mL stem cell factor (SCF), 10 ηg/mL, interleukin-6 (IL-6), and 5 ηg/mL, interleukin-3 (IL-3). Cell viability will be determined by the trypan blue exclusion test and around 1×10^7 cells will be isolated from the rat. Cell suspension will be seeded into a growth medium enriched with SCF, IL-6, and IL-3 at a density of 3.5×10^6 /mL for 24 hours prior to downstream application and will be maintained at 37˚C in 5% CO_2 for 24 hours. Then HSC will be tested for different concentrations of plant latex (15.625, 31.25, 62.5, 125, 250, 500 and 1000 µg/mL) following the procedure described under 3.7.4 including MTT assay and percentage proliferation of cells will be calculated.[60] Experiments with each latex concentration will be conducted in triplicates on the same batch of cells.

Animal study

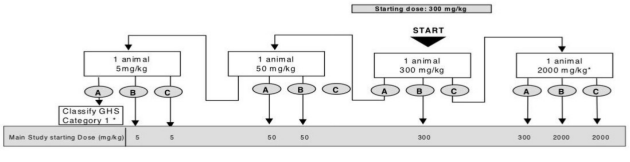
Experimental rats - Wistar rats handling and grouping

A total of 75 (The number may reduce to 55 if the limited study is applied) healthy Wistar rats (Species - *Rattus norvegicus*) of both sexes (30 males and 45 non-pregnant females) aged between 8 to 12 weeks, weighing 200 to 250 g will be recruited in the study by maintaining them under standard laboratory conditions (temperature: 22 ± 2°C, photoperiod: 12 hours natural light and 12 hours dark, relative humidity: 40 to 70%).[61-63] They will be acclimatized for a week in the animal house of the MRI and allowed to access food and water *ad libitum*. Animals will be housed in standard cages with sawdust as bedding. They will be fed with a standard rat formula prepared based on the WHO formula using locally available feed ingredients.[63,64] They will be identified by color markings on their body or identification number. All the animals will be housed and treated in accordance with the internationally accepted laboratory animal use and care.[65]

Acute toxicity (Fixed dose procedure)

The oral acute toxicity of the latex will be determined according to the OECD 420 guidelines, 2001 using female rats.[61] They will be observed for changes in physical characteristics, signs of behavioral toxicity, and mortality.

In the sighting study, the latex will be administered orally to single animals in a sequential manner (Figure 2). The starting dose of the main study will be selected according to OECD 420; 2001.[61] In the absence of previously published data on the toxicity of latex, the starting dose will be 300 mg/kg body weight. A period of 24 hours will be allowed between the dosing of each animal. They will be observed for 14 days without treatment.



(Source: https://read.oecd-ilibrary.org/environment/test-no-420-acute-oral-toxicity-fixed-dose-procedure_9789264070943-en#page9)

Figure 2: Acute oral toxicity fixed-dose procedure (Flow chart for the sighting study)

In the main study, a total of 5 female animals will be recruited for each dose level including 1 animal from the sighting study dosed at the selected dose level, with an additional 4 animals. A period of 3 days will be kept between dosing at each dose level. The study will be carried out depending on the starting dose (Figure 3).

The animals will be closely observed for signs of behavioral toxicity and mortality after the first 30 minutes of dosing and hourly for the next 4 hours. Then they will be observed after 4 hours and thereafter twice daily (at 9.00 a.m. and 3.00 p.m.) for 14 days.[61,66] The behavioral toxicity signs and mortality will be recorded separately for each animal in each phase.

Sub-acute toxicity test (Figure 4)

Overnight fasted (but having access to drinking water at will) rats will be randomly grouped into five, including 10 rats per each (Five female and five male).[62,66] Intravenous (IV) injections of phenylhydrazine (10 mg/kg body weight) will be used to induce hemolytic anemia in rats.[67-70]

The experimental latex doses will be administered orally by Gavage as a daily single dose.

A satellite group (An additional group of Wistar rats, including 5 males and 5 females in the top dose group for observation of reversibility, persistence, or delayed occurrence of toxic effects) will be kept under observation for 14 days post-treatment.

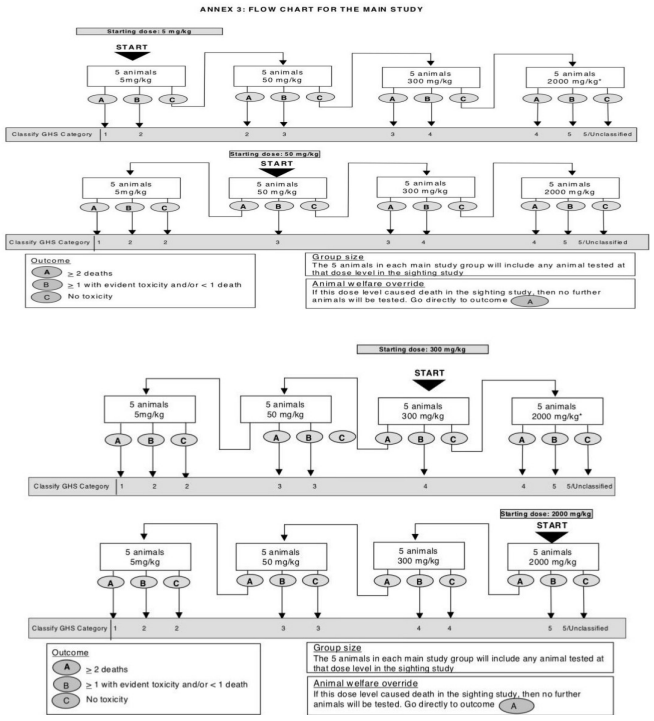
A limit test will be applied if the highest dose level (at least 1000 mg/kg bw) produces no observable toxic effects, whereas the other two dose levels will not be considered.

The general observations of physical and behavioral signs will be recorded once a day, at the same time. All animals will be observed for signs of morbidity and mortality twice daily (at 9.00 a.m. and 3.00 p.m.). The detailed clinical observations of all animals will be recorded once prior to the first exposure and once a week thereafter.[47,62,66,71,72]

Detection of anti-anemic and erythropoietin (EPO)-like the effect of P. marsupium latex on wistar rats

- *Collection of blood samples*

Blood samples will be collected from overnight fasted animals in each experimental group of sub-acute study for the analysis of serum electrolytes, liver and real functions, erythropoietin, testosterone, thyroxine, reticulocyte count, hemoglobin and other hematological parameters and preparation of blood films. Animals will be mildly sedated using an inhalant anesthetic agent and the tail of each will be inserted into a flask containing slightly warm water (40°C) for 1 minute. Then they will be placed in a rat holder and blood will be obtained from the lateral tail vein of each rat using a syringe and needle. Blood samples will be collected on days 0, 4, 11, 18, 25 and 32 in the sub-acute toxicity study into a plain tube (without anticoagulants) and an ethylenediamine–tetraacetate (EDTA) containing tube separately.[32,62,67,72,73] The 1.5 mL of blood will be collected on day 0 and 32 and while 1-mL of blood will be collected on day 4, 11, 18 and 25.



(Source: https://read.oecd-ilibrary.org/environment/test-no-420-acute-oral-toxicity-fixed-dose-procedure_9789264070943-en#page9)

Figure 3: Acute oral toxicity fixed-dose procedure (Flowchart for the main study)

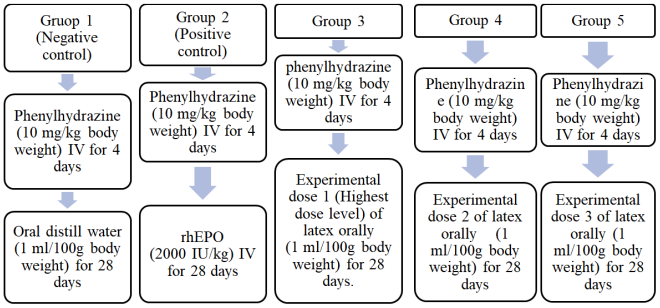


Figure 4: Flowchart for the sub-acute toxicity test

- *Preparation of blood films*

Blood films will be prepared according to the Wedge method described in Houwen, 2002, on days 0, 4, 11, 18, 25, and 32.[74] Then, they will be fixed and stained with Leishman stain and observed under light microscopy for abnormalities in red cell shape, such as fragments (schistocytes, helmet cells, keratocytes), sickle cells, target cells, spherocytes, tear drop cells (dacrocytes) and red cell agglutination. [26,30,75]

- *Quantitative analysis of serum electrolytes*

The serum will be used to detect Na^+, K^+, Ca^{2+}, Fe^{2+}, Mg^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+}, and Co^{2+} concentrations using an automated method. The results on days 0 and 32 will be reported as means ± standard error of the mean (SEM) of three independent analyses.

- *Hematological parameters*

Hematological parameters will be analyzed using an automated hematology analyzer on days 0, 4, 11, 18, 25, and 32. The reticulocyte count, PCV, total and differential WBC, RBC, hemoglobin concentration (Hb), MCV, MCHC, platelet count (PLT), MPV and red cell distribution width RDW will be included as the hematological parameters.[32,62,69,72,76]
The absolute WBC counts will be calculated using the following formula.[77]

Absolute count = WBC count x 1000 x percent of WBC type (in decimal)

- *Ranal and liver functions*

Renal parameters (BUN and Creatinine), AST and ALT levels will be measured using an automatic analyzer at the beginning (Day 0) and day 32.[78]

- *Serum erythropoietin concentration*

Serum erythropoietin concentration will be determined quantitatively using ELISA in accordance with the recommendation from the manufacturer of the rat EPO ELISA kit (https://www.sigmaaldrich.com/LK/en/product/sigma/rab1897) on days 0, 4, 11, 18, 25, and 32. [11,68]

- *Serum thyroxin concentration*

Total thyroxine concentration (On days 0 and 32) will be quantified by an ELISA kit (https://www.mybiosource.com/rat-elisa-kits/thyroxine-t4/261867) according to the manufacturer's instructions.[62,79,80]

- *Serum testosterone concentration*

According to the manufacturer's instructions, testosterone concentration (On days 0 and 32) will be measured using a commercially available ELISA kit (https://www.mybiosource.com/t-rat-elisa-kits/testosterone/282195).[22,81]

Investigation of toxic effects of P. marsupium latex on wistar rats

- *Food and water consumption*

Food and water consumption of each animal in sub-acute studies will be measured weekly and recorded.[62]

- *Body weight*

The initial body weight of all Wistar rats recruited in acute and sub-acute toxicity studies will be measured on day 0 of the experiment, then weekly, and on the last day (Day 32) of the experiment in grams.[61,62]
The percentage change in body weight will be calculated as follows.[73]
(Final body weight - initial body weight) g/(Final body weight) g × 100

Relative organ weight

On the last day of the study (Day 32) overnight fasted animals will be sacrificed humanely by CO_2 inhalation. Then the organs (kidney, liver, heart, and spleen) will be promptly excised. They will be trimmed, blotted with clean tissue paper, and weighed in grams. The organs will be observed for any abnormalities and lesions. The relative organ weight will be calculated as follows.[61,62,73,82]
Relative organ weight = Organ weight (g) / Body weight (g)

- *Histopathology of organs*

Tissue samples of the kidney, liver, heart, and spleen obtained from the sub-acute toxicity study will be fixed in 10% formalin for 24-48 hours. After that, relevant sections will be obtained from the organs, dehydrated in a graded ethanol series from 50 to 100%, and kept in each concentration for 2 ½ hours. Then the tissues will be immersed in three xylene solutions for 1½ hours in each and subsequently will be kept for 2 hours in liquid paraffin. Finally, the tissue samples will be embedded in paraffin wax solution, and 4-5µm paraffin-embedded sections will be prepared using a microtome (Sakura ACCU-cut SRM 200). The tissue sections will be mounted using poly egg albumin coated glass slides, which will be allowed to dry for 1 hour. After that, the tissue sections will be deparaffinized in xylene and rehydrated in descending concentrations of ethanol series by immersing them for 2 to 3 min in 100, 90, 80, and 70% ethanol and distilled water. The slides will be stained with Harris Hematoxylin for 8-10 minutes. Then the slides will be washed in water, followed by several dips in 1% acid alcohol to remove excess blue color. Then, the slides will be kept in running tap water for about 10 minutes. After washing the slides, they will be stained with Eosin for 30 seconds, followed by washing them with water. The sections will be dehydrated in 70, 90, and 100% ethanol and finally, the sections will be cleared in xylene, mounted, and examined under a light microscope (with × 40 objective lenses). Randomly selected fields of stained tissue will be taken to study the histopathology of the organ.[62,83,84] A consultant histopathologist will observe the pathological changes of the tissue sections in test groups in comparison to the control groups. Finally, a corresponding photomicrograph will be taken and the observations will be recorded.

- *Animal behavior*

This will be observed on the basis of the ethograms described in Casarrubea *et al*. [85] and Abou-Ismail and Mahboub.[86] The observation will be done twice daily (at 9.00 am and 3.00 pm).

Investigation of *In-vitro* Cytotoxic Effects of *P. marsupium* Latex

Cytotoxicity on rat bone-marrow-derived hematopoietic stem cells (HSC)

This will be tested in the same stem cell cultures where the hematopoietic effect is investigated (Section 3.5.1). Then, HSC will be tested for different concentrations of plant latex (15.625, 31.25, 62.5, 125, 250, 500, 1000 µg/mL) following the procedure described under 3.7.4, including MTT assay. The percentage inhibition will be calculated using the following formula, and IC_{50} will be calculated.[60] Experiments with each latex concentration will be conducted in triplicates on the same batch of cells.

Percentage cell inhibition = mean OD (control) − mean OD (treatment)/mean OD (control) x100

Normal human cell lines for cytotoxicity testing

Normal cell lines of the human embryonic kidney (HEK 293), human liver (Hep3B), human spleen (PMC2), and human heart (HAEC) will be used in testing the cytotoxicity of plant latex. Cell lines will be purchased from the American Type Cell Collection (ATCC).

The cells will be maintained in DMEM supplemented with 10% FBS and 20 mL each of penicillin and streptomycin in a humidified atmosphere of 5% CO_2 and 95% air, in a CO_2 incubator.[87,88]

Preparation of plant latex

The latex will be dissolved in 100% dimethyl sulfoxide (DMSO) or phosphate buffer saline (PBS) to a final stock concentration of 1000 µg/mL and stored at -20°C for future use. The stock solution will be diluted with DMEM supplemented with 10% FBS into the desired concentration series (15.625, 31.25, 62.5, 125, 250, 500, 1000 µg/mL).[88]

Cell culture and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

All cell lines will be cultured (5×10^3 cells/well) in 96-well tissue culture plates for 24 hours. After removing media, cells will be treated with 1ml of the above-mentioned concentrations of plant latex and incubated overnight at 37°C with 5% CO_2. Cells treated with 10% FBS will be the negative control. After incubation, the test solution will be removed from each well and washed in PBS. Then the cells will be incubated in serum-free DMEM and MTT dye (5 mg/mL in PBS) will be added to each. The plates will be incubated for 4 hours and the medium will be removed. The cells will be incubated for 15 minutes with 100 mL of 1% DMSO. The absorbance of the MTT will be determined at 570 and 630 ηm (reference) using an ELISA reader. The percentage inhibition will be calculated using the following formula, and IC_{50}, defined as the concentration of the test substance at which cell viability is decreased to 50%, will be calculated.[88,89]

Percentage cell inhibition = mean OD (control) − mean OD (treatment)/mean OD (control) x100

Experiments with each latex concentration will be conducted in triplicates on the same batch of cells.

Embryotoxicity Test

Maintenance of adult zebrafish

Maintenance and breeding of zebrafish (Species - *Danio rerio*) adults will be done according to the standard OECD guidelines.[90] Fish with visible signs of infections or who have undergone any pharmaceutical treatment for 2 months before spawning will be excluded from the study. Adult fish will be maintained in 1 ft x 1 ft aquaria with 12-hour light and 12-hour dark cycle at 27 ± 1°C with a pH of 7 ± 0.2 and fed twice daily with a standard artificial fish diet and artemia.[91,92]

Egg spawning and collection

Eggs will be obtained from spawning zebrafish adults in breeding tanks. A few hours before the onset of darkness on the day prior to the test, males and females will be placed in the tank with a sex ratio of 2 males, 4 females. Spawning will be induced in the morning. Fertilized eggs will be collected after 30 minutes of spawning from a minimum of three breeding groups. Then, the eggs will be randomly selected from breeding groups, rinsed a few times in system water, and placed in fresh fish water in a petri dish. Unfertilized or necrotic embryos will be removed.[90,92,93]

Fish embryo acute toxicity test (FET)

The embryo acute toxicity test will be referred to the OECD guideline No. 236.[90.] The range-finding test will be carried out using 10 embryos per concentration, including one embryo per well in 24-well plates. Eggs will be transferred to each well (at 24 hours post-fertilization) containing 2 mL of test concentration and 4 eggs in dilution water will be kept as an internal plate control on each of the plates. Ten eggs in dilution water will be maintained as a negative control. Another 10 embryos will be the positive control with 4mg/L 3,4-dichloroaniline. Seven concentrations of plant latex in distilled water (15.625, 31.25, 62.5, 125, 250, 500, 1000 µg/mL) will be tested on separate plates.[94] Plates will be sealed with parafilm and embryos will be kept in an incubator at 26 ± 1°C with a photoperiod of 14 hours light: 10-hour dark. Each test will be performed in triplicate. The embryo development will be monitored at 24-hour intervals for 96 hours. Four observations will be recorded as indicators of lethality, including coagulation of fertilized eggs, lack of somite formation, lack of detachment of the tail-bud from the yolk sac, and lack of heartbeat using an inverted microscope. The number of heartbeats will be counted for 15 seconds and then the heart rate will be calculated by multiplying it by 4. The appropriate concentration range of the plant latex will be determined.

The definitive test will be performed based on the concentration range determined in the range-finding test. Twenty embryos per concentration, including one embryo per well in 24-well plates will be utilized following the procedure described in the range finding test and lethal concentration (LC_{50}) will be calculated.[91,92]

Analysis of Chemical Composition

Qualitative analysis of phytochemicals

The latex will be analyzed qualitatively for carbohydrates, alkaloids, flavonoids, tannins, saponins, glycosides, and steroids.[3-5,63]

Functional group analysis

This will be analyzed by fourier transform infrared (FTIR).[95]

Quantitative analysis of hematopoietic vitamins

Vitamin B_6, B_2, B_{12}, B_9, A, E, and C in the plant latex will be quantified by high-performance liquid chromatography (HPLC) and titrimetric method. The data will be reported as means ± SEM of three independent analyses.[96-100]

Quantitative analysis of plant hemoglobin

Hemoglobin concentration in the plant latex will be quantified by a commercially available hemoglobin assay kit (https://www.sigmaaldrich.com/LK/en/product/sigma/mak115?gclid=EAIaIQobChMIno3gt82q9gIVvZJmAh2gZAAqE AAYASAAEgJM-_D_BwE) following the manufacturers' guidelines.[43] The data will be reported as means ± SEM of three independent analyses.

Quantitative analysis of metal content

Na, K, Fe, Mg, Ca, Zn, Mn, Cu, and Co of the plant latex will be analyzed quantitively using inductively coupled plasma mass spectrometry (ICP-MS).[67,101] The data will be reported as means±SEM of three independent analyses.

Web tool for further analysis

A web tool (SwissADME) (http://www.swissadme.ch/) will be used which allows the computing of physicochemical descriptors as well as the prediction of ADME parameters (absorption, distribution, metabolism, and excretion, pharmacokinetic properties, druglike nature, and medicinal chemistry friendliness of one or multiple small molecules to support drug discovery.

The Benefits To Institutions And Community

Benefits to Institutions

The identification of the phytochemical composition of the plant latex will be carried out using standard chemical tests. The quantitative analysis of hematopoietic vitamins will be carried out using the HPLC and titrimetric methods, while the quantitative analysis of metals will be carried out using ICP-MS. The functional groups of active components in the plant latex will be analyzed using FTIR. Cytotoxicity, embryotoxicity, histopathology, and hematology testing will also be carried out while the study involved with Wistar rats will be done at the animal center, where animal handling and maintenance facilities are available. Utilizing available facilities and establishing some facilities/techniques in these laboratories will facilitate the further understanding of herbal medicine and its applications in hematological diseases.

Benefits to the Community

As anemia has become a moderate public health problem in Sri Lanka, it is worth exploring and further understanding the nature of available traditional medicines in treating anemia. The majority of people rely on herbal medicines for anemia because, the other available treatment options might be expensive and often thought to be associated with some side effects. Most of these medicinal plants have been ingested blindly by many people without prior knowledge of their mechanism of relief as well as associated safety or toxicity risks. Therefore, it is important to identify effective, low-cost, less toxic, and feasible natural plant sources as a remedy for anemia, especially in countries categorized as 'developing countries. The outcomes of our study will guide and enlighten the people who rely on these natural products in treating anemia and also fulfill the need for scientific documentation on the safety or toxicity profile of this natural treatment.

Assessment of hematological parameters in our study will be useful in determining the systemic deleterious effects of the plant latex as these laboratory investigations report to be highly sensitive, accurate, and reliable. On the other hand, if we discover a similar action, structure, and considerable concentration of EPO in this plant latex, it would be beneficial in the way of treating anemia-associated disease conditions such as chronic renal failure, myelodysplasia, rheumatoid arthritis, HIV, and cancer as a replacement for recombinant human EPO (rhEPO).[102] This will be beneficial in the way of reducing the high cost of rhEPO treatment as well.

Study-Related Injury

This study will use Wistar rats and zebrafish embryos as they are biologically and genetically the same as humans in many processes. Embryotoxicity will be assessed using fertilized fish embryos and hemolytic anemia will be induced in Wistar rats using phenylhydrazine as an induction model. Euthanasia is necessary at the end of the treatment process to observe the toxic effect of plant latex on the organ level. Carbon dioxide euthanasia will be performed as it is a standardized method of euthanasia and it will be performed by skilled personnel in the method in a professional and compassionate manner. Death will be confirmed by the same skilled personnel, recognizing cessation of vital signs. Therefore, the scientific objectives of the study will be achieved making the endpoint humane by using rapid unconsciousness without pain or distress.

Conclusion

This research study will shed light on the potential anti-anemic properties of *P. marsupium* latex, suggesting its role in combating anemia. However, it is crucial to acknowledge the cytotoxic and embryotoxic effects of latex. The findings of the study will emphasize the importance of further investigation into the therapeutic and adverse effects of *P. marsupium* latex. Ultimately, a comprehensive understanding of the balance between efficacy and safety will be essential for linking the therapeutic potential of *P. marsupium* latex in clinical settings, offering new research pathways for the management of anemia and related disorders.

Future research findings will be focused on explaining the specific pathways involved in its anti-anemic actions while also exploring strategies to mitigate its cytotoxic and embryotoxic impacts.

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PEER-REVIEWED CERTIFICATION

During the review of this manuscript, a double-blind peer-review policy has been followed. The author(s) of this manuscript received review comments from a minimum of two peer-reviewers. Author(s) submitted revised manuscript as per the comments of the assigned reviewers. On the basis of revision(s) done by the author(s) and compliance to the Reviewers' comments on the manuscript, Editor(s) has approved the revised manuscript for final publication.