# EFFECTS OF ETHANOL EXTRACT OF *JUSTICIA SECUNDA* LEAVES ON HAEMATOLOGICAL INDICES AND ANTIOXIDANT STATUS IN PHENYLHYDRAZINE-INDUCED ANEMIC RATS

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**OCTOBER, 2018**

**CERTIFICATION**

This is to certify that this work has been submitted to Department of Biochemistry, Faculty of Biological Sciences, University of Nigeria, Nsukka by OFFOR, CHINEDUM ROLAND with Registration number PG/M.Sc/16/80510 in partial fulfillment of the requirement for the award of Master of Science (M.Sc.) degree in Pharmacological Biochemistry. The work embodied in this report is original and has not been submitted in part or full for any other diploma or degree of this or any other university.

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## DEDICATION

This work is dedicated to Almighty God who is the Author and the finisher of my faith.

For seeing me through. Obinigwe daalu!

## ACKNOWLEDEGMENTS

The researcher heartly appreciates God Almighty for His guidance, wisdom and protection throughout this my postgraduate studies especially in actualizing this research work.

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My Siblings, Uncles, Aunts, colleagues and friends you are not left out.

Thanks to you all and God bless you.

**ABSTRACT**

Plants used for traditional medicine contain a wide range of substances which can be used to treat various diseases. The study evaluated the effects of ethanol extract of *Justicia secunda* leaves on haematological indices and antioxidant status in phenylhydrazine-induced anemic rats. The preliminary quantitative phytochemicals analysis of the ethanol extract of *Justicia secunda* leaves were carried out in order to ascertain the presence of plant secondary metabolites. The air-dried leaves were pulverized and extracted with ethanol to give the plant extract. Phytochemical analysis of the extract revealed the presence of flavonoids, alkaloids, terpenoids, tannins, steroids, saponins, glycosides, polyphenol and phenols. Acute toxicity tests of the extracts were carried out and the result showed that the extract was well tolerated by the rats as neither death nor signs of toxicity were observed up to the dose of 5000mg/kg body weight of the rats. The effects of the extract on haematological indices, antioxidant parameters as well as some serum electrolytes and vitamins concentrations of phenylhydrazine-induced rats were evaluated. There were significant increase ($P<0.05$) in total red blood cell count and haemogloblin concentrations as well as PCV in the extract-treated rats when compared with the controls. However, the extract caused significant increases in the antioxidant parameters ($P<0.05$) of extract treated groups of rats when compared with the positive control group. There was significant decrease in lipid peroxidation parameter in (MDA concentration) when the extract-treated groups were compared with the positive control group (anemic rats). The extract significantly increased ($P<0.05$) the serum electrolytes concentrations of the treated groups when compared to the control group. There were also significant changes in the treated groups form the control and untreated group. These results showed that the ethanol extract of *Justicia secunda* leaves significantly reduced the anemic condition in the rats and restored some of the changes in biochemical parameters of the phenylhydrazine-induced anemic rats. Results obtained from the present study indicate that the leaf extract of *Justicia secunda* possesses antianemic and antioxidant activities and this supports the therapeutic use of the plant in traditional medicine for the treatment of anemia.

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# CHAPTER ONE

# INTRODUCTION

Anemia is the most common blood disorder, affecting about a third of the global population. Iron-deficiency anemia affects nearly 1 billion people. In 2013, anemia due to iron deficiency resulted in about 183,000 deaths – down from 213,000 deaths in 1990. Anemia is a condition in which the blood has a lower than normal number of red blood cells. In other words, it is defined as decrease in the total amount of red blood cells (RBCs) or hemoglobin in the blood, or a lowered ability of the blood to carry oxygen (Janz *et al*., 2013). This condition can also occur if the red blood cells do not contain enough haemoglobin (an iron-rich protein that gives blood its red colour).

Haemoglobin helps red blood cells carry oxygen from the lungs to the rest of the body. With severe or long-lasting anemia, the lack of oxygen in the blood can damage the heart, brain and other organs in the body. Very severe anemia may even cause death. When anemia comes on slowly, the symptoms are often vague and may includeweakness, shortness of breath and a poor ability to exercise. Anemia that comes on quickly often has greater symptoms, which may include confusion, feeling like one is going to pass out, loss of consciousness, or increased thirst. Anemia must be significant before a person becomes noticeably pale. Additional symptoms may occur depending on the underlying cause (Bhutta*et al.,* 2013).The three main types of anemia are due to blood loss, decreased red blood cell production, and increased red blood cell breakdown. Causes of blood loss include trauma and gastrointestinal bleeding, among others. Causes of decreased production include iron deficiency, a lack of vitamin B12, thalassemia, and a number of neoplasms of the bone marrow. Causes of increased breakdown include a number of genetic conditions such as sickle cell anemia, infections such as malaria, and certain autoimmune diseases. It can also be classified based on the size of red blood cells and amount of hemoglobin in each cell. If the cells are small, it is microcytic anemia. If they are large, it is macrocytic anemia while if they are normal sized, it is normocytic anemia (Bhutta *et al.,* 2013).Medicinal plants are the most common sources of drugs used in traditional medicine (Sofowora, 2008). Despite improvement in health care delivery system,medicinal plants play a vital role in both human and animal health care system and about 60% of the world population depends on herbs for their primary health care (Ezuruike *et al*.,

2014). The reason for this may be due to high cost of orthodox drugs, side effects and unavailability of orthodox medicine and personnel in remote areas. Poverty and cultural inclination may have contributed to the trend (Akah *et al*., 2009).

*Justicia secunda* is an evergreen, perennial plant with stems that sometimes become more or less woody. *Justicia secundaVahl* belonging to the family of Acanthacae is known as "Bloodroot" and "Sanguinaria" in Barbados and Venezuela respectively (Carrington *et al.,* 2012). In the eastern part of Nigeria, it is called "Osisi ka dibia".

The folkloric uses of the plant include wound healing, anemia and abdominal pains (Kone *et al.,* 2012 ; N'guessan *et al*., 2010). In view of the use of this plant to manage anemia in folkloric medicine, this studywas therefore carried out with a view to giving scientific credence to the tradomedicinal use of the plant.

### 1.1 Description of *Justicia secunda*

They are evergreen perennials and shrubs with leaves which are often strongly veined; but they are primarily cultivated for their showy tubular flowers in shades of white, cream, yellow, orange or pink.*Justicia secunda* can grow from 90 - 200cm tall. They are not hardy below 7 °C (45 °F), so may be grown under glass in frost-prone areas (Kanchanapoom*et al*., 2005). There are also some other species of *Justicia* in existence and they include *Justicia Americana*,*Justicia ovate Justicia brandegeeana*, *Justicia carnea* and *Justicia procumbens. Justicia americana* (American water-willow), is native to eastern North America and north to southern Ontario.

*Justicia brandegeeana* (formerly *Beloperone guttata*, commonly called shrimp plant) is native to Mexico.

*Justicia carnea* (formerly *Jacobinia carnea*, common names including Brazilian plume flower, flamingo flower, and jacobinia) is native to South America, southern Brazil, Paraguay and northern Argentina. It is hardy to −2 °C but will often recover in the spring after freezing back.*Justicia procumbens* is procumbent herb with angular stems, swollen at nodes, small ovate leaves, small purple flowers in terminal spikes, inserted didynamous stamens, and shortly bilobed stigmas. In South-Eastern Nigeria, Congo and South Cote-d'Ivoire the leaf decoction is consumed by Jehovah Witness believers in the management of anemia (Kanchanapoom *et al*., 2005.The anti-sickling, haematic, antimicrobial and anti-hypertensive activities of *Justicia secundaVahl* have been reported (Carrington *et al.,* 2012).

Phytochemical screening of the plant has shown the presence of tannins, flavonoids, alkaloids, quinines and anthocyanins (Theiler *et al.,* 2010). Luteolin, aurantamide acetate, auranamide, quindoline and pyrrolidone derivatives secundarellone A, B and C have been isolated in *J. secunda* (Bedoya*et al*., 2008). Notwithstanding the popular uses of *J.secunda* in folkloric medicine, only few pharmacological studies have been done on the plant (Bourdy*et al.,* 2004).

**Figure1:***Justicia secunda* plant

**Source:** (www.inaturalist.org)

**1.1.2Taxonomy of *Justicia seunda* Vahl plant.**

| | |
|---|---|
| **Kingdom** | *Plantae* |
| **Phylum** | *Angiosperms* |
| **Class** | *Eudicots* |
| **Order** | *Lamiales* |
| **Family** | *Acanthaceae* |
| **Sub-family** | *Acanthoideae* |
| **Tribes** | *Justicieae* |
| **Genus** | *Justicia* |
| **Specie** | *secunda* |

**Source: (Daniel, 2011).**

### 1.1.3 Previous works done on the plant

#### 1.1.3.1 Ethnopharmacological information for the species of *Justicia*

Several species of *Justicia* are widely used in folk medicine for the treatment of respiratory and gastrointestinal diseases (Anand,2008) as well as inflammation (including applications in rheumatism and arthritis). The plants are also utilized for their effects on the central nervous system as hallucinogens, somniferous agents, sedatives, depressors, and treatments for epilepsy and other mental disorders,(Cantuti-Castelvetri,2000). Other species are popularly used in the treatment of headache and fever (eight occurrences, which may be associated with their sedative and analgesic properties), cancer (Badmadi, 2003), diabetes (Anand,2008), and HIV (Alcami,2008).Whole plant and aerial parts are usually used in folk medicine. Extracts made from only the leaves are the most used, followed by those extracts made from only the roots. Some species are used as mixtures. For example, traditional physicians around Kotagiri village near Ootacamund use a mixture of the powdered roots of *Cassia occidentalis* L., Caesalpineae, *Derris brevipes* var. *coriacea*, Papillionaceae, and *Justicia simplex* D. Don, Acanthaceae, to control fertility. Administration of this mixture for a few days after menstruation prevents conception without any toxic effects. The number of pregnancies among treated women was significantly less than that of the control group. These results indicate the abortifacient nature of the roots of these plants (Badami *et al*., 2003). The species *Justicia pectoralis* Jacq. is

used as the major component in a mixture to treat various diseases. Moreover, *Justicia insularis* T. Anderson is used as an infusion mixed with the leaves of *Ambrosia maritime* L., Compositae.

### 1.1.3.2 Pharmacological investigationsof species of *Justicia*

Some species show antitumoral activity against different cancer cell lines (seven occurrences). An ethanol extract of *Justicia neesii* Ramamoorthy (Acanthaceae) exhibited anticancer activity against P388 lymphocytic leukemia in mice. A methanol extract of the whole plant of *Justicia procumbens* L. showed significant inhibitory activity *in vivo* against P-388 lymphocytic leukemia growth and *in vitro* cytotoxicity in the 9-KB (human nasopharyngeal carcinoma) cell culture assay (Chen *et al*., 1995). Some species also showed inhibition of human cancer cell lines, mainly toward human cervical carcinoma (*Justicia ciliata* Jaqc.), T 47D and HeLa human cell lines (*Justicia spicigera* Schltdl.), and human ovarian cancer cell line (*Justicia rhodoptera* Baker), as well as prevention of some tumoral cell growth (*Justicia patentiflora* Hemsl.). The activity of popularly used whole-plant extracts of *J. procumbens* and *J. nesii* and leaf extracts of *J. specigera* as anticancer agents was confirmed by employing the same parts of the plant.

However, the anticancer properties of *Justicia adhatoda* L. have not yet been confirmed pharmacologically.The whole-plant extract of *J. spicigera* contains cytotoxic factors for leukemic cells and has no proliferative activity on normal hematopoietic progenitor cells. The plant extract induces apoptosis in the human leukemia cell line TF-1, but not in the bcl-2 transfectant cell line TB-1(N'Guessan *et al.,* 2010). These data suggest a strong correlation between the cytotoxic effect and cell proliferation. The results indicate that the infusion of the aerial parts of *J. spicigera* does not contain any hematopoietic activity, induces apoptosis inhibited by bcl-2, and is linked to cell proliferation.Some species show antiviral activity (five occurrences, *i.e., Justicia extensa* T. Anderson, *Justicia gendarussa*Burm. f., *J. procumbens, Justicia reptans* Sw., and *Justicia valida* Ridl.) against *in vitro* HIV type 1 reverse transcriptase, HIV replication, and vesicular stomatitis virus.

However, the species popularly used as antiviral agents, *Justicia betonica* L. and *Justicia flav*a (Vahl) Vahl, were either not included in pharmacological studies, or were tested but did not show antiviral activity. Crude water extracts of the aerial parts of *J. gendarussa* proved to be strongly active against *in vitro* HIV type 1 reverse transcriptase. Based on these observations, this species might be further explored for its antiviral indications.*J. pectoralis* showed high antibacterial activity against *E. coli, E. faecalis,* and *S. epidermidis.* Moreover, this species shows positive antimosquito tests, which were observed on the growth and development of IV-stage larvae of *Aedes aegypt*i mosquitoes. A brief exposure to concentrations of 0.05 to 0.50 mg/mL of the plant extract is required to produce 100% larvicidal activity.

The extracts of *J. pectoralis* were found to be the most toxic larvicide among the species of *Justicia* extracts tested. Extracts of *J. pectoralis* have estrogenic, progestagenic, and anti-inflammatory effects, explaining the plant's traditional use in menopause and PMS therapies.The methanol extract of the whole plant of *J. procumbens* exhibited 50% inhibitory activity toward the arachidonic acid-induced aggregation of rabbit platelets (Chen *et al*., 1995; Chen *et al*., 1996). The antiplatelet aggregation activity can be related to the popular use of extracts obtained from *Justicia ansellian*a (Nees) T. Anderson in the treatment of heart disease.The ethanol extract of the leaves of *J. gendarussa* showed a higher paw edema inhibition than aspirin-treated rats in the FCA-induced and the collagen-induced arthritic models. These pharmacological results align with the popular use of *J. gendarussa* in the treatment of arthritis and rheumatism. The species *J. spicigera* is popularly used as an anti-inflammatory agent, and this activity was also pharmacologically confirmed.The popular use of *J. pectoralis* in the treatment of epilepsy and anxiety was confirmed with the ethanol extract of the leaves. The ethanol extracts of *J. pectoralis, Justicia aurea* Schltdl., and *Justicia albobracteata* Leonard were tested *in vitro* for their ability to inhibit GABA- transaminase (GABA-T) or to bind to the GABAA-benzodiazepine receptor, two principal drug targets in epilepsy and anxiety. A significant positive correlation between GABA-T inhibition and the relative frequency of use for epilepsy was observed. Moreover, an even stronger correlation between GABAA binding and the relative frequency of use for shock was observed. Thus the Q'eqchi' traditional knowledge of *J. pectoralis, J. aurea*, and *J. albobracteata* is associated with the plant's antiepileptic and anxiolytic activities.The pharmacological studies of some species were not based on their use in

folk medicine. Extracts of the whole plant of *Justicia prostrata* Gamble showed antiulcer activity. The aqueous extract was more active than the alcoholic extract when tests were made using the aspirin-induced pylorus ligated rat model. The antiulcerogenic activities of both extracts were compared with the drug Rantidine, an H2-receptor antagonist. Alcoholic extracts of *J. anselliana* showed allelopathic properties. The aerial part of the plant produced more significant effects on the growth parameters of the cowpea plant (*Vigna unguiculata* (L.) Walp., Leguminosae), such as germination, elongation, and the weight, than extracts of the root (Ahanchede *et al*., 2004). Likewise, the popular use of the leaves of *Justicia schimperiana* (Hochst. ex Nees) T. Anderson in the treatment of liver disease may be related to the hepatoprotective activity of the leaf extracts of the plant.

However, the hepatoprotective activity of *J. adhatoda* was not studied despide its popular use. In addition, some other species, such as *J. betonica, Justicia calycina* (Nees) V.A.W.Graham, *Justicia diffusa* Willd., *Justicia dumetorum* Morong, *J. flava, Justicia ghiesbreghtiana* Lem., *Justicia ideogenes, J. insularis, Justicia plectrantus, Justicia purpurea* L., *Justicia secunda* Vahl, *Justicia sericea* Ruiz and Pav., and *J. simplex*, showed a variety of popular uses and have no yet been studied pharmacologically.

### 1.1.3.3 Compounds isolated from species of *Justicia*

A great diversity of chemical classes is found in the species of *Justicia*, mainly alkaloids, lignans, flavonoids, and terpenoids (iridods, diterpenoids, and triterpenoids). Other chemical classes have been isolated from species of *Justicia*, such as essential oils, vitamins, fatty acids (docosanoic acid), and salicylic acid (Angonese *et al*., 1992; Al-Juaid and Abdel-Mojib, 2004). The steroids campesterol, stigmasterol, sitosterol, and sitosterol-D-glucoside were isolated from the leaves and roots of *J. flava, J. spicigera*, and *J. gendarussa* (Olaniyi, 1980; Wahi *et al*., 1974; Domínguez *et al*., 1990; Amborabé *et al*., 2002; Deepak *et al*., 2002; Rajakumar and Shivana, 2009). The literature describes the allelopathy effect of the sterols and triterpenes. Both of the chemical classes isolated from the alcoholic extract of the aerial parts of *J. anselliana* showed allelopathic effects on cowpea plants (Kpoviessi *et al*., 2006). The allelopathic effects of the leaf and root extracts of *J. anselliana* have also been described. Experiment shows a coumarin, flavonoids, alkaloids, and triterpenoidal glycosides isolated from the species of *Justicia*. Only

one coumarin, umbeliferone (Abdel-Ghani*et al*., 2001), and a small variety of flavonoids (Agra *et al*.,2007), alkaloids (Alonso-Castro *et al*., 2011), and triterpenoidal glycosides (Awad *et al*., 2009) were identified.Leaf extracts from *J. reptans* display a clear virucidal effect on HIV, which was attributed to two glycosylated flavonoids that have not yet been identified (Bedoya, 2008). Compounds of this chemical class have been previously reported to display anti-HIV properties including reverse transcriptase or integrase inhibition, but this is the first time that they are described as virucides (Kumar *et al*., 2005). Pharmacological tests using the ethanol extract from *J. reptans* indicated inhibition of HIV replication.A large variety of lignans has been isolated from species of *Justicia*. Lignans are a large group of natural products that show diverse biological effects. Lignans may serve as lead compounds for the development of new therapeutic agents with cytotoxic activity (Fukamiya and Lee, 1986; Hui *et al*., 1986). For example, lignans obtained from *J. pectoralis* are cytotoxic to leukemia and solid tumor cell lines (Hui *et al*., 1986).Lignans also show antiangiogenic, antileishmanial, antifungal, hypolipidemic, antiasthmatic (Vasilev and Ionkova, 2005), antiviral (Asano *et al*., 1996), antineoplastic (Gordaliza *et al*., 2000), antifeedant (Bedoya *et al*., 2008), insecticidal, cardiotonic, antidepressant (Ghosal *et al*., 1979), analgesic, antiplatelet (Chen *et al*., 1996), and anti-inflammatory (Navarro *et al*., 2004) indications, as well as activity as lipid peroxidation inhibitors. Potent anti-inflammatory activities were described for lignan glycosides isolated from *J. ciliata* (Day *et al*., 2000) and phenolic compounds isolated from *J. prostrata* (Sanmugapriya *et al*., 2005).Many lignans contain an arylnaphthalide skeleton and are found in relatively high proportions (Rajasekhar and Subbaraju, 2000). For example, jusmicranthin was isolated from a chloroform extract of *J. neesii*, giving a mass yield of 0.025%. The dry leaves of *J. extensa* contain approximately 1% of justicidin P, which exists at 25 ºC as two rotamers (Wang and Ripka, 1983). Some arylnaphthalide lignans are glycosylated derivatives. Other miscellaneous-type lignans are also found in species of *Justicia*.

**Biological activity of compounds isolated from species of *Justicia***

Some compounds show biological activities related to those observed in the species from which they are isolated. Coumarin umbeliferone, isolated from hydroalcoholic extract of the leaves of *J. pectoralis*, showed anti-inflammatory, antinociceptive, and bronchodilator activities, which are related to the estrogenic, progestogenic, and anti-inflammatory activities of this species and its

popular use in the treatment of bronchitis. This species is also popularly used in the treatment of respiratory diseases(Anand, 2008). The vasodilator activity of the flavonoid is related to the anti-hypertensive activity of the *Justicia cataractae* Leonard. Apigenin has been reported to exert anti-inflammatory effects such as lowering oxidative stress and forestalling the expression of several inflammatory factors (Sawatzky *et al*., 2006). The flavonoid vitexin is a potent anti-inflammatory agent, inhibiting the 5-lipoxygenase pathway, which, together with the COX-2 pathway, is very important in producing and maintaining inflammation (Sridhar *et al*., 2006). Compoundswere isolated from the ethanol extract of *J. gendarussa*, which is used in the treatment of inflammation, rheumatism, and arthritis in folk medicine. The antimicrobial and anti-inflammatory activities of flavonoid, as well as its effects on macrophage regulation and reduction in blood glucose levels are related to the popular uses of *J. spicigera* in giardicidal, anti-inflammatory, anticancer, and antidiabetes therapies. Alkaloid, also isolated from *J. spicigera*, is used as an anti-inflammatory agent.Alkaloids and show bronchodilator activity and were isolated from *J. adhatoda,* which is popularly used in the treatment of bronchitis. The antifertility activity of triterpenoidal glycoside is related to the popular use of *J. simplex* as an abortifacient and to control fertility. Alkaloids show antitumor activity and were isolated from *J. betonica*, however, this species is popularly used in the treatment of diarrhea, inflammations, and HIV/AIDS, not toward cancer.Elenoside, isolated from *Justicia hyssopifolia* L., is the most pharmacologically studied arylnaphthalene lignan in the genus *Justicia.* This compound shows sedative, muscle relaxant, cytotoxic, antiviral, insecticidal, cardiotonic, analgesic, lipid peroxidation inhibitory, anti-inflammatory, and stimulant activities and exhibits significant central nervous system depressant properties in rats. Its anxiolytic action, inducing sedation and muscle relaxation (Navarro *et al*., 2001a), is similar to other tranquilizer drugs (Irwin, 1968) such as the action of sedative-hypnotic barbiturates (Navarro *et al*., 2004).

The cytotoxic activity of was verified in human cancer cell lines in a range of concentrations from $10^{-5}$ to $10^{-4}$ M, with an LD50 of 305 mg/kg in mice and central depressive properties at doses of 25, 50, and 100 mg/kg. No lethality was observed for five days following administration of this compound (Alonso *et al*., 1997). As a consequence, this compound behaves as a sedative with broad-spectrum cytotoxicity (Navarro *et al*., 2001), also showing cytotoxic effects toward leukemia cell lines (Navarro *et al*., 2001).Other lignans isolated from

species of *Justicia* show a smaller spectrum of biological activity. The antiplatelet aggregation activity of lignans and are related to the pharmacological tests of the methanol extract of *J. procumbens*. Lignans showed strong antiviral activity against vesicular stomatitis virus and low cytotoxicity against cultured rabbit lung cells (RL-33) (Asano *et al*., 1996). Lignans and showed inhibition of secondary aggregation induced by adrenaline (Wu *et al*., 2007).

Moreover, these compounds showed an inhibitory effect on cyclooxygenase-1 (COX-1), with an antiplatelet effect partially due to the suppression of COX-1 activity and reduced thromboxane formation.Lignaninhibits human hepatitis B viral replication. This compound is isolated from *J. flava*, which is popularly used in the treatment of HIV/AIDS in Uganda. Lignans show antiviral activities. These compounds were isolated from *J. extensa, J. betonica*, and *J. procumbens,* and also show the same biological activities. Conversely, lignans show antiviral activity, but were isolated from species that did not show this activity. A larger investigation of the extracts of these species is required to explore their antiviral activities.The antitumor activity of lignans are related to the popular uses of *J. procumbens, J. ciliata, J. rhodoptera,* and *J. patentiflora* as anticancer therapies. Lignans isolated from *J. ciliata*, showed significant citotoxic effects toward a number of cancer cell types (human hepatomacellular carcinoma, human cervical carcinoma, human colorectal adenocarcinoma, human colorectal carcinoma, and human breast cancer) (Day *et al*., 2002). Lignan also displayed potent cytotoxic effects against T-24, CaSki, SiHa, HT-3, PLC/PRF/5, and 212 cells *in vitro* (Day *et al*., 1999). Lignan exhibited low cytotoxicity against three human tumor cell lines: A-549 (human lung carcinoma), MCF-7 (human breast carcinoma), and HT-29 (human colon adenocarcinoma) (Subbaraju *et al*., 1991). Lignan is included in a wide variety of cancer chemotherapy protocols and was used as a precursor for the semi-synthesis of anticancer therapeutics (Canel *et al*., 2000). Lignans show antitumoral activity, but they were isolated from species that did not show this activity. The data warrant a larger exploration of the extracts of these species for their anticancer properties.

#### 1.1.3.4 Biological screening of the activity of *Justicia secunda* Vahl plant.

Biological screening of the activity of Justicia secunda Vahl. extracts and fractions against several Gram-positive and Gram-negative bacterial strains of Enterobacteriaceae and in the brine

shrimp Artemia salina Linn. assay, is reported (Kpoviessi *et al.,* 2006). The ethanol extract of the aerial parts of the plant was sequentially partitioned with n-hexane (FHX) and chloroform (FCH), remaining a water solution (FOH). All fractions showed bactericidal activity against Gram-positive bacteria Bacillus cereus (ATCC 9634) and Staphylococcus aureus (ATCC 6538P), while they were inactive against Gram-negative strains Escherichia coli (ATCC 0389), Klebsiella pneumoniae (ATCC 10031), Proteus vulgaris (ATCC 9920) and Salmonella typhimurium (ATCC 14028). The water-soluble fraction (FOH) showed activity in the brine shrimp bioassay, yielding a LC50 value of 37.93mg/ ml. The original FHX and FCH fractions were subjected to short column chromatography. Three fractions from FHX and four fractions from FCH were active against Grampositive bacterial strains, and they were inactive against the Gram-negative ones. Antibacterial activity, Artemia salina Linn, ethnobotanic use, Justicia secunda Vahl., LC50 value, toxicity test.

**1.2 Clinical presentation of anemia.**

Anemia goes undetected in many people and symptoms can be minor. The symptoms can be related to an underlying cause or the anemia itself. Most commonly, people with anemia report feelings of weakness or fatigue, and sometimes poor concentration. They may also report shortness of breath on exertion. In very severe anemia, the body may compensate for the lack of oxygen-carrying capability of the blood by increasing cardiac output. The patient may have symptoms related to this, such as palpitations, angina (if pre-existing heart disease is present), intermittent claudication of the legs, and symptoms of heart failure. On examination, the signs exhibited may include pallor (pale skin, lining mucosa, conjunctiva and nail beds), but this is not a reliable sign (Guagnozzi and Lucendo, 2014).

There may be signs of specific causes of anemia, e.g., koilonychia (in iron deficiency), jaundice (when anemia results from abnormal break down of red blood cells; in hemolytic anemia), bone deformities (found in thalassemia major) or leg ulcers (seen in sickle-cell disease). In severe anemia, there may be signs of a hyperdynamic circulation: tachycardia (a fast heart rate), bounding pulse, flow murmurs, and cardiac ventricular hypertrophy (enlargement). There may be signs of heart failure. Pica, the consumption of non-food items such as ice, but also paper, wax, or grass, and even hair or dirt, may be a symptom of iron deficiency, although it occurs often in those who have normal levels of hemoglobin. Chronic anemia may result in behavioral

disturbances in children as a direct result of impaired neurological development in infants, and reduced academic performance in children of school age. Restless legs syndrome is more common in those with iron-deficiency anemia (Stein *et al*., 2016).

### 1.2.1 Etiologial factors of anemia

The causes of anemia may be classified as impaired red blood cell (RBC) production; increased RBC destruction (hemolytic anemia) blood loss and fluid overload (hypervolemia). Several of these may interplay to cause anemia eventually. Indeed, the most common cause of anemia is blood loss, but this usually does not cause any lasting symptoms unless a relatively impaired RBC production develops, in turn most commonly by iron deficiency (World Health Organization, 2008).

### 1.2.2 Disturbance of proliferation and maturation of erythroblasts:

Pernicious anemia is a form of megaloblastic anemia due to vitamin $B_{12}$ deficiency dependent on impaired absorption of vitamin $B_{12}$. Iron deficiency anemia, resulting from deficient heme synthesis leading to iron deficiency anemia. Iron is essential for the production of hemoglobin. The depletion of iron stores may result from blood loss, decreased intake, impaired absorption, or increased demand. Iron-deficiency anemia could result from occult gastrointestinal bleeding (Idris et al., 2005).

### 1.2.3 Influence of hormones as a cause of anemia in adolescence

In adolescence, hemoglobin levels are admittedly higher in males than in females because prostaglandins(PGE) facilitate erythropoietic activity, both directly(PGE1) and via cyclic AMP (PGE 2). Androgens stimulate erythropoietin action by increasing or facilitating its production in the erythroid stem cells. Conversely, estrogens inhibit the effects of erythropoietin (Santos *et al*., 2012).Due to changes in the nutritional requirements of adolescents at menarche in girls and as a result of the hormonal changes at puberty in boys-hemoglobin levels differ as a function of gender, age or stage of sexual maturity (Romilda *et al*., 2014).In women of reproductive age, menstrual bleeding defines anemia, sometimes requiring daily oral iron supplementation. Women, in whom menstrual bleeding is excessive, either with respect to the number of bleeding days or to the amount of flow and the occurrence of menstrual clots, need to be monitored

continuously for as long as dysfunctional uterine bleeding is present, a period in which iron supplementation may indeed be required (Dallmann and Reeves, 2001).

### 1.2.4 Blood loss

Anemia of prematurity from frequent blood sampling for laboratory testing combined with insufficient RBC production. Trauma or surgery, causing acute blood loss can cause anemia. Gastrointestinal tract lesions, causing either acute bleeds (e.g. variceal lesions, peptic ulcers) or chronic blood loss (e.g. angiodysplasia) and Infection by intestinal nematodes feeding on blood, such as hookworms and the whipworm *Trichuristrichiura* can cause anemia (Gyorkos *et al.*, 2011).

### 1.2.5 Fluid overload

Fluid overload (hypervolemia) causes decreased hemoglobin concentration and apparent anemia. General causes of hypervolemia include excessive sodium or fluid intake, sodium or water retention and fluid shift into the intravascular space (Verma and Chera, 2017).

### 1.2.6 Intestinal inflammation

Certain gastrointestinal disorders can cause anemia. The mechanisms involved are multi-factorial and not limited to mal-absorption but mainly related to chronic intestinal inflammation, which causes dysregulation of hepcidin that leads to decreased access of iron to the circulation.

## 1.3Pathogenesis of anemia

Anemia is a common comorbidity in patients with heart failure and is associated with worse long-term outcomes (Anand and Tang, 2008). Although the mechanisms involved in the development of anemia in patients with heart failure are unclear, data suggest that renal dysfunction, neurohormonal, and proinflammatory cytokine activation in heart failure lead to anemia of chronic disease with defective iron utilization, inappropriate erythropoietin responsiveness, and depressed bone marrow function(Anand, 2008). Likewise, the mechanisms by which anemia worsens heart failure outcomes are also uncertain and may be related to increased myocardial workload to compensate for reduced tissue oxygen delivery leading to unfavorable cardiac remodeling, to the effects of factors that cause anemia in patients with heart

failure or because of aggravation of other comorbidities seen in these patients(Anand, 2008). Patients with heart failure and anemia are more likely to be older, have diabetes mellitus, chronic kidney disease, more severe heart failure, lower blood pressure, and greater neurohormonal and proinflammatory cytokine activation (Anand, 2008). Most multivariable analyses, apart from a few exceptions, have reported that anemia is an independent predictor of mortality and morbidity in a variety of patients with heart failure including men and women, patients with acute decompensated and chronic heart failure, and those with impaired or preserved ejection fraction (Anand and Tang, 2008). Thus, correcting anemia may be an important therapeutic target to improve long-term outcomes in such patients. Although the results of several small studies in patients with anemia and heart failure with reduced ejection fraction have shown that erythropoiesis-stimulating agents increase hemoglobin and have some beneficial effects on clinical outcomes,1 the neutral results of treating anemic patients with darbepoetin in the large, more definitive the Reduction of Events by Darbepoetin Alfa in Heart Failure (RED-HF) trial3 indicate that we need to better understand the pathogenesis of anemia and whether it is just a marker of the severity of heart failure.

## 1.4 Pathophysiology of anemia

### 1.4.1 Erythrocyte life cycle

Erythroid precursors develop in bone marrow at rates usually determined by the requirement for sufficient circulating Hb to oxygenate tissues adequately. Erythroid precursors differentiate sequentially from stem cells to progenitor cells to erythroblasts to normoblasts in a process requiring growth factors and cytokines. This process of differentiation requires several days. Normally, erythroid precursors are released into circulation as reticulocytes (Liang and Ghaffari, 2016).

Reticulocytes are so called because of the reticular meshwork of rRNA they harbor. They remain in the circulation for approximately 1 day before they mature into erythrocytes, after the digestion of RNA by reticuloendothelial cells. The mature erythrocyte remains in circulation for about 120 days before being engulfed and destroyed by phagocytic cells of the reticuloendothelial system (Liang and Ghaffari, 2016).

Erythrocytes are highly deformable and increase their diameter from 7 µm to 13 µm when they traverse capillaries with a 3-µm diameter. They possess a negative charge on their surface, which

may serve to discourage phagocytosis. Because erythrocytes have no nucleus, they lack a Krebs cycle and rely on glycolysis via the Embden-Meyerhof and pentose pathways for energy. Many enzymes required by the aerobic and anaerobic glycolytic pathways decrease within the cell as it ages. In addition, the aging cell has a decrease in potassium concentration and an increase in sodium concentration. These factors contribute to the demise of the erythrocyte at the end of its 120-day lifespan (De Loughery, 2014).

### 1.4.2 Response to anemia

The physiologic response to anemia varies according to acuity and the type of insult. Gradual onset may allow for compensatory mechanisms to take place. With anemia due to acute blood loss, a reduction in oxygen-carrying capacity occurs along with a decrease in intravascular volume, with resultant hypoxia and hypovolemia. Hypovolemia leads to hypotension, which is detected by stretch receptors in the carotid bulb, aortic arch, heart, and lungs. These receptors transmit impulses along afferent fibers of the vagus and glossopharyngeal nerves to the medulla oblongata, cerebral cortex, and pituitary gland (Veng-Pedersen*et al*., 2002).

In the medulla, sympathetic outflow is enhanced, while parasympathetic activity is diminished. Increased sympathetic outflow leads to norepinephrine release from sympathetic nerve endings and discharge of epinephrine and norepinephrine from the adrenal medulla. Sympathetic connection to the hypothalamic nuclei increases antidiuretic hormone (ADH) secretion from the pituitary gland. ADH increases free water reabsorption in the distal collecting tubules. In response to decreased renal perfusion, juxtaglomerular cells in the afferent arterioles release renin into the renal circulation, leading to increased angiotensin I, which is converted by angiotensin-converting enzyme (ACE) to angiotensin II (Adamson and Longo, 2001).

Angiotensin II has a potent pressor effect on arteriolar smooth muscle. Angiotensin II also stimulates the zona glomerulosa of the adrenal cortex to produce aldosterone. Aldosterone increases sodium reabsorption from the proximal tubules of the kidney, thus increasing intravascular volume. The primary effect of the sympathetic nervous system is to maintain perfusion to the tissues by increasing systemic vascular resistance (SVR). The augmented venous tone increases the preload and, hence, the end-diastolic volume, which increases stroke volume. Therefore, stroke volume, heart rate, and SVR all are maximized by the sympathetic nervous system. Oxygen delivery is enhanced by the increased blood flow (Kuku*et al*., 2009).

### 1.5 Epidemiology of anemia

Sickle cell disease is common in regions of Africa, India, Saudi Arabia, and the Mediterranean basin. The thalassemias are the most common genetic blood diseases and are found in Southeast Asia and in areas where sickle cell disease is common(Glassberg, 2018).

#### 1.5.1 Race-related demographics

Certain races and ethnic groups have an increased prevalence of genetic factors associated with certain anemias. (Gill *et al*.,2007). Diseases such as the hemoglobinopathies, thalassemia, and G-6-PD deficiency have different morbidity and mortality in different populations due to differences in the genetic abnormality producing the disorder. Race is a factor in nutritional anemias and anemia associated with untreated chronic illnesses to the extent that socioeconomic advantages are distributed along racial lines in a given area; socioeconomic advantages that positively affect diet and the availability of health care lead to a decreased prevalence of these types of anemia(Oliveira*et al*., 2007).

Similarly, anemia of chronic disorders is commonplace in populations with a high incidence of chronic infectous disease (eg, malaria, tuberculosis, acquired immunodeficiency syndrome [AIDS]), and this is at least in part worsened by the socioeconomic status of these populations and their limited access to adequate health care.

#### 1.5.2 Sex-related demographics

Overall, anemia is twice as prevalent in females as in males. This difference is significantly greater during the childbearing years due to pregnancies and menses. Approximately 65% of body iron is incorporated into circulating Hb. One gram of Hb contains 3.46 mg of iron (1 mL of blood with an Hb concentration of 15 g/dL = 0.5 mg of iron). Each healthy pregnancy depletes the mother of approximately 500 mg of iron (Bhutta *et al*.,2013). While a man must absorb about 1 mg of iron to maintain equilibrium, a premenopausal woman must absorb an average of 2 mg daily. Further, because women eat less food than men, they must be more than twice as efficient as men in the absorption of iron to avoid iron deficiency. Women have a markedly lower incidence of X-linked anemia, such as G-6-PD deficiency and sex-linked sideroblastic anemia, than men do. In addition, in the younger age groups, males have a higher incidence of acute anemia from traumatic causes (Idris *et al*., 2005).

### 1.5.3 Age-related demographics

Previously, severe, genetically acquired anemias (eg, sickle cell disease, thalassemia, Fanconi syndrome) were more commonly found in children because they did not survive to adulthood. However, with improvement in medical care and breakthroughs in transfusion and iron chelation therapy, in addition to fetal hemoglobin modifiers, the life expectancy of persons with these diseases has been significantly prolonged (Bhutta *et al*.,2013).

Acute anemia has a bimodal frequency distribution, affecting mostly young adults and persons in their late fifties. Causes among young adults include trauma, menstrual and ectopic bleeding, and problems of acute hemolysis. During their childbearing years, women are more likely to become iron deficient. In people aged 50-65 years, acute anemia is usually the result of acute blood loss in addition to a chronic anemic state(Arokiyaraj *et al*., 2007). This is the case in uterine and GI bleeding. Neoplasia increases in prevalence with each decade of life and can produce anemia from bleeding, from the invasion of bone marrow with tumor, or from the development of anemia associated with chronic disorders(Rothman, 2008). The use of aspirin, nonsteroidal anti-inflammatory drugs (NSAIDs), and warfarin also increases with age and can produce GI bleeding.

## 1.6Treatment of anemia

Hydroxyurea represents the only major breakthrough in pharmacotherapy of anemia within the past 20 years and is the only drug that is approved by the U.S. Food and Drug Administration (FDA) for treatment of adults with anemia. The clinical management of anemia includes medullar transplantation, repeated blood transfusion to stabilize the patient’s Hb level, and the use of chemical agents which interfere with the mechanism or kinetics of the anemia process. Unfortunately, all currently proposed therapies are quite expensive and have attendant risk factors in terms of clinical use. Therefore, there is a need for more definite and effective treatments for the anemia (Angelilo-Scherrer *et al*., 2008).

## 1.7 Causes of anemia

Anaemia has three main causes: blood loss, lack of red blood cell production, or high rates of red blood cell destruction. These may be due to a number of diseases, conditions, or other factors (Levin, 2007).

- **Blood loss**

Blood loss is the most common cause of anaemia, especially iron-deficiency anaemia. Heavy menstrual periods or bleeding in the digestive or urinary tract can cause blood loss. Surgery, trauma, or cancer can also lead to blood loss (Ananda, 2008).

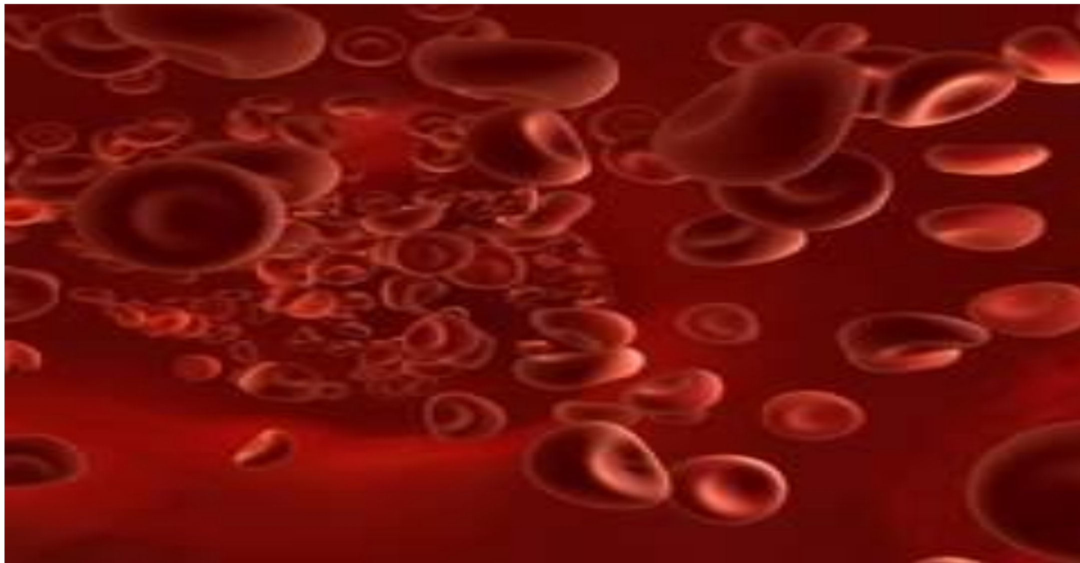


**Figure 2: Picture of red blood cells** (Rifkind et al., 2002).

- **Lack of red blood cell production.** Both acquired and inherited conditions and factors can prevent your body from making enough red blood cells. Examples of acquired conditions and factors that can cause this include diet, hormones, some chronic diseases and pregnancy. Aplastic anaemia can also prevent your body from making enough red blood cells – this condition can be acquired or inherited. A diet that lacks iron, folic acid, or vitamin B12, or conditions that make it hard for your body to absorb nutrients, can also prevent the body from making enough red blood cells (Ducharme, 2014). The body needs the hormone erythropoietin to make red blood cells and a low level of this hormone may cause anaemia. Chronic diseases, like kidney disease and cancer, can make it hard for the body to make enough red blood cells, and those with HIV/AIDS can develop anaemia due to infections or medicines used to treat the disease. Anaemia can occur during pregnancy due to low levels of iron and folic acid and changes in the blood (Levin, 2007).

- **High rates of red blood cell destruction**. There are various conditions that can cause your body to destroy too many red blood cells such as an enlarged spleen, sickle cell anaemia, thalassaemia and a lack of certain enzymes. Anaemia is a common condition. It occurs across all age, racial and ethnic groups. Both men and women can have anemia, but women of childbearing age are at higher risk for the condition as they lose blood from menstruation. Anaemia can also develop during pregnancy due to low levels of iron and folic acid and changes in the blood. Infants younger than two years are also at risk for anaemia because they may not get enough iron in their diet (Ananda, 2008).

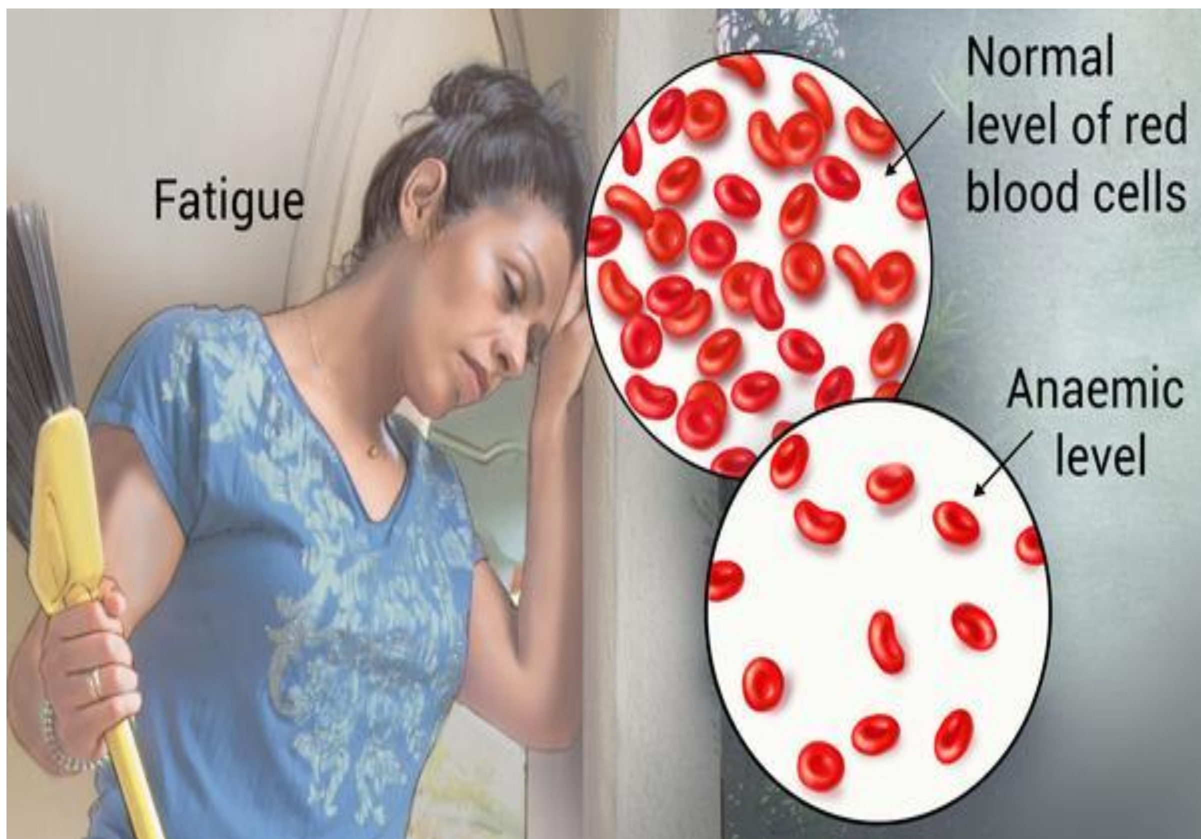



**Figure:3** Picture of normal level of red blood cells and anemic level (Rifkind et al., 2002).

Hemoglobin is a main part of red blood cells and binds oxygen. In the presence of too few or abnormal red blood cells, or abnormal hemoglobin, the cells in your body will not get enough oxygen. Symptoms of anemia like fatigue occur because organs aren't getting what they need to function properly.

Anemia is the most common blood condition in the U.S. It affects about 5.6% of the people in the U.S. Women, young children, and people with chronic diseases are at increased risk of anemia. Important factors to remember are:

- Certain forms of anemia are hereditary and infants may be affected from the time of birth.
- Women in the childbearing years are particularly susceptible to iron-deficiency anemia because of the blood loss from menstruation and the increased blood supply demands during pregnancy.
- Older adults also may have a greater risk of developing anemia because of poor diet and other medical conditions.

There are many types of anemia. All are very different in their causes and treatments. Iron-deficiency anemia, the most common type, is very treatable with diet changes and iron supplements. Some forms of anemia -- like the mild anemia that develops during pregnancy -- are even considered normal. However, some types of anemia may present lifelong health problems (Ananda, 2008).

#### 1.7.1.1 Sickle cell anemia

This is an inherited disorder that, in the U.S. affects mainly African-Americans and Hispanic Americans. Red blood cells become crescent-shaped because of a genetic defect. They break down rapidly, so oxygen does not get to the body's organs, causing anemia. The crescent-shaped red blood cells can also get stuck in tiny blood vessels, causing pain (O’Mearar, 2014).

#### 1.7.1.2 Iron-deficiency anemia

This occurs because of a lack of the mineral iron in the body. Bone marrow in the center of the bone needs iron to make hemoglobin, the part of the red blood cell that transports oxygen to the body's organs. Without adequate iron, the body cannot produce enough hemoglobin for red blood cells (White, 2014). The result is iron-deficiency anemia. This type of anemia can be caused by:

- An iron-poor diet, especially in infants, children, teens, vegans, and vegetarians
- The metabolic demands of pregnancy and breastfeeding that deplete a woman's iron stores
- Menstruation

- Frequent blood donation
- Endurance training
- Digestive conditions such as Crohn's disease or surgical removal of part of the stomach or small intestine
- Certain drugs, foods, and caffeinated drinks

#### 1.7.3.2 Vitamin-deficiency anemia

May occur when vitamin B12 and folate are deficient. These two vitamins are needed to make red blood cells. Conditions leading to anemia caused by vitamin deficiency include:

- Megaloblastic anemia: Vitamin B12 or folate or both are deficient
- Pernicious anemia: Poor vitamin B12 absorption
- Dietary deficiency: Eating little or no meat may cause a lack of vitamin B12, while overcooking or eating too few vegetables may cause a folate deficiency.
- Other causes of vitamin deficiency: pregnancy, certain medications, alcohol abuse, intestinal diseases such as tropical sprue and celiac disease

During early pregnancy, sufficient folic acid can help prevent the fetus from developing neural tube defects such as spina bifida.

#### 1.7.2.0 Bone marrow and stem cell problems

May prevent the body from producing enough red blood cells. Some of the stem cells found in bone marrow develop into red blood cells. If stem cells are too few, defective, or replaced by other cells such as metastatic cancer cells, anemia may result. Anemias resulting from bone marrow or stem cell problems include:

- Aplastic anemia occurs when there's a marked reduction in the number of stem cells or absence of these cells. Aplastic anemia can be inherited, can occur without apparent cause, or can occur when the bone marrow is injured by medications, radiation, chemotherapy, or infection.

- Thalassemia occurs when the red cells can't mature and grow properly. Thalassemia is an inherited condition that typically affects people of Mediterranean, African, Middle Eastern, and Southeast Asian descent. This condition can range in severity from mild to life-threatening; the most severe form is called Cooley's anemia.
- Lead exposure is toxic to the bone marrow, leading to fewer red blood cells. Lead poisoning occurs in adults from work-related exposure and in children who eat paint chips, for example. Improperly glazed pottery can also taint food and liquids with lead (Tardif *et al*., 2014).

#### 1.7.2.1 Anemia associated with other conditions

Usually occurs when there are too few hormones necessary for red blood cell production. Conditions causing this type of anemia include the following:

- Advanced kidney disease
- Hypothyroidism
- Other chronic diseases, such as cancer, infection, lupus, diabetes, and rheumatoid arthritis
- Old age

### 1.7.3 Anemia Caused by Destruction of Red Blood Cells

When red blood cells are fragile and cannot withstand the routine stress of the circulatory system, they may rupture prematurely, causing hemolytic anemia (Angelilo-Scherrer *et al*., 2008). Hemolytic anemia can be present at birth or later. Sometimes there is no known cause. Known causes of hemolytic anemia may include:

- Inherited conditions, such as sickle cell anemia and thalassemia
- Stressors such as infections, drugs, snake or spider venom, or certain foods
- Toxins from advanced liver or kidney disease
- Inappropriate attack by the immune system (called hemolytic disease of the newborn when it occurs in the fetus of a pregnant woman)
- Vascular grafts, prosthetic heart valves, tumors, severe burns, exposure to certain chemicals, severe hypertension, and clotting disorders

- In rare cases, an enlarged spleen can trap red blood cells and destroy them before their circulating time is up.

### 1.8 Management ofAnemia with Herbs

Medicinal plants are the most common sources of drugs used in traditional medicine. Despite improvement in health care delivery system, medicinal plants play a vital role in both human and animal health care systems and about 60% of world population depends on herbs for their primary healthcare. The reason for this may be due to high cost of orthodox drugs, side effects and unavailability of orthodox medicine and personnel in remote areas. Poverty and cultural inclination may have contributed to the trend.

Some of the herbs used for the management of anemia are:

- **Nettle**

Nettle, commonly known as stinging nettle, is a herb that contains very high levels iron. For centuries, this plant has been extensively used as a source of medicine, food, and fiber. It has been used in the preparation of nettle tonic, tea, or supplement to help recover from anemia. It is also a rich source of vitamins A (retinol), B group, C (ascorbic acid), and K, which improve iron absorption in the body. The powered leaf extract has been used as an anti-hemorrhagic agent to reduce excessive menstrual flow and nose bleedings. In folk medicine, it is used for the treatment of anemia and considered as blood builders (Ahmed *et al*., 2014).

- **Parsley**

Parsley is a popular green herb native to the Mediterranean and is widely used in western cuisine, commonly used as a garnish. It is an excellent treatment to overcome anemia as it contains a significant concentration of iron and folic acid. Vitamin C present in parsley helps your body to absorb the iron efficiently. Parsley is full of antioxidants, which may prevent cadmium toxicity and has a profound effect on the recovery of anemia.Doctors often suggest drinking parsley juice on tea for persons who find it difficult to take iron supplements. Parsley is an effective medicinal plant to increase iron content in the body and recover from anemia (Allam *et al*., 2016).

- **Dandelion**

Dandelion is a herbaceous perennial plant that has medicinal and culinary uses. Dandelion has been used as a remedy for anemia, purifying the blood, and providing immune modulation. It is a rich source of vitamins and minerals and is particularly high in vitamins A and C and iron. It contains more iron and calcium than spinach. Dandelion has traditionally been used to fortify and cleanse the blood. It may help bring levels of hemoglobin into normal range in mild cases of anemia.Iron-deficiency anemia is mainly caused due to either the lack of iron-rich foods in your diet, or because your body is unable to efficiently absorb the iron present in foods. Dandelion leaves not only contain high levels of iron, but also enhance the body's ability to absorb this important mineral (Modaresi *et al*., 2012).

- **Alfalfa**

Alfalfa is rich in vitamins and phytochemicals. It also contains high levels of beta-carotene and vitamins C, E, and K. The presence of vitamin C in alfalfa helps the body to absorb the iron in food. Physicians practicing traditional Chinese and Ayurvedic medicines have used young alfalfa leaves to treat disorders. It can be consumed in a variety of ways such as sprouts on sandwiches, alfalfa seeds in salads, or it can even be cooked as a vegetable. Alfalfa has many healing properties including its ability to detoxify the body due to its high chlorophyll content. Aalfalfa has been used for treating many conditions by 19th-century physicians in the United States who also used it as a herbal therapy for anemia. The results obtained in the study demonstrate that alfalfa concentrate is an effective and more palatable alternative to iron and folic acid supplements for treating anemia in adolescent girls (Alfalfa, 2015).

- **Watercress**

Watercress is an aquatic plant and one of the oldest known leaf vegetables consumed by humans. It is rich in vitamin K and contains considerable amounts of vitamin A, C, riboflavin, vitamin B6, calcium, manganese, and folate. It contains more iron than spinach, more calcium than milk, and more vitamin C than oranges. It also has very low concentrations of toxic metals making watercress an effective herb to increase iron content in blood and completely safe for human

consumption (Kisten *et al*.,2015). Watercress supplement included in the diet has shown beneficial changes that were greater and more significant in smokers than in non-smokers. Plasma lutein and beta-carotene increased significantly by 100% and 33%, respectively, after watercress supplementation(Gill *et al*.,2007).

- **Sorghum**

Sorghum, also known as great millet, is a grass species cultivated for its grain. In parts of West Africa, it is used as a herbal treatment for anemia. Recent research has found that supplementing with sorghum bicolor extract along with iron and folic acid before surgery can decrease anemia (Tayo*et al*., 2017). Sorghum bicolor extract is from the leaf of the sorghum bicolor strain grown in South West Nigeria. The extract has been shown to have properties that can increase red blood cells. People with preoperative anemia can include sorghum bicolor extract as a simple and low-cost intervention (Tayo*et al*., 2017).

**1.9Phenylhydrazine**

Phenylhydrazine (PHZ) is an antipyretic drug that was first characterized by Herman Emil Fisher in 1875. This drug is well known for its ability to produce hemolysis in rats and humans (Dornfest *et al.,*1992). Due to such effects, PHZ was remarked as a potent drug to meet blood disorders. PHZ is known to decrease hemoglobin levels, RBC (Red Blood Cell) count and PCV (Pack Cell Volume) whereas; it induces increase in MCV (Mean Cell Volume), MCH (Mean Cell Hemoglobin) and MCHC (Mean Corpuscular Hemoglobin Concentration) levels (Shukla *et al.,* 2012).

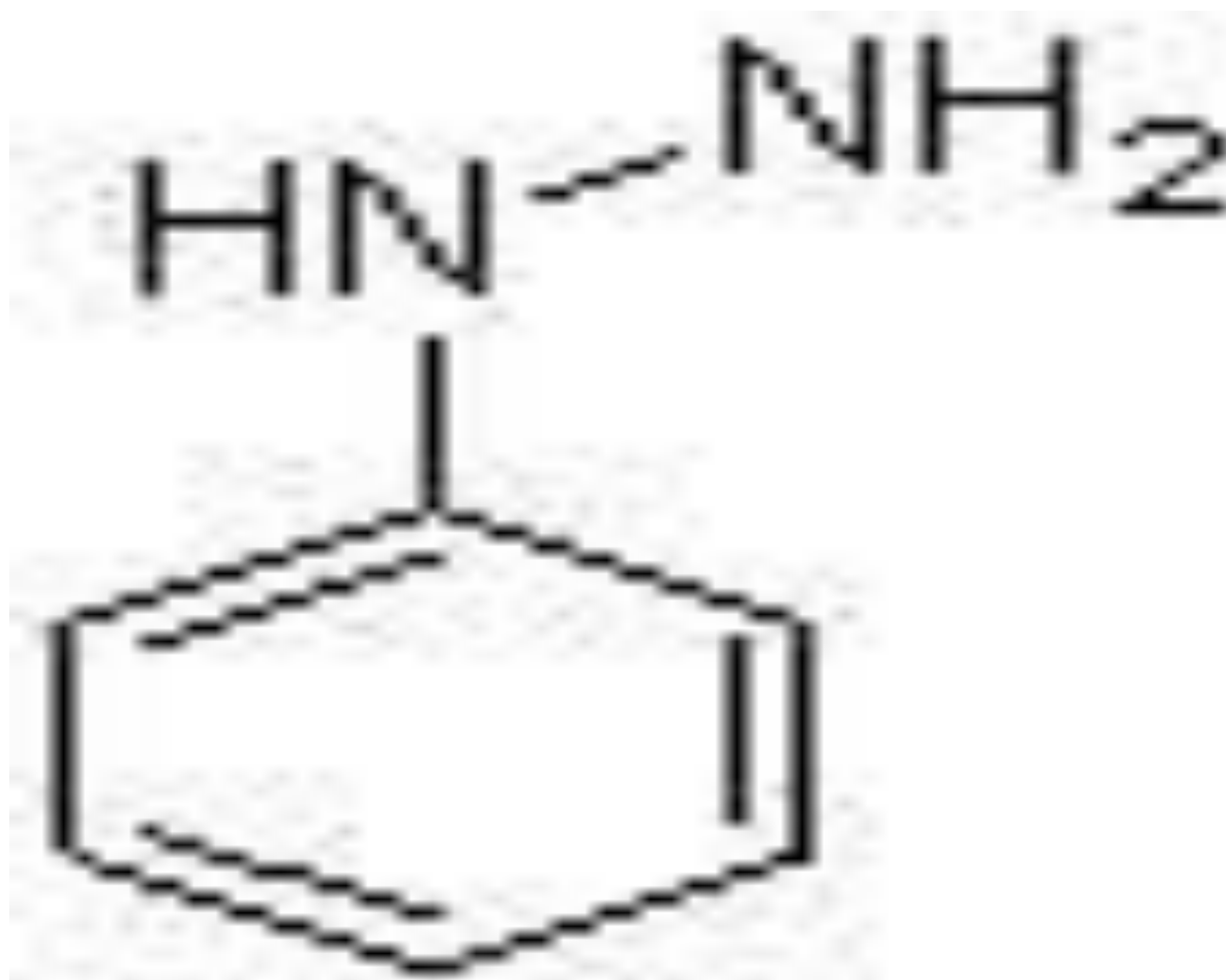



**Figure .4 Structure of Phenylhydrazine (Ferrli et al., 1997)**

**1.9.1 Mechanism of phenylhydrazine induced toxicity haemolytic anemia.**

Phenylhydrazine (PHZ) –induces hemolytic anemia to study erythropoietin regenerative response through clinical, pathological, and morphological studies. PHZ is absorbed by the inhalation, oral and dermal routs hemotoxicant PHZ causes oxidative stress within erythrocytes resulting oxidation of oxyhemoglobin leading to the formation of methemoglobin which is subsequently converted into irreversible hemichromes that lead to the precipitation of hemoglobin in the form of Heinz bodies. PHZ causes damage in skeletal protein, lipid peroxidation, ATP depletion, cation Imbalances, and reduced membrane deformability. All these symptoms show hemolytic anemia (Liszkowski *et al*., 2014).

### 1.9.2 Alteration of iron metabolism

In mammals iron metabolism is altered by haemolytic anemia induced by Phenylhydrazine. Phenylhydrazine (PHZ)- induced anemia increases the iron absorption that induces the expression of iron transport genes (Dcytb, DMT1-IRE and Ireg) in the duodenum so the expression of Dcytb, DMT1-IRE and Ireg1 mRNA was enhanced in the duodenum of PHZ-treated mice. The patterns of gene expression in the Duodenum can be seen by RT-PCR analyses. Dcytb and Ireg1 genes are also involved in iron metabolism in spleen and liver of the PHZ-treated mice. During a period of acute haemolysis the catabolic and anabolic pathways of haemoglobin in the spleen must be regulated to maintain a balance in systemic iron homeostasis by measuring the expression of transferrin receptor (TFR1) and haem oxygenase (HO1). HO1 is an important inducible enzyme involved in haem degradation and also causes iron efflux from cell. The expression of Ireg1, TFR1 and HO1will increased in the spleen of the PHZtreated mice. In PHZ-treated mice the level of Dcytb in spleen will also be increased. Finally local demand and supply of Fe will increases erythropoeitic activity of the spleen so the size of spleen will increase it will cause the splenomegaly. Liver also plays an important role in maintaining body iron homeostasis. We can examine the hepatic expression of several relevant genes following PHZinduced haemolysis. The expression of TFR1 in liver was significantly increased in PHZtreated mice, while hepcidin expression will decrease.

### 1.9.3 Effect of PHZ on immune system

PHZ-induced anemia is also responsible for immune activation10. In this respect, PHZ can cross red blood cells and binds with circulating autologous antibodies8. This antigen- antibody complex is recognized by macrophage receptors which triggers phagocytosis in the spleen and liver. This indicates that damaged cells are removed intact by the spleen. Apart from blood storage and immune competence, the spleen also acts as the main erythrophagocytic organ in rodents and rabbits which are suffering from PHZ induced haemolytic anemia (Madhu KeLi. *et al.*, 2003).

### 1.9.4 Effect of PHZ on JAK-STAT pathway

PHZ also affects the EPO receptors of JAK-STAT pathway which is responsible for the maturation of red blood cells. After Phenylhydrazine-induced anemia, EpoRHM mice failed to respond with efficient splenic stress erythropoisis (Madhu KeLi. *et al*., 2003). The erythropoietin receptor which is a member of the cytokine receptor family, upon erythropoietin binding, this receptor activates Jak2 tyrosine kinase which activates different intracellular pathways including: Ras/MAP kinase, phosphatidylinositol 3-kinase and STAT transcription factors. The stimulated erythropoietin receptor appears to have a role in erythroid cell survival. Defects in the erythropoietin receptor may produce erythroleukemia and familial erythrocytosis. Disregulation of this cytokine may affect the growth of certain tumors.

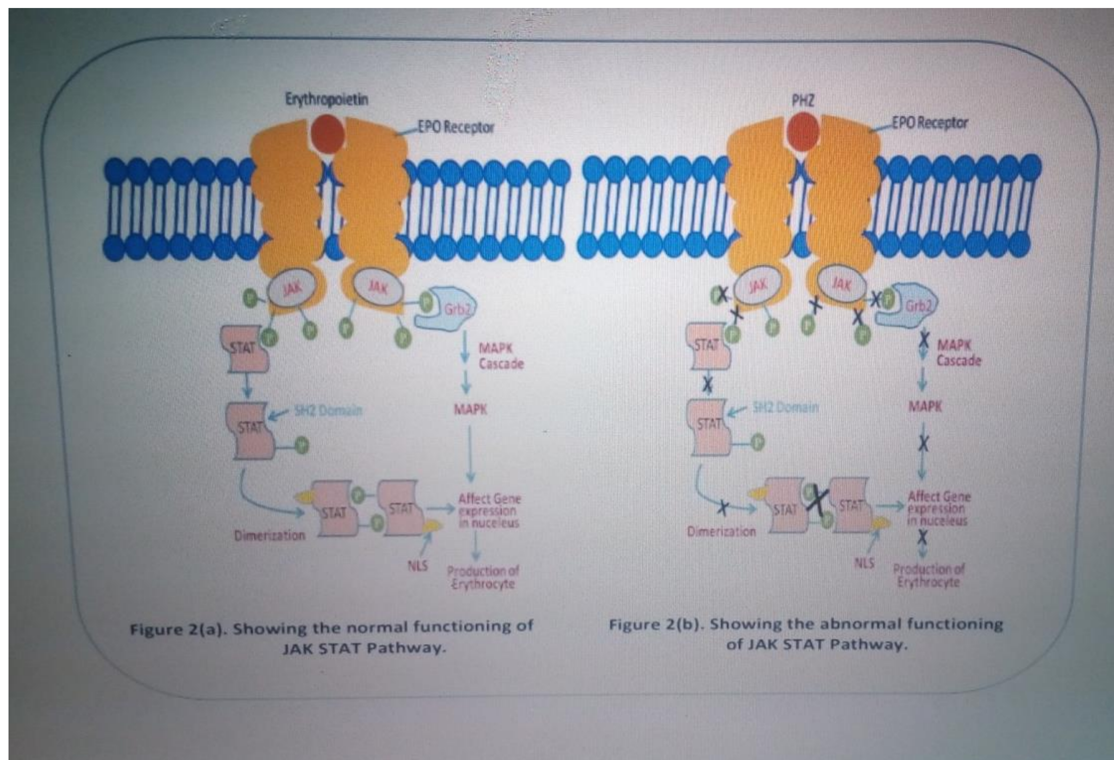


Figure 2(a). Showing the normal functioning of JAK STAT Pathway.
Figure 2(b). Showing the abnormal functioning of JAK STAT Pathway.

**Figure 5: Effect of PHZ on JAK-STAT pathway (Madhu KeLi. *et al*., 2003).**

### 1.9.5 Genotoxic effect of phenylhydrazine

PHZ create single strand DNA damage from lung tissue extracts and mouse liver through alkaline elution rate method11. In an experiment, the liver DNA from PHZ treated rats was

analyzed by electrophoresis and found to be markedly fragmented(Ferrali M. *et al*.,1997). PHZ is absorbed by the inhalation, oral and dermal routs. After absorption it causes oxidative stress in RBCs and generates reactive oxygen species (ROS) in the RBCs this ROS reacts with haemoglobin and changes the oxyhaemoglobin in to methaemoglobin, hemichromes and other haemoglobin breakdown products such as Heinz bodies.This compound seems to be very useful in models studying mechanism of hemolytic anaemia. PHZ induces a reactive oxygen species formation which results in Peroxidation of lipid and oxidative degradation of spectrin in the membrane Skelton. This chemical has potential for skin and eye irritation in human and animals. After that PHZ translocates the phosphatidylserine from inner to outer of the plasma membrane and causes the membrane lipid peroxidation due to lipid peroxidation RBCs enter in the spleen and uptake by the macrophages. It is signal for Phagocytosis of cell under programmed death by macrophages. It will cause haemolytic anemia. Apart from haemolytic anemia PHZ also alters the iron metabolism by increasing the expression of ferrous transporter (DMT1) in the spleen, duodenum and liver. DMTI transporter promotes the expression of genes related to iron metabolism such as ferric reductase DCytb, Ireg1 and DMT1 in human and mice. Expression level is checked by Northern blot, RT-PCR and immunocytochemistry. Increased mRNA expression of DCytb, DMT1, Ireg1 and IFR1 in spleen and liver will increase the iron demand resulting stimulation of erythropoisis so the size of spleen will increase it will causes the splenomegaly. PHZ also affects the EPO receptors of JAK-STAT pathway which is responsible for the maturation of red blood cells. Phenylhydrazine-induced anemia, EpoR-HM mice failed to respond with efficient splenic stress erythropoisis.

## 1.10CONCEPTS ASSOCIATED WITH ANEMIA

### 1.10.1 Free Radicals

A free radical can be defined as any molecular species capable of independent existence that contains an unpaired electron in an atomic orbital. The presence of an unpaired electron results in certain common properties that are shared by most radicals. Many radicals are unstable and highly reactive. They can either donate an electron to or accept an electron from other molecules, therefore behaving as oxidants or reductants (Aruoma O.I, 2003). The most important oxygen-

containing free radicals in many disease states are hydroxyl radical, superoxide anion radical, hydrogen peroxide, oxygen singlet, hypochlorite, nitric oxide radical, and peroxynitrite radical. These are highly reactive species, capable in the nucleus, and in the membranes of cells of damaging biologically relevant molecules such as DNA, proteins, carbohydrates, and lipids (Young, 2001).Free radicals attack important macromolecules leading to cell damage and homeostatic disruption. Targets of free radicals include all kinds of molecules in the body. Among them, lipids, nucleic acids, and proteins are the major targets.

### 1.10.2 Production of free radicals in the human body

Free radicals and other ROS are derived either from normal essential metabolic processes in the human body or from external sources such as exposure to X-rays, ozone, cigarette smoking, air pollutants, and industrial chemicals (Bagchi*et al.*, 1998). Free radical formation occurs continuously in the cells as a consequence of both enzymatic and nonenzymatic reactions. Enzymatic reactions, which serve as source of free radicals, include those involved in the respiratory chain, in phagocytosis, in prostaglandin synthesis, and in the cytochrome P-450 system (Bagchi*et al*, 1998). Free radicals can also be formed in nonenzymatic reactions of oxygen with organic compounds as well as those initiated by ionizing reactions.

**Some internally generated sources of free radicals are**

- Mitochondria
- Xanthine oxidase
- Peroxisomes
- Inflammation
- Phagocytosis
- Arachidonate pathways
- Exercise
- Ischemia/reperfusion injury
- Some externally generated sources of free radicals are:
- Cigarette smoke
- Environmental pollutants
- Radiation

- Certain drugs, pesticides
- Industrial solvents
- Ozone

### 1.10.3 Free radicals in biology

Free radical reactions are expected to produce progressive adverse changes that accumulate with age throughout the body. Such "normal" changes with age are relatively common to all. However, superimposed on this common pattern are patterns influenced by genetics and environmental differences that modulate free radical damage. These are manifested as diseases at certain ages determined by genetic and environmental factors. Cancer and atherosclerosis, two major causes of death, are salient "free radical" diseases. Cancer initiation and promotion is associated with chromosomal defects and oncogene activation. It is possible that endogenous free radical reactions, like those initiated by ionizing radiation, may result in tumor formation. The highly significant correlation between consumption of fats and oils and death rates from leukemia and malignant neoplasia of the breast, ovaries, and rectum among persons over 55 years may be a reflection of greater lipid peroxidation (Bagchi*et al.*, 1998). Studies on atherosclerosis reveal the probability that the disease may be due to free radical reactions involving diet-derived lipids in the arterial wall and serum to yield peroxides and other substances. These compounds induce endothelial cell injury and produce changes in the arterial walls.

## 1.11Concept of oxidative stress

The term is used to describe the condition of oxidative damage resulting when the critical balance between free radical generation and antioxidant defenses is unfavorable(Mc Cord, 2000). Oxidative stress, arising as a result of an imbalance between free radical production and antioxidant defenses, is associated with damage to a wide range of molecular species including lipids, proteins, and nucleic acids(Mc Cord, 2000). Short-term oxidative stress may occur in tissues injured by trauma, infection, heat injury, hypertoxia, toxins, and excessive exercise. These injured tissues produce increased radical generating enzymes (e.g., xanthine oxidase, lipogenase, cyclooxygenase) activation of phagocytes, release of free iron, copper ions, or a disruption of the electron transport chains of oxidative phosphorylation, producing excess ROS.

The initiation, promotion, and progression of cancer, as well as the side-effects of radiation and chemotherapy, have been linked to the imbalance between ROS and the antioxidant defense system. ROS have been implicated in the induction and complications of diabetes mellitus, age-related eye disease, and neurodegenerative diseases such as Parkinson's disease.

### 1.11.1Oxidative stress and human diseases

#### 1.11.1.2 Oxidative stress

Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Disturbances in the normal redoxstate of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA. Oxidative stress from oxidative metabolism causes base damage, as well as strand breaks in DNA. Base damage is mostly indirect and caused by reactive oxygen species (ROS) generated, e.g. $O_2^-$ (superoxide radical), OH (hydroxyl radical) and $H_2O_2$ (hydrogen peroxide) (Chandra *et al., 2015*). Further, some reactive oxidative species act as cellular messengers in redox signaling. Thus, oxidative stress can cause disruptions in normal mechanisms of cellular signaling.

In humans, oxidative stress is thought to be involved in the development of ADHD (Joseph . *et al*.,2015), cancer (Halliwell, and Barry 2007), Parkinson's disease, Lafora disease, Alzheimer's disease, atherosclerosis, heart failure, myocardial infarction, fragile X syndrome, sickle-cell disease, lichen planus, vitiligo, autism, infection, chronic fatigue syndrome, and depressionand seems to be characteristic of individuals with Asperger syndrome(Parellada *et al.*, 2012). However, reactive oxygen species can be beneficial, as they are used by the immune system as a way to attack and kill pathogens. Short-term oxidative stress may also be important in prevention of aging by induction of a process named mitohormesis(Gems *et al*.,2008).

### 1.11.2 Chemical and biological effects of Oxidative stress

Chemically, oxidative stress is associated with increased production of oxidizing species or a significant decrease in the effectiveness of antioxidant defenses, such as glutathione(Schafer F.Q *et al.,* 2001). The effects of oxidative stress depend upon the size of these changes, with a

cell being able to overcome small perturbations and regain its original state. However, more severe oxidative stress can cause cell death, and even moderate oxidation can trigger apoptosis, while more intense stresses may cause necrosis(Lennon *et al*., 1991).

Production of reactive oxygen species is a particularly destructive aspect of oxidative stress. Such species include free radicals and peroxides. Some of the less reactive of these species (such as superoxide) can be converted by oxidoreduction reactions with transition metals or other redox cycling compounds (including quinones) into more aggressive radical species that can cause extensive cellular damage (Valko *et al*., 2005).Most long-term effects are caused by damage to DNA (Evans *et al*., 2004) DNA damage induced by ionizing radiation is similar to oxidative stress, and these lesions have been implicated in aging and cancer. Biological effects of single-base damage by radiation or oxidation, such as 8-oxoguanine and thymine glycol, have been extensively studied. Recently the focus has shifted to some of the more complex lesions. Tandem DNA lesions are formed at substantial frequency by ionizing radiation and metal-catalyzed $H_2O_2$ reactions. Under anoxic conditions, the predominant double-base lesion is a species in which C8 of guanine is linked to the 5-methyl group of an adjacent 3'-thymine (G [8, 5- Me] T) (Colis *et al*., 2008). Most of these oxygen-derived species are produced by normal aerobic metabolism. Normal cellular defense mechanisms destroy most of these. Repair of oxidative damages to DNA is frequent and ongoing, largely keeping up with newly induced damages. In rat urine about 74,000 oxidative DNA adducts per cell per day are excreted. (Helbock *et al*., 1998). However, there is a steady state level of oxidative damages, as well, in the DNA of a cell. There are about 24,000 oxidative DNA adducts per cell in young rats and 66,000 adduct per cell in old rats (Helbock *et al*., 1998). Likewise, any damage to cells is constantly repaired. However, under the severe levels of oxidative stress that cause necrosis, the damage causes ATP depletion, preventing controlled apoptotic death and causing the cell to simply fall apart (Lelli*et al., 1998*).

Polyunsaturated fatty acids, particularly arachidonic acid and linoleic acid, are primary targets for free radical and singlet oxygen oxidations. For example, in tissues and cells, the free radical oxidation of linoleic acid produces racemic mixtures of 13-hydroxy-9*Z*,11*E*-octadecadienoic acid, 13-hydroxy-9*E*,11*E*-octadecadienoic acid, 9-hydroxy-10*E*,12-*E*-octadecadienoic acid (9-EE-HODE), and 11-hydroxy-9*Z*,12-*Z*-octadecadienoic acid as well as 4-Hydroxynonenal while

singlet oxygen attacks linoleic acid to produce (presumed but not yet proven to be racemic mixtures of) 13-hydroxy-9*Z*,11*E*-octadecadienoic acid, 9-hydroxy-10*E*,12-*Z*-octadecadienoic acid, 10-hydroxy-8*E*,12*Z*-octadecadienoic acid, and 12-hydroxy-9*Z*-13-*E*-octadecadienoic (*Akazawa-Ogawa et al.,*2015). Similar attacks on arachidonic acid produce a far larger set of products including various isoprostanes, hydroperoxy- and hydroxy- eicosatetraenoates, and 4-hydroxyalkenals (Riahi *et al*., 2010). While many of these products are used as markers of oxidative stress, the products derived from linoleic acid appear far more predominant than arachidonic acid products and therefore easier to identify and quantify in, for example, atheromatous plaques.Certain linoleic acid products have also been proposed to be markers for specific types of oxidative stress. For example, the presence of racemic 9-HODE and 9-EE-HODE mixtures reflects free radical oxidation of linoleic acid whereas the presence of racemic 10-hydroxy-8*E*,12*Z*-octadecadienoic acid and 12-hydroxy-9*Z*-13-*E*-octadecadienoic acid reflects singlet oxygen attack on linoleic acid(Oleo .2015). In addition to serving as markers, the linoleic and arachidonic acid products can contribute to tissue and/or DNA damage but also act as signals to stimulate pathways which function to combat oxidative stress. (Kyung-Jin *et al.,* 2011).

A role of oxidative stress has been postulated in many conditions, including anthersclerosis, inflammatory condition, certain cancers, and the process of aging. Oxidative stress is now thought to make a significant contribution to all inflammatory diseases (arthritis, vasculitis, glomerulonephritis, lupus erythematous, adult respiratory diseases syndrome), ischemic diseases (heart diseases, stroke, intestinal ischema), hemochromatosis, acquired immunodeficiency syndrome, emphysema, organ transplantation, gastric ulcers, hypertension and preeclampsia, neurological disorder (Alzheimer's disease, Parkinson's disease, muscular dystrophy), alcoholism, smoking-related diseases, and many others(Rao*et al*., 2006). An excess of oxidative stress can lead to the oxidation of lipids and proteins, which is associated with changes in their structure and functions.

### 1.11.3Cardiovascular diseases

Heart diseases continue to be the biggest killer, responsible for about half of all the deaths. The oxidative events may affect cardiovascular diseases therefore; it has potential to provide enormous benefits to the health and lifespan (Rao*et al*., 2006). Poly unsaturated fatty acids occur as a major part of the low density lipoproteins (LDL) in blood and oxidation of these lipid

components in LDL play a vital role in atherosclerosis. The three most important cell types in the vessel wall are endothelial cells; smooth muscle cell and macrophage can release free radical, which affect lipid peroxidation (Rao *et al*., 2006). With continued high level of oxidized lipids, blood vessel damage to the reaction process continues and can lead to generation of foam cells and plaque the symptoms of atherosclerosis. Oxidized LDL is antherogenic and is thought to be important in the formation of anthersclerosis plaques. Furthermore, oxidized LDL is cytotoxic and can directly damage endothelial cells. Antioxidants like B-carotene or vitamin E play a vital role in the prevention of various cardiovascular diseases.

### 1.11.4Free radical and aging

The human body is in constant battle to keep from aging. Research suggests that free radical damage to cells leads to the pathological changes associated with aging (Ashok *et al.,*1999). An increasing number of diseases or disorders, as well as aging process itself, demonstrate link either directly or indirectly to these reactive and potentially destructive molecules(Cantuti-Castelvetri I, *et al*., 2000). The major mechanism of aging attributes to DNA or the accumulation of cellular and functional damage (Cantuti-Castelvetri, *et al*., 2000). Reduction of free radicals or decreasing their rate of production may delay aging. Some of the nutritional antioxidants will retard the aging process and prevent disease. Based on these studies, it appears that increased oxidative stress commonly occurs during the aging process, and antioxidant status may significantly influence the effects of oxidative damage associated with advancing age. Research suggests that free radicals have a significant influence on aging, that free radical damage can be controlled with adequate antioxidant defense, and that optimal intake of antioxidant nutrient may contribute to enhanced quality of life. Recent research indicates that antioxidant may even positively influence life span.

## 1.12 Oxidative damage to protein and DNA

### 1.12.1 Oxidative damage to protein

Proteins can be oxidatively modified in three ways: oxidative modification of specific amino acid, free radical mediated peptide cleavage, and formation of protein cross-linkage due to reaction with lipid peroxidation products. Protein containing amino acids such as methionine, cystein, arginine, and histidine seem to be the most vulnerable to oxidation (Cantuti-Castelvetri*et*

*al*., 2000). Free radical mediated protein modification increases susceptibility to enzyme proteolysis. Oxidative damage to protein products may affect the activity of enzymes, receptors, and membrane transport. Oxidatively damaged protein products may contain very reactive groups that may contribute to damage to membrane and many cellular functions. Peroxyl radical is usually considered to be free radical species for the oxidation of proteins. ROS can damage proteins and produce carbonyls and other amino acids modification including formation of methionine sulfoxide and protein carbonyls and other amino acids modification including formation of methionine sulfoxide and protein peroxide. Protein oxidation affects the alteration of signal transduction mechanism, enzyme activity, heat stability, and proteolysis susceptibility, which leads to aging.

### 1.12.2Lipid peroxidation

Oxidative stress and oxidative modification of biomolecules are involved in a number of physiological and pathophysiological processes such as aging, artheroscleosis, inflammation and carcinogenesis, and drug toxicity. Lipid peroxidation is a free radical process involving a source of secondary free radical, which further can act as second messenger or can directly react with other biomolecule, enhancing biochemical lesions (Mruthunjaya *et al*., 2007). Lipid peroxidation occurs on polysaturated fatty acid located on the cell membranes and it further proceeds with radical chain reaction. Hydroxyl radical is thought to initiate ROS and remove hydrogen atom, thus producing lipid radical and further converted into diene conjugate. Further, by addition of oxygen it forms peroxyl radical; this highly reactive radical attacks another fatty acid forming lipid hydroperoxide (LOOH) and a new radical (Neuzil *et al*., 1997). Thus lipid peroxidation is propagated. Due to lipid peroxidation, a number of compounds are formed, for example, alkanes, malanoaldehyde, and isoprotanes. These compounds are used as markers in lipid peroxidation assay and have been verified in many diseases such as neurogenerative diseases, ischemic reperfusion injury, and diabetes (Lovell, 1995).

### 1.12.3Oxidative damage to DNA

Many experiments clearly provide evidences that DNA and RNA are susceptible to oxidative damage. It has been reported that especially in aging and cancer, DNA is considered as a major target (Woo*et al*., 1998). Oxidative nucleotide as glycol, dTG, and 8-hydroxy-2-deoxyguanosine

is found to be increased during oxidative damage to DNA under UV radiation or free radical damage. It has been reported that mitochondrial DNA are more susceptible to oxidative damage that have role in many diseases including cancer. It has been suggested that 8-hydroxy-2-deoxyguanosine can be used as biological marker for oxidative stress (Hattori*et al.*,1997).

### 1.13Antioxidants

An antioxidant is a molecule stable enough to donate an electron to a rampaging free radical and neutralize it, thus reducing its capacity to damage. These antioxidants delay or inhibit cellular damage mainly through their free radical scavenging property (Halliwell, 1995). These low-molecular-weight antioxidants can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged. Some of such antioxidants, including glutathione, ubiquinol, and uric acid, are produced during normal metabolism in the body (Shi*et al.*, 1999). Other lighter antioxidants are found in the diet. Although there are several enzymes system within the body that scavenge free radicals, the principle micronutrient (vitamins) antioxidants are vitamin E (α-tocopherol), vitamin C (ascorbic acid), and B-carotene (Levine*et al.*, 1991). The body cannot manufacture these micronutrients, so they must be supplied in the diet.

#### 1.13.1 History

The term antioxidant originally was used to refer specifically to a chemical that prevented the consumption of oxygen. In the late 19th and early 20th century, extensive study was devoted to the uses of antioxidants in important industrial processes, such as the prevention of metal corrosion, the vulcanization of rubber, and the polymerization of fuels in the fouling of internal combustion engines (Wolf,2005).Early research on the role of antioxidants in biology focused on their use in preventing the oxidation of unsaturated fats, which is the cause of rancidity (German J, 1999). Antioxidant activity could be measured simply by placing the fat in a closed container with oxygen and measuring the rate of oxygen consumption. However, it was the identification of vitamins A, C, and E as antioxidants that revolutionized the field and led to the realization of the importance of antioxidants in the biochemistry of living organisms (Wolf,2005). The possible mechanisms of action of antioxidants were first explored when it was recognized that a substance with antioxidative activity is likely to be one that is itself readily oxidized(Wolf,2005). (Research into how vitamin E prevents the process of lipid peroxidation led to the identification of

antioxidants as reducing agents that prevent oxidative reactions, often by scavenging ROS before they can damage cells (Wolf,2005).

### 1.13.2 Antioxidant defense system

Antioxidants act as radical scavenger, hydrogen donor, electron donor, peroxide decomposer, singlet oxygen quencher, enzyme inhibitor, synergist, and metal-chelating agents. Both enzymatic and nonenzymatic antioxidants exist in the intracellular and extracellular environment to detoxify ROS (Zelko*et al.*, 2002).

### 1.13.3 Mechanism of action of antioxidants

Two principle mechanisms of action have been proposed for antioxidants (Rice-Evans*et al.*, 1993). The first is a chain- breaking mechanism by which the primary antioxidant donates an electron to the free radical present in the systems. The second mechanism involves removal of ROS/reactive nitrogen species initiators (secondary antioxidants) by quenching chain-initiating catalyst. Antioxidants may exert their effect on biological systems by different mechanisms including electron donation, metal ion chelation, co-antioxidants, or by gene expression regulation (Krinsky, 1992).

### 1.13.4 Levels of antioxidant action

The antioxidants acting in the defense systems act at different levels such as preventive, radical scavenging, repair and de novo, and the fourth line of defense, i.e., the adaptation.The first line of defense is the preventive antioxidants, which suppress the formation of free radicals. Although the precise mechanism and site of radical formation *in vivo* are not well elucidated yet, the metal-induced decompositions of hydroperoxides and hydrogen peroxide must be one of the important sources. To suppress such reactions, some antioxidants reduce hydroperoxides and hydrogen peroxide beforehand to alcohols and water, respectively, without generation of free radicals and some proteins sequester metal ions.

### 1.13.5 Glutathione peroxidase,

Glutathione peroxidase, glutathione-s-transferase, phospholipid hydroperoxide glutathione peroxidase (PHGPX), and peroxidase are known to decompose lipid hydroperoxides to

corresponding alcohols. PHGPX is unique in that it can reduce hydroperoxides of phospholipids integrated into biomembranes. Glutathione peroxidase and catalase reduce hydrogen peroxide to water.The second line of defense *is* the antioxidants that scavenge the active radicals to suppress chain initiation and/or break the chain propagation reactions. Various endogenous radical-scavenging antioxidants are known: some are hydrophilic and others are lipophilic. Vitamin C, uric acid, bilirubin, albumin, and thiols are hydrophilic, radical-scavenging antioxidants, while vitamin E and ubiquinol are lipophilic radical-scavenging antioxidants. Vitamin E is accepted as the most potent radical-scavenging lipophilic antioxidant.The third line of defense is the repair and de novo antioxidants. The proteolytic enzymes, proteinases, proteases, and peptidases, present in the cytosol and in the mitochondria of mammalian cells, recognize, degrade, and remove oxidatively modified proteins and prevent the accumulation of oxidized proteins.The DNA repair systems also play an important role in the total defense system against oxidative damage. Various kinds of enzymes such as glycosylases and nucleases, which repair the damaged DNA, are known.There is another important function called adaptation where the signal for the production and reactions of free radicals induces formation and transport of the appropriate antioxidant to the right site (Niki, 1993).

## 1.14.0ENZYMATIC

### 1.14.1 Types of antioxidants

Cells are protected against oxidative stress by an interacting network of antioxidant enzymes (Zelko*et al*, 2002). Here, the superoxide released by processes such as oxidative phosphorylation is first converted to hydrogen peroxide and then further reduced to give water. This detoxification pathway is the result of multiple enzymes, with superoxide dismutases catalyzing the first step and then catalases and various peroxidases removing hydrogen peroxide (Magnenat*et al*., 1998).

### 1.14.2 Superoxide dismutase

Superoxide dismutases (SODs) are a class of closely related enzymes that catalyze the breakdown of the superoxide anion into oxygen and hydrogen peroxide (Zelko *et al*, 2002). SOD enzymes are present in almost all aerobic cells and in extracellular fluids(Johnson*et al*.,2005). There are three major families of superoxide dismutase, depending on the metal cofactor: Cu/Zn

(which binds both copper and zinc), Fe and Mn types (which bind either iron or manganese), and finally the Ni type which binds nickel (Wuerges *et al*., 2004). In higher plants, SOD isozymes have been localized in different cell compartments. Mn-SOD is present in mitochondria and peroxisomes. Fe-SOD has been found mainly in chloroplasts but has also been detected in peroxisomes, and CuZn-SOD has been localized in cytosol, chloroplasts, peroxisomes, and apoplast (Corpas*et al*., 2006).In humans (as in all other mammals and most chordates), three forms of superoxide dismutase are present. SOD1 is located in the cytoplasm, SOD2 in the mitochondria, and SOD3 is extracellular. The first is a dimer (consists of two units), while the others are tetramers (four subunits). SOD1 and SOD3 contain copper and zinc, while SOD2 has manganese in its reactive center (Cao *et al*., 2008).

### 1.14.3.Catalase

Catalase is a common enzyme found in nearly all living organisms, which are exposed to oxygen, where it functions to catalyze the decomposition of hydrogen peroxide to water and oxygen (Chelikani*et al.*, 2004). Hydrogen peroxide is a harmful by-product of many normal metabolic processes: to prevent damage, it must be quickly converted into other, less dangerous substances. To this end, catalase is frequently used by cells to rapidly catalyze the decomposition of hydrogen peroxide into less reactive gaseous oxygen and water molecules(Gaetani*et al*., 1996). All known animals use catalase in every organ, with particularly high concentrations occurring in the liver(Eisner*et al*.,1999).

### 1.14.4 Glutathione systems

The glutathione system includes glutathione, glutathione reductase, glutathione peroxidases, and glutathione S-transferases. This system is found in animals, plants, and microorganisms(Brigelius-Flohe, 1999).Glutathione peroxidase is an enzyme containing four selenium-cofactors that catalyze the breakdown of hydrogen peroxide and organic hydroperoxides. There are at least four different glutathione peroxidase isozymes in animals (Brigelius-Flohe, 1999). Glutathione peroxidase 1 is the most abundant and is a very efficient scavenger of hydrogen peroxide, while glutathione peroxidase 4 is most active with lipid hydroperoxides. The glutathione S-transferases show high activity with lipid peroxides. These enzymes are at particularly high levels in the liver and also serve in detoxification metabolism (Hayes*et al.*, 2005).

## 1.15 NONENZYMATIC

### 1.15.1 ASCORBIC ACID

Ascorbic acid or “vitamin C” is a monosaccharide antioxidantfound in both animals and plants. As it cannot be synthesized in humans and must be obtained from the diet, it is a vitamin (Smirnoff N, 2001).Most other animals are able to produce this compound in their bodies and do not require it in their diets. In cells, it is maintained in its reduced form by reaction with glutathione, which can be catalyzed by protein disulfide isomerase and glutaredoxins (Padayatty*et al*., 2003). Ascorbic acid is a reducing agent and can reduce and thereby neutralize ROS such as hydrogen peroxide(Padayatty*et al*., 2003). In addition to its direct antioxidant effects, ascorbic acid is also a substrate for the antioxidant enzyme ascorbate peroxidase, a function that is particularly important in stress resistance in plants (Shigeoka*et al*., 2002).

### 1.15.2 Glutathione

Glutathione is a cysteine-containing peptide found in mostforms of aerobic life (Padayatty*et al*., 2003).It is not required in the diet and is instead synthesized in cells from its constituent amino acids. Glutathione has antioxidant properties since the thiol group in its cysteine moiety is a reducing agent and can be reversibly oxidized and reduced (Meister, 1998). In cells, glutathione is maintained in the reduced form by the enzyme glutathione reductase and in turn reduces other metabolites and enzyme systems as well as reacting directly with oxidants. Due to its high concentration and central role in maintaining the cell's redox state, glutathione is one of the most important cellular antioxidants (Meister, 1998). In some organisms, glutathione is replaced by other thiols, such as by mycothiol in the actinomycetes, or by trypanothione in the kinetoplastids (Nassar*et al*.,2007).

### 1.15.3 Melatonin

Melatonin, also known chemically as N-acetyl-5-methoxytryptamine, is a naturally occurring hormone found in animals and in some other living organisms, including algae (Caniato*et al*.,2003). Melatonin is a powerful antioxidant that can easily cross cell membranes and the blood–brain barrier (Reiter*et al*., 1997).Unlike other antioxidants, melatonin does not undergo redox cycling, which is the ability of a molecule to undergo repeated reduction and oxidation.

Melatonin, once oxidized, cannot be reduced to its former state because it forms several stable end-products upon reacting with free radicals. Therefore, it has been referred to as a terminal (or suicidal) antioxidant (Tan, 2000).

### 1.15.4 Tocopherols and tocotrienols (Vitamin E)

Vitamin E is the collective name for a set of eight related tocopherols and tocotrienols, which are fat-soluble vitamins with antioxidant properties (Herrera*et al*, 2001). Of these, α-tocopherol has been most studied as it has the highest bioavailability, with the body preferentially absorbing and metabolizing this form (Brigelius-Flohe R *et al*.,1999).It has been claimed that the α-tocopherol form is the most important lipid-soluble antioxidant, and that it protects membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction (Traber*et al*.,2007). This removes the free radical intermediates and prevents the propagation reaction from continuing. This reaction produces oxidized α-tocopheroxyl radicals that can be recycled back to the active reduced form through reduction by other antioxidants, such as ascorbate, retinol, or ubiquinol(Wang*et al*.,1999).

### 1.15.5 Uric acid

Uric acid accounts for roughly half the antioxidant ability of plasma. In fact, uric acid may have substituted for ascorbate in human evolution (Jaeschke *et al*., 2002).However, like ascorbate, uric acid can also mediate the production of active oxygen species.

## 1.16 PLANTS AS SOURCE OF ANTIOXIDANTS

Synthetic and natural food antioxidants are used routinely in foods and medicine especially those containing oils and fats to protect the food against oxidation. There are a number of synthetic phenolic antioxidants, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) being prominent examples. These compounds have been widely uses as antioxidants in food industry, cosmetics, and therapeutic industry. However, some physical properties of BHT and BHA such as their high volatility and instability at elevated temperature, strict legislation on the use of synthetic food additives, carcinogenic nature of some synthetic antioxidants, and consumer preferences have shifted the attention of manufacturers from synthetic to natural antioxidants (Papas, 1999). In view of increasing risk factors of human to various deadly

diseases, there has been a global trend toward the use of natural substance present in medicinal plants and dietary plats as therapeutic antioxidants. It has been reported that there is an inverse relationship between the dietary intake of antioxidant-rich food and medicinal plants and incidence of human diseases. The use of natural antioxidants in food, cosmetic, and therapeutic industry would be promising alternative for synthetic antioxidants in respect of low cost, highly compatible with dietary intake and no harmful effects inside the human body. Many antioxidant compounds, naturally occurring in plant sources have been identified as free radical or active oxygen scavengers(Brown*et al*., 1995). Attempts have been made to study the antioxidant potential of a wide variety of vegetables like potato, spinach, tomatoes, and legumes(Furuta *et al*., 1997). There are several reports showing antioxidant potential of fruits(Wang *et al*, 1996). Strong antioxidants activities have been found in berries, cherries, citrus, prunes, and olives. Green and black teas have been extensively studied in the recent past for antioxidant properties since they contain up to 30% of the dry weight as phenolic compounds (Papas,1999).

## 1.17ANTIOXIDANT POTENTIAL OF INDIAN FUNCTIONAL FOODS

### 1.17.1 Concepts of functional foods and nutraceuticals

In the last decade, preventive medicine has undergone a great advance, especially in developed countries. Research has demonstrated that nutrition plays a crucial role in the prevention of chronic diseases, as most of them can be related to diet. Functional food enters the concept of considering food not only necessary for living but also as a source of mental and physical well-being, contributing to the prevention and reduction of risk factors for several diseases or enhancing certain physiological functions(Devasagayam*et al*.,2004). A food can be regarded as functional if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way which is relevant to either the state of well being and health or reduction of the risk of a disease. The beneficial effects could be either maintenance or promotion of a state of well being or health and/or a reduction of risk of a pathologic process or a disease (Ruysschaert *et al*., 2009). Whole foods represent the simplest example of functional food. Broccoli, carrots, and tomatoes are considered functional foods because of their high contents of physiologically active components (sulforaphen, B-carotene, and lycopene, respectively). Green vegetables and spices like mustard and turmeric, used

extensively in Indian cuisine, also can fall under this category (Krishnaswamy, 1996). "Nutraceutical" is a term coined in 1979 by Stephen DeFelice (DeFelice, 1992). It is defined "as a food or parts of food that provide medical or health benefits, including the prevention and treatment of disease." Nutraceuticals may range from isolated nutrients, dietary supplements, and diets to genetically engineered "designer" food, herbal products, and processed products such as cereals, soups, and beverages. A nutraceutical is any non toxic food extract supplement that has scientifically proven health benefits for both the treatment and prevention of disease (Dillard*et al*., 2000). The increasing interest in nutraceuticals reflects the fact that consumers hear about epidemiological studies indicating that a specific diet or component of the diet is associated with a lower risk for a certain disease. The major active nutraceutical ingredients in plants are flavonoids. As is typical for phenolic compounds, they can act as potent antioxidants and metal chelators. They also have long been recognized to possess anti-inflammatory, antiallergic, hepatoprotective, antithrombotic, antiviral, and anticarcinogenic activities (Tapas*et al*., 2008).

## 1.18. AIM OF STUDY

The aim of this research work was to investigate the *in vivo* antianamic and antioxidant activities of the ethanol leaf extract of *Justicia secunda* in rats.

### 1.18.1 SPECIFIC OBJECTIVES

**The specific objectives of this study were:**

- To determine the quantitative phytochemicalconstituent of the ethanol leaf extract of *Justicia secunda*.
- To determine the median lethal dose ($LD_{50}$) of the ethanol leaf extract of *Justicia secunda.*
- To determine in vivo anti-anemic activities of the ethanol leaf extract of *Justicia secunda*.
- To determine the effect of the ethanol leaf extract on the antioxidant status of anemic rat.

# CHAPTER TWO

# 2.0 MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 Plant materials

The leaves of *Justicia secunda* were collected from a garden in Garriki, Enugu on 15th June, 2018. The botanical identification of the sample was authenticated by Mr. A.Ozioko of the Bioresource Department and Conservation Program (BDCP), Research Centre, Nsukka.

### 2.1.2 Animals

Adult rats of between 10 and 16 weeks with the average weight of 160 ± 13g were obtained from the Animal House of the Faculty of Biological Sciences, University of Nigeria, Nsukka. The animals were acclimatized for 7 days under standard environmental conditions, with a 12 hour light/dark cycle maintained on a regular feed (vital feed) and water *ad libitum*.

### 2.1.3 Equipment

Water Bath (Gallenkamp, England), Chemical Balance (Gallenkamp, England), Conicaln Flask (Pyrex, England), Beakers (Pyrex, England), Test tubes (Pyrex, England),Hamilton Syringe, 100-jl(Pye, Unican 293, England), Conical flask (Pyrex, England), pH Meter (Pye, Unican 293, England), Refrigrator (Kelvinator), Adjustable Micropippette (Perfect, U.S.A),VIS Spectrophoto meter (Kelvinator, Germany), Centrifuge(3,500rpm,PIC,England).

### 2.1.4 Chemicals and reagents

All chemicals used in this study were of the analytical grade and products of May and Baker, England and Merck, Darmstadt, Germany. QCA, Spain, Teco (TC) USA.

## 2.2 METHODS

### 2.2.1 Preparation of *Justicia secunda leaves* extract

**Extraction procedure:** The leaves of*Justicia secunda* were air dried under shed and pulverized using a mechanical grinder (Mettler, Germany). A known weight of the pulverized leaves (1000g) was macerated in 5 liters of ethanol using a maceration flask. The mixture was left for 72 hours with occasional stirring, after which it was filtered into a flat bottomed flask using a

muslin cloth. Further filtration was achieved using of Whatman No .1 filter paper so as to remove fine residues. The filtrates were concentrated using a rotary evaporator at 45°C to obtain the crude ethanol extract of the plant. The concentrated extract was stored in a labeled sterile screw-capped bottle at 4°C until needed.

### 2.2.2 Qantitative phytochemical study

The preliminary quantitative phytochemical analysis of the ethanol extract of *Justicia secunda* leaves were carried out in order to ascertain the presence or absence of some plant secondary metabolites. The determination was done by using standard conventional protocols as Illustrated by Harborne (1998); Trease and Evans (1989).

#### 2.2.2.1 Determination of Phenol and Polyphenol:

The sample (0.1ml solution) was taken into triplicate tubes, 2.5ml of distilled $H_20$ and 0.125ml of folinciocalteus was added to the mixture then followed by 1ml of 20% of $NaCO_3$ each. They were allowed to stand for 30mins and centrifuged for 5mins and the absorbance was taken at the wave length of 650nM and the process was also used for polyphenol but the absorbance was read at the wave length of 760nM both against the blank.

$$\text{Phenol} = \frac{\text{Abs} - \text{blk}}{\text{Slope}} \times \frac{\text{volume of solvent}}{\text{wt of sample}}$$

#### 2.2.2.2 Determination of taninns:

The sample (0.1ml) was taken into triplicate tubes, 2.4mls of methanol was added to each of the tubes followed by 0.15ml of 0.1m ferrium chloride in 0.1m HCL and 0.15ml of 0.0008molar potassium ferricyanide mixed and the absorbance was taken at the wave length of 720nM against the blank.

$$\text{Tannin} = \frac{\text{Abs} - \text{blk}}{\text{Slope}} \times \frac{\text{volume of solvent}}{\text{wt of sample}}$$

#### 2.2.2.3 Determination of saponins:

The sample (0.1ml solution) was taken into triplicate tubes and 2mls of ethyl acetate was added to each of the tubes followed by 1ml of 0.5% anyslaldehyde in ethyl acetate mixed then followed

by 1ml of 50% sulphuric acid in ethyl acetate mixed. The test tubes were incubated at $60^0C$ in an electrical oven for 20mins and made up to 5mls with ethyl acetate and the absorbance was taken at the wave length of 430nM against the blank.

$$\text{Saponin} = \frac{\text{Abs} - \text{blk}}{\text{Slope}} \times \frac{\text{volume of solvent}}{\text{wt of sample}}$$

**2.2.2.4 Determination of flavonoids:**

The sample (0.1ml)was taken into triplicate tubes and 1.9mls of ethanol was added to each of the tubes followed by 0.1ml of 1.0molar sodium acetate and 2.8mls of distilled $H_20$ and 0.1ml of 10% alluminium chloride shaken vigorously for 1 min and allowed to stand for 30mins and the absorbance was taken at the wave 415nM against the blank.

$$\text{Flavonoid} = \frac{\text{Abs} - \text{blk}}{\text{Slope}} \times \frac{\text{volume of solvent}}{\text{wt of sample}}$$

**2.2.2.5 Determination of alkaloids:**

This was determined according to the method of El-Olemyl (1994). A total of 0.1ml solution of the sample extract was taken into triplicate tubes and 2.5mls of 60% $H_2SO_4$(aq) was added to each of the tubes followed by 2.5mls of 0.5% formaldehyde in 60% $H_2SO_4$ acid mix and was allowed to stand for 3hours. Then the absorbance was taken at the wavelength of 565nM against the blank.

$$\text{Alkaloid} = \frac{\text{Abs} - \text{blk}}{\text{Slope}} \times \frac{\text{volume of solvent}}{\text{wt of sample}}$$

**2.2.2.6 Determination of terpenoids**

A total of 0.1ml solution of the sample extract was taken with a micropipette into triplicate tubes and 0.9ml of ethanol was added with 1ml of 5% phosphomolybdic acid solution and 1ml concentrated sulphuric acid mixed and was allowed to stand for 30mins then followed by the addition of 2mls of ethanol. Then the absorbance was read at 700nm against the blank.

$$\text{Terpenoid} = \frac{\text{Abs} - \text{blk}}{\text{Slope}} \times \frac{\text{volume of solvent}}{\text{wt of sample}}$$

### 2.2.2.7 Determination of steroids

The sample (0.2ml solution) was taken using micropipette into a test tube with the addition of 2ml of alcoholic potassium hydroxide boiled in a water bath for 30mins, then it was allowed to cool and 5mls of n-hexane was added followed by 2ml of distilled water, it was then shaken vigorously for 5mins and centrifuged for 5mins. After this 1ml of the supernatant was transferred into triplicate tubes and was evaporated to dryness in a water bath after which 2mls of ethanol was added to the residues then 2mls of steroid coloured reagents were mixed with the solution and was allowed to stand for 30mins, it's absorbance was read at the wavelength 550nm against the blank.

$$\text{Steroids} = \frac{\text{Abs} - \text{blk}}{\text{Slope}} \times \frac{\text{volume of solvent}}{\text{wt of sample}}$$

### 2.2.2.8 Determination of glycosides

The sample (0.1ml solution) was taken into triplicate tubes and 0.9ml of distilled water was added followed by 1ml of alkaline copper reagent and boiled for 8mins in a water bath, then it was allowed to cool and 1ml of 5% phosphomoblic acid reagent was added mixed and 7mls of distilled was added and mixed, then the absorbance was read at 420nm and 680nm.

$$\text{Glycoside} = \frac{\text{Abs} - \text{blk}}{\text{Slope}} \times \frac{\text{volume of solvent}}{\text{wt of sample}}$$

### 2.2.3Acute Toxicity Studies ($LD_{50}$)

The acute toxicity study of the crude ethanol extract, ethyl acetate and ethanol fractions were estimated in mice using the method of Lorke (1983). The tests involved two phases. The first phase was determination of the toxic range. The mice were placed in six groups of 3 mice each and were given 10, 100 and 1000 mg/kg body weight of the extract solubilized in 2.5%, v/v propylene glycol in water. The treated mice were observed for 24 h for number of deaths. The death pattern in the first phase determined the doses used for the second phase. In the second phase, the mice received 1600, 2900 and 5000 mg/kg body weight of the extract. The treated animals were observed for 24 h for lethality or signs of acute intoxication.

**2.2.4. Experimental Design:** The study was carried out in stages as follows:

**2.2.4.1 Animal Grouping**

Thirty six male Wistar albino rats weighing (50-250g) were housed in wire meshed cage under standard conditions (temperature 25-29$^0$C, 12/12 hrs light-darkness cycles) starting from the time of acclimatization till the end of the experiments. They had continuous access to food and water during the entire period of experimentation.

**Animal treatment and experimental design:**

A total number of thirty six male Wistar rats were used for this experiment for both the haematology, oxidative stress parameter and anti-oxidant vitamins. The animals were divided into six groups in this format:

Group 1 = Normal control

Group 2 = Positive control (induced with phenylhydrazin and untreated)

Group 3 = Standard control (induced with phenylhydrazin and treated with hematic suspension).

Group 4 = induced with phenylhydrazin and treated with 200 mg/kg b.w. of the extract.

Group 5 = induced with phenylhydrazin and treated with 400 mg/kg b.w. of the extract

Group 6 = induced with phenylhydrazin and treated with 600 mg/kg b.w. of the extract

On day four, 4ml of blood samples were collected by ocular puncture from each of the animal model using capillary tube and evaluated for blood parameters(white blood cell count, re blood cell count, packed cell volume, hemaegloblin and platelets) using Sahli's hemoglobinometer for Hb and Neubars chamber for RBC count. A total of thirty six adult male Wister rats with weight range of 50-250 g were randomly assigned to five groups, group I as the control and II-V as test groups. The blood sample was transferred into properly labelled, EDTA sample bottles. The anti-coagulated blood was used for the determination of erythrocyte count, Packed Cell Volume (PCV), Haemoglobin (HB), White Blood Cell (WBC), and platelets with the aid of an automatic haematology analyzer (Mindray Hematology analyzer, BC-2300).

**2.2.5 Haematological assay**

**2.2.5.1 Determination of the Red blood cells (RBC) count**

**Principle**

The red blood cells count was determined by the method of Ochei and Kolhatkar (2008). The blood specimen was diluted 1:200 with RBC diluting fluid and cells were counted under high power (40X) objective of a microscope by using a counting chamber. The number of cells was calculated and reported as the number of red cells / cu.mm of whole blood. The requirements are the following:

1. Microscope
2. Haemocytometer
3. RBC pipette
4. RBC diluting fluid (Sodium citrate)

**Procedure**

Whole blood (20 µl) added to 3.98 ml of 10 % sodium citrate and mixed well. After 5 minutes, the first few drops were discarded by holding the pipette vertically and the counting chamber was charged with the fluid. It was allowed to settle for 3 minutes. By switching to low power (10X) objective the centre large squares with 25 small squares were adjusted to light and then adjusted to high power (10X) objective. The red blood cells in the four corner squares and one central square were counted.

$$\text{Total RBC (mm/l)} = N \times \frac{1}{0.2} \times \frac{1}{0.1} \times 200$$

N = numbers of cells counted

0.1 = depth of the chamber

0.2 = area counted

200 = dilution factor

**2.2.5.2 Determination of white blood cells (WBC) count**

The white blood cell count was determination following the method described by Ochei and Kolhatkar (2008). The glacial acetic acid lyses the red cells while the gentian violet slightly stains the nuclei of the leucocyte. The blood specimen was diluted 1:20 in a WBC pipette with the diluting fluid and the cells were counted under low power microscope by using a counting chamber. The number of cells in undiluted blood was reported as the number of white cells/cu.nm of whole blood. The additional requirements are the following:

**Procedure**

Whole blood (20 µl) was added to 380 µl of diluting fluid (acetic acid, with gentian violet) and mixed. The counting chamber was charged with the well mixed diluted blood (after discarding the first five drops) with the aid of a pipette. Cells were allowed to settle in a moist chamber for 3 minutes. The four corners of the chamber were visualized under a low power (10X) objective microscope and the cells were counted in all the four marked corner squares.

$$\text{Total WBC (mm}^3) = \frac{N \times 20}{0.1 \times A}$$

N = unmbers of cells counted

0.1 = depth of the chamber

A = area counted

20 = dilution factor

### 2.2.5.3 Determination of the Packed Cell Volume (PCV)

Packed cell volume (PCV) was estimated as described by Ochei and Kolhatkar (2008). Blood sample was taken with a heparinised capillary tube, cleaned and sealed with plasticine. The filled tubes were placed in the microhaematocrit centrifuge and spun at 10,000 rpm for 5 minutes. Spunned tubes were placed into a specially designed scale and the PCV was read as a percentage.

$$\text{PVC \%} = \frac{\text{Packed RBC column height}}{\text{Total blood volumn height}} \times 100$$

### 2.2.5.4 Determination of the haemoglobin (HB) concentration

Heamoglobin (HB) concentration was determined using cyanomethaglobin technique as outlined by Ochei and Kolhatkar (2008).

**Principle**

Drabkin’s solution which contains potassium ferricyanide, potassium cyanide and potassium dihydrogen phosphate was mixed with the haemoglobin. The ferricyanide forms methaemoglobin which is converted to cyanmethaemogloin by the cyanide. The cyanmethaemoglobin produces a colour which is measured colorimetrically.

**Procedure**

Whole blood (20 µl) was added to 4 ml of Drabkin's solution in a test tube in a 1:250 dilution. This was well mixed, allowed to stand for 10 minutes at room temperature and the absorbance was read with colorimetrically at 540 nm with Drabkin's solution as a blank.

Heamoglobin (HB) g/dl= Reading of test × 36.8

### 2.2.6 Determination of the non-enzymatic and enzymatic antioxidants

#### 2.2.6.1 Assay of superoxide dismutase (SOD)

Superoxide dismutase activity was assayed by the method of Arthur and Boyne (1985) as contained in the Randox Kit used.

**Principle**

The method employed xanthine and xanthine oxidase to generate superoxide radicals which reacted with 2(-4- iodophenyl)-3(- 4 – nitrophenol), – 5 – phenyltetrazolium chloride (INT) to form a red formazan dye. The superoxide dismutase activity were measured by the degree of inhibition of this reaction. One unit of SOD is that which caused a 50 % inhibition of the rate of reduction of INT under the conductions of the assay.

The requirements we the following:

1. R1a (Mixed substrate)
2. R1b (Buffer)
3. R2 (Xanthine Oxidase)
4. Standard

**Procedure**

Into three test tubes labeled test, serum, standard and blank were added 15 µl of serum, 15 µl of standard and 15 µl distilled water respectively. Then, R1 (500 µl) was added and mix well. Xanthine oxidase (75 µl) was added, mix again and the initial absorbance taken after 30 sec. The finial absorbance was taken after 3 mins at wavelength 505 nm and units of SOD per gramme haemoglobin were extrapolated from a standard curve.

Superoxide dismutase (SOD) $= \frac{\text{Abs of sampe} \quad - \quad \text{abs of blk}}{\text{Abs} \times \text{conc of std}}$

### 2.2.6.2 Assay of catalase

The activity of catalase was assayed by the method of Sinha (1972). Dichromate in acetic acid was reduced to chromic acetate, when heated in presence of hydrogen peroxide with the formation of per chromic acid as an unstable intermediate. The chromic acetate formed was measured at 570 nm. Catalase was allowed to split and $H_2O_2$ was determined by measuring chromic acetate colorimetrically. The requirements are the following:

1. Phosphate buffer, 0.01 M, pH 7
2. Hydrogen peroxide, 0.2 M
3. Potassium dichromate, 5 %
4. Dichromate acetic acid reagent: Potassium dichromate and glacial acetic acid were mixed in the ratio 1:3.
5. Standard hydrogen peroxide 0.2 M

**Procedure**

The distilled water (0.9 ml) and 0.1 ml of plasma in a test tube were added 2 ml of $H_2O_2$ and 2 ml phosphate buffer. The reaction was initiated by adding 2 ml of dichromate acetic acid reagent to 1 ml portion of this mixture. Absorbance of the reaction was taken in 30 secs intervals for 2 mins. The activity of catalase was expressed as U/ml of plasma (U – micromoles of $H_2O_2$ utilised/ second).

Catalase activity (U/ml) = 0.23 x log Abs 1

Abs 2

0.00693

where Abs 1 is absorbance at t = 0 seconds

Abs 2 is absorbance at t 30 seconds

### 2.2.6.3 Determination of the glutathione concentration

**Principles**

This was determined according to the method of King and Wootton (1959). Blood sample (0.1 ml) was mixed with (0.9 ml) of distilled water in a beaker; 0.02 ml of sodium sulphate was also added, shaken and allowed to stand for 2 min at room temperature. This was filled by the

addition of 0.02 ml of lithium sulphate 20 %, 0.2 ml of 20 % $NaCO_3$ and 0.2 ml of phosphor- 18-tungstic acid were to the beaker, it was shaken and allowed to stand for 4min while observing for maximum colour development. Then, 2.5 ml of 20 % sodium sulphate was also added and the absorbance was measured at 680 nm within 10 min. A blank, 0.1 ml of $H_2O$ was used replace of blood was also set up and glutathione concentration was calculated from a standard cysteine curve.

$$\text{Glutathione concentration} = \frac{\text{Abs of sample}}{\text{Abs of std} \times \text{Conc}}$$

**2.2.7 Estimation of the extent of lipid peroxidation (malondialdehyde)**

**Principles**

Lipid peroxidation was estimated by measuring spectrophotometerically the level of the lipid peroxidation product, malondialdehhyde (MDA) as described by Wallin *et al.* (1993). Lipid degradation occurs forming such products as malondialdehyde (from fatty acids with two or more double bonds), ethane and pentane (from the n – terminal carbons of 3 and 6 fatty acids, respectively) malondialdehhyde reacts with thiobarbituric acid to form a red or pink coloured complex which in acid solution absorbs maximally at 532 nm.

$$\text{MDA} + 2\text{TBA} \rightarrow \text{MDA: TBA adduct} + H_2O$$

**Procedure**

Into three test tubes labeled test, serum and blank were added 10 µl of serum, and 10 µl distilled water respectively. Then, 0.5 ml of 25 % TCA (trichloroacetic acid) and 0.5 ml of 1 % TBA (thiobarbituric acid) in 0.3 % NaOH were added. The mixture was boiled for 40 minutes in a water bath and cooled in cold water. Then, 0.1 ml of 20 % sodium dodecyl sulfate (SDS) was added to the cooled solution and mixed properly. The absorbance was taken at wavelengths of 532 nm and 600 nm against a blank.

$$\text{MDA} = \frac{\text{abs 1} - \text{abs 2}}{0.00693}$$

### 2.2.8 Serum Electrolytes

**Sodium Ion ($Na^{+}$)**

Sodium ion was determined using the method of Trinder (1951) and Maruna (1958) as outlined in Teco Kit.

**Principle**

Sodium is precipitated as the triple salt, sodium magnesium uranyl acetate with the excess uranium then being reacted with ferrocyanide, producing a chromophore whose absorbance varies inversely as the concentration of sodium in the test specimen.

**Reagents**

Filtrate reagent: uranyl acetate (2.1 mM) and magnesium acetate (20 mM)

Acid reagent: A diluted acetic acid.

Sodium colour reagent: potassium ferrocyanide

Sodium standard, sodium chloride solution: (150 mEq/L of sodium)

**Procedure**

Labeling of four test tubes were done thus: blank, standard, control and test sample One milliliter (1.0 ml) of filtrate reagent 2.1mM (uranyl acetate 2.1 mM and magnesium 20mM acetate in ethyl alcohol) was pipetted out into the tubes followed by the addition of 50 µl of sample to all the tubes and distilled water was used as blank. All the mixtures in the test tubes were vigorously shaken for 3 minutes. The reaction mixtures were then centrifuged at a high speed (1500 g) for 10 minutes. The colour development was carried out thus: Labelling of test tubes corresponding to the above filtrate tubes was done. The corresponding test tubes were set up. One milliliter (1.0 ml) of acid reagent was pipetted into all the tubes, followed by the addition of 50 µl of supernatant and 50 µl of colour reagentto respective tubes. The contents of the test tubes were mixed and the absorbace was read at 550 nm.

Sodium ion was calculated using the following formula:

$$\text{Sodium ion (mEq/L)} = \frac{Abs\ of\ blank - Abs\ of\ S}{Abs\ of\ blank - abs\ of\ STD} \text{ X Conc. of STD (mEq/L)}$$

Abs = Absorbance

S = Sample

STD = Standard

**Potassium Ion ($K^+$)**

Potassium ion concentration was determined using the method of Terri and Sesin (1985) as described in Teco diagnostic kit

**Principle**

The amount of potassium was determined by using Sodium tetraphenylboron in a specifically prepared mixture to produce a colloidal suspension, the turbidity of which was proportional to potassium concentration in the range of 2-7 mEq/L

**Procedure**

Three test tubes were labeled thus: standard, control and test sample. One milliliter (1.0 ml) of potassium reagent (2.1 Mm sodium tetraphenylboron (2.1 mM)) was pipetted to all the tubes, 0.01 ml (10 µl) of samples was added to the respective tubes, mixed and incubated at room temperature for 3 minutes. After 3 minutes, colorimeter was zeroed with reagent blank and all the tubes were read at 500 nm.

$$\text{Potassium ion (mEq/L)} = \frac{Abs\ of\ unknown}{Abs\ of\ blank} \text{ X Conc. of STD (mEq/L)}$$

STD = Standard

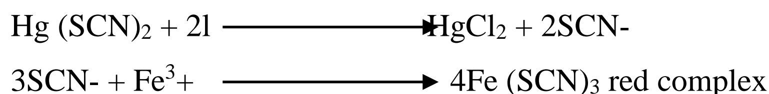
Potassium standard: Equivalent to 4 mEq/L

**Chloride Ion Concentration**

The concentration of chloride ion was determined using the method of Skeggs and Hochstrasser (1964) as outlined in Teco kit.

**Principle**

Chloride ions form a soluble, non-ionized compound. When reacted with mercuric ions, they would displace thiocyanate ions from non-ionized mercuric thiocyanate. The released of the colour produced was directly proportional to the chloride ion concentration.



$Hg\ (SCN)_2 + 2l \longrightarrow HgCl_2 + 2SCN-$

$3SCN- + Fe^3+ \longrightarrow 4Fe\ (SCN)_3$ red complex

Reagents: Mercuric Nitrate (0.058mM), Mercuric Thiocyanate (1.75mM), Mercuric Chloride (0.74mM) and Ferric Nitrate (22.3 mM)

Chloride Calibrator: Sodium Chloride (100 mEq/L)

**Procedure**

Two test tubes were labelled blank and calibrator. One and half milliliter (1.5ml) of chloride reagent (mercuric nitrate 0.058mM, mercuric thiocyanate 1.75mM, mercuric chloride 0.74 mM and ferric nitrate 22.3 mM in dilute acid and methanol) was pipetted to each tube. This was followed by the addition of 10 µl of calibrator/sample to respective tubes and then mixed. They were incubated at room temperature for at least five minutes and absorbance read at 480 nm.

$$\text{Chloride ion conc. (m Eq/L} = \frac{\text{Abs. of unknown x conc. calibrator}}{\text{Abs. of calibrator}}$$

## 2.6.0 Determination of Vitamins

**Determination of VitaminC**

**Principle**

Vitamin C was estimated by the method (Omaye *et al*.,1962). Ascorbic acid is oxidized and converted to diketoglutamic acid, a strong acid solution and forms a diphenyl hydrazine by reacting with 2, 4-dinitrophenylhydrazine. The hydrazine red colour with can be measured corimetrically at wavelength of 500 nm.

**Reagents**

1. 2,4-Dinitrophenylhydrazine-thiourea-copper sulphate reagent (DTC): 0.4 gm thiourea, 0.05 gm copper sulphate and 3.0 gm DNPH were dissolved in 100.0 ml of 9N H2SO4.
2. 10 % TCA.
3. 65 % H2SO4.

   Standard Vitamin C solution: 10 mg of Vitamin C was dissolved in 100 ml of 5 % TCA.

**Procedure**

The serum (1.0 ml) and 1.0 ml of blank was added to test tubes. Then, 1 ml of 10 % TCA and 0.5 ml chloroform were added and centrifuged for 15 minutes. After that, 1 ml of the supernatant was pipetted into a test tube and 0.4 ml of combined reagent was added. This was boiled for 1 hour, cooled and 2 ml of $H_2S0_4$ was added. It was allowed to stand for 30 minutes. The absorbance was measured at 540 nm.

$$\text{Vitamine C} = \frac{\text{Abs of sample} \times \text{Conc of std}}{\text{Abs of Conc}}$$

**Determination of Vitamin E**

Vitamin E was estimated by the method of Baker *et al*.,(1980). The method involves the reduction of ferric ions to ferrous ions by α-tocopherol and the formation of a red coloured complex with 2,2'-dipyridyl. Absorbance of the chromophore was measured at 520 nm.

**Reagents**

1. Petroleum ether 60-80°C
2. Double distilled ethanol
3. 2,2'-Dipyridyl solution: 0.2 % in ethanol
4. Ferric chloride solution: 0.5 % in ethanol
5. Stock standard: 10 mg of Vitamin E in 100 ml distilled ethanol
6. Working standard: The stock solution was diluted indistilled ethanol to a concentration of 10 μg/mL.

**Procedure**

The serum (1.0 ml) and 1.0 ml of blank was added to test tubes with 2.0mls petroleum ether and 1.6 ml ethanol. The tubes were centrifuged and the supernatant was mixed with 0.2 ml each of 2,2'-dipyridyl and ferric chloride. The tubes were kept in the dark for five minutes. An intense red colour was developed. To all the tubes, 4.0 ml water was added and mixed well. Standard Vitamin E in the range of 10-100μg were taken and treated similarly along with the blank containing only the reagent. The colour in the aqueous layer was read at 520 nm. The values are expressed as mg/ gm of dried sample.

$$\text{Vitamine E} = \frac{\text{Abs of sample} \times \text{Conc of std}}{\text{Abs of std}}$$

**2.7 STATISTICAL ANALYSIS**

Data were reported as means ± SEM, where appropriate. Both one and two way analyses of variance (ANOVA) were used to analyse the experimental data and Duncan multiple test range was used to compare the group means obtained after each treatment with control measurements. Differences were considered significant when $p \leq 0.05$. Using Statistical Package for Social Sciences (SPSS) version 16.

# CHAPTER THREE

## 3.0 RESULTS

### 3.1 Percentage yield of ethanol extract of *Justicia secunda*leaves

Table1 represents the percentage yield of the ethanol extract of the*Justicia secunda*leaves. One thousand grams (1000g) of dried crude sample of *Justicia secunda*powder gave a percentage yield of 8.23%

**Table 1: Percentage yield of *Justicia secunda*leafextract**

| Weight of crude sample (g) | Weight of extract (g) | Percentage yield (%) |
|---|---|---|
| **1000** | **82.26** | **8.23** |

### 3.2 Quantitative phytochemical composition of the ethanol extract of *Justicia secunda*

Table.2 shows the results of the quantitative phytochemical analysis of ethanol leaf extract of*Justicia secunda*. The leaf ethanol extract contained respectively: tannins (1.86 ± 1.54 mg/100g), terpenoids (13.83 ± 1.50 mg/100g), steroids (1.76.10 ± 1.56mg/100g), phenols (4.35 ±0.84mg/100g), alkaloids (24.00± 18.06mg/100g), flavonoids (13.78 ± 1.41 mg/100g), saponins (134.64 ± 48.64 mg/100g), glycoside (0.85 ± 0.14 mg/100g) and polyphenol (3.31 ± 0.84 mg/100g),

**Table 2: Quantitative phytochemical composition of the ethanol extract of *Justicia secunda* leaves**

| Phytochemical Constituents | Concentration (mg/100 g) (Mean + SE) |
|---|---|
| Tannins | 1.86± 1.54 |
| Terpenoids | 13.83 ± 1.50 |
| Steroids | 1.76 ± 1.56 |
| Phenols | 4.35± 0.84 |
| Alkaloids | 24.00± 18.06 |
| Flavonoids | 13.78± 1.41 |
| Saponins | 134.64± 48.64 |
| Glycoside | 0.85 ± 0.14 |
| Polyphenol | 3.31 ± 0.84 |

**Results are expressed in Means ± SE (n = 3).**

### 3.3 Results of the acute toxicity test of the ethanol extract of *Justicia secunda*

Table 3 shows the acute toxicity test results of ethanol leaf extract of*Justicia secundda.* The result showed that the extract was not lethal even at the highest dosage (5000 mg/kg body weight) administered. The median lethal dose ($LD_{50}$) of the extract should therefore be over 5000 mg/kg body weight.

**Table 3: Results of phase I and phase II of the acute toxicity test of the ethanol extract of *Justicia secunda***

| Phases/groups | Dosage of extract (mg/kg body weight) | Mortality rate |
|---|---|---|
| **Phase I** | | |
| Group 1 | 10 | 0/3 |
| Group 2 | 100 | 0/3 |
| Group 3 | 1000 | 0/3 |
| **Phase II** | | |
| Group 1 | 1600 | 0/3 |
| Group 2 | 2900 | 0/3 |
| Group 3 | 5000 | 0/3 |

**3.4 Effect of the ethanol extract of the *Justicia secunda*on some haematological indices in phenylhydrazin-induced anemic rats.**

The result (Table.4) revealed that PCVof groups 4 and 5 exhibited non-significant (P>0.05) difference when compared to group 1. However, there was a significant (P<0.05) difference in increase at 32.33% against 28.83% when group 6 was compared to group 2, but group 1 was significantly (P>0.05) higher at 38.17% than group2.

In the analysis of white blood cells (WBC) count, it revealed that groups 4 and 6 had no significant (P>0.05) difference when compared to groups 1, 2, 3 and 5. However, group 5 showed significant (P<0.05) difference in decrease at 7833.33x10mm$^3$ when compared to groups 1 and 2 at 8933.33 x10mm$^3$ and 10916 x10mm$^3$ respectively. Similarly, groups 6 was non-significantly (P>0.05) higher than groups 2 and lower than group 3.

The analysis of red blood cell count (RBC) revealed that groups 4, 5 and 6 were non-significantly (P>0.05) difference compared to group 2 and group 3. However, group 4, 5 and 6 shows significance (P<0.05) difference compared to group1.

Analysis of result of platelet count, revealed that groups 4, 5 and 6 were non- significantly (P>0.05) different compared to groups 1, 2 and 3. Similarly, groups 4 and 5 were non-significantly (P>0.05) higher than group 2 and lower than group 1.

The Hb concentration had no significant (P>0.05) difference when compared amongst the groups.

**Table 4: Effect of the ethanol extract of *Justicia secunda* on some haematological indices in Phenylhydrazin-induced anemic rats.**

| Treatment Groups | Haematological Indices | | | | |
|---|---|---|---|---|---|
| | PCV(%) | Hb(g/dl) | RBC($\times10^6$) | WBC($\times10mm^3$) | Platelet(×10mm3) |
| Group 1 | $38.17 \pm 3.19^d$ | $19.32\pm 4.00^a$ | $6.08\pm 0.36^b$ | $8933.33\pm 817.00^{bc}$ | $142.67 \pm 8.00^c$ |
| Group 2 | $28.83 \pm 1.60^a$ | $18.58 \pm 2.88^a$ | $5.07\pm 0.48^a$ | $10916\pm 875.02^a$ | $112.00\pm 10.00^a$ |
| Group 3 | $33.83 \pm 2.48^b$ | $18.12\pm 4.72^a$ | $5.38\pm 0.66^a$ | $9133.33\pm 1269.12^c$ | $143.00\pm9.27^c$ |
| Group 4 | $31.50 \pm 2.43^{ab}$ | $19.78\pm 2.00^a$ | $5.15\pm 0.45^a$ | $8100.00\pm 874.07^{abc}$ | $127.00\pm 5.14^b$ |
| Group 5 | $31.33 \pm 3.44^{ab}$ | $21.84\pm 4.77^a$ | $5.03\pm 0.39^a$ | $7833.33\pm 999.33^{ab}$ | $123.83\pm 7.52^b$ |
| Group 6 | $32.33 \pm 2.34^b$ | $20.15\pm 3.14^a$ | $5.42\pm 1.00^a$ | $8200.00\pm 473.29^{abc}$ | $125.67\pm 5.13^b$ |

**Results are expressed in means ± SE (n = 6)**

Mean values with different letters as superscripts across the column are considered significant at $p < 0.05$.

Group 1 = Normal control

Group 2 = Positive control (induced with phenylhydrazin and untreated)

Group 3 = Standard control (induced with phenylhydrazin and treated with hematic suspension).

Group 4 = induced with phenylhydrazin and treated with 200 mg/kg b.w. of the extract.

Group 5 = induced with phenylhydrazin and treated with 400 mg/kg b.w. of the extract

Group 6 = induced with phenylhydrazin and treated with 600 mg/kg b.w. of the extract

### 3.5 Effect of ethanol extract of *Justicia secunda* leaves on MDA and Antioxidant Parametersin phenylhydrazin-inducedanemic rats.

Table 5 shows the pro-and anti-oxidant indices in rats treated with extract of *Justicia secunda* leafextract. MDA results revealed that there was no significant ($p > 0.05$) difference when group 4 was compared to group 5. However, MDA concentration of groups 4 and 5 was found to be non-significant ($p > 0.05$) compared to group 3. Compared to group 1, MDA concentration of groups 4, 5 and 6 was found to be significantly ($p < 0.05$) higher but significantly ($p < 0.05$) lower when compared to group 2.

SOD activity of group 4, 5 and 6 rats was non-significant ($p > 0.05$) when compared to groups 1, 2 and 3 but was found to be significantly ($p < 0.05$) higher compared to group 2.

The catalase activity of group 4 showed no significant ($p > 0.05$) difference compared to all the groups but groups 5 and 6 was observed to be significant ($p < 0.05$) compared to groups 1 and 2.

The Gluthatione concentration was significantly decreased ($p < 0.05$) in difference when groups 4 and 5 at 21.00UL/L and 22.38 UL/L respectively were compared to groups 1, 2 and 3 at 26.23, 18.89 and 21.16UL/L respectively. However group 6 was non-significantly ($p > 0.05$) different compared to groups 3 and 5.

GSH concentrations of groups 3, 4, 5 and 6 was found to be non-significantly ($p > 0.05$) lower when compared to group 1, but non-significantly ($p > 0.05$) higher when compared to group 2.

The result of GPx shows that group 4 was non-significant ($p>0.05$) different when compared to group 1. However groups 5 and 6 were significant ($p < 0.05$) when compared to groups 1 and 2. But groups 4, 5 and 6 were non-significantly ($p > 0.05$) higher than group 2 and lower than group 1.

**Table 5: Effect of the ethanol extract of *Justicia secunda* leaves on MDA and antioxidant parameters in phenylhydrazin-induced anemic rats.**

| Treatment Group | Antioxidant Levels | | | |
|---|---|---|---|---|
| | MDA Mmol/mg protein | SOD Activity(U/L) | Catalase Activity(U/L) | GSH Conc.(UL/L) |
| Group 1 | 6.46±0.89[b] | 38.64±3.00 [ab] | 23.36±2.14 [d] | 26.23±1.42[d] |
| Group 2 | 7.68±0.56 [a] | 30.00±2.12 [a] | 15.64±2.47 a | 18.89±2.18 [a] |
| Group 3 | 5.45±0.77 [a] | 36.24±1.53 [d] | 21.70±1.09[cd] | 21.65±2.21[b] |
| Group 4 | 5.01±0.17 [a] | 32.06±2.47[bc] | 19.66±2.00 [b] | 21.00±1.60 [b] |
| Group 5 | 5.67±0.50 [ab] | 32.83±2.00 [bc] | 19.95±2.00 [b] | 22.38±1.21 [b] |
| Group 6 | 5.57±0.86 [b] | 33.73±2.66 [d] | 21.41±0.96 [b] | 22.16±1.66 [b] |

Results are expressed in Means ± SE (n = 6)

Mean values with different letters as superscripts across the column are considered significant at $p < 0.05$

Group 1 = Normal control

Group 2 = Positive control (induced with phenylhydrazine and untreated)

Group 3 = Standard control (induced with phenylhydrazine and treated with hematic suspension).

Group 4 = induced with Phenylhydrazine and treated with 200 mg/kg b.w. of the extract.

Group 5 = induced with Phenylhydrazine and treated with 400 mg/kg b.w. of the extract

Group 6 = induced with Phenylhydrazine and treated with 600 mg/kg b.w. of the extract

**3.6: Effect of the ethanol extract of *Justicia secunda* leaves on electrolyte concentrations phenylhydrazine-induced anemic rats.**

Table 6 shows the electrolyte concentrations of *Justicia seconda* leaf extract. It reveals that the potassium ion ($K^+$) concentration was not significant ($P > 0.05$) when group 4 was compared to groups 1, and 3. However, group 4 was found to be significantly higher ($P < 0.05$) than group 2. Group 5 is non- significantly ($P > 0.05$) higher than groups 1 and 6 but significantly higher than groups 2 and 3 ($p < 0.05$). However, group 6 was found to be significantly higher ($P < 0.05$) when compared to groups 2, and 3 but non- significance when compared to group 1 ($P > 0.05$)

From the table also, sodium ion ($Na^+$) concentrations revealed that there was no significance difference ($P>0.05$) when group 4 was compared to groups 3, 5 and 6. However, group 4 was found to be significantly higher ($P < 0.05$) than group 2. Group 5 was not significant ($P>0.05$) when compared to group 3, 4 and 6, but significantly higher ($P < 0.05$) than groups 2. Group 6 was not significant ($P>0.05$) when compared to group 3, 4 and 5, however it was found to be significantly higher ($P < 0.05$) groups 2.

Also, chloride ion ($Cl^-$) concentration shows that group 4 was insignificant ($P>0.05$) when compared to groups 2, 5 and 6. In group 5, it was found to be non significant ($P>0.05$) when compared to groups 3, 4 and 6. However, group 5 was significantly ($P < 0.05$) higher than group 2. Group 6 was also insignificant ($P>0.05$) when compared to group 1. In the same way, group 6 was significantly ($P < 0.05$) higher than group 2.

**Table 6: Effect of the ethanol extract of *Justicia secunda* leaves on electrolyte concentrations in phenylhydrazine-induced anemic rats.**

| Treatment Groups | Electrolyte Concentrations | | |
|---|---|---|---|
| | $K^+$ (MEq/L) | $Na^+$ (MEq/L) | $Cl^-$ (MEq/L) |
| Group 1 | $0.43 \pm 0.07^b$ | $163.34 \pm 14.23^d$ | $1.00 \pm 0.10^b$ |
| Group 2 | $0.25 \pm 0.05^a$ | $130.64 \pm 9.00^a$ | $0.33 \pm 0.07^a$ |
| Group 3 | $0.40 \pm 0.03^b$ | $154 \pm 7.95^{cd}$ | $0.45 \pm 0.05^b$ |
| Group 4 | $1.00 \pm 0.05^a$ | $142.21 \pm 7.38^b$ | $0.36 \pm 0.06^b$ |
| Group 5 | $0.09 \pm 0.02^b$ | $149.27 \pm 8.00^{bc}$ | $0.38 \pm 0.03^b$ |
| Group 6 | $0.38 \pm 0.10^b$ | $149.16 \pm 3.00^c$ | $0.39 \pm 0.02^{cd}$ |

**Results are expressed in means ± SE (n = 6)**

Mean values with different letters as superscripts across the column are considered significant at $p < 0.05$.

Group 1 = Normal control

Group 2 = Positive control (induced with Phenylhydrazine and untreated)

Group 3 = Standard control (induced with Phenylhydrazine and treated with hematinic suspension).

Group 4 = induced with Phenylhydrazine and treated with 200 mg/kg b.w. of the extract.

Group 5 = induced with Phenylhydrazine and treated with 400 mg/kg b.w. of the extract

Group 6 = induced with Phenylhydrazine and treated with 600 mg/kg b.w. of the extract

**3.7: Effect of the ethanol extract of *Justicia secunda*leaveson vitamins concentrations phenylhydrazine-induced anemic rats.**

The Vit. E and Vit. C concentrations weresignificantly ($p<0.05$) different when groups 4, 5 and 6 were compared to groups 1, 2 and 3. However, the value in group 6 was significantly ($p<0.05$) higher than group 2, but lower than group 1.

**Table 7: Effect of the ethanol extract of *Justicia secunda*leaveson Vitamins concentrations in phenylhydrazine-induced anemic rats**

| Treatment Group | | |
|---|---|---|
| | **Vit. C (mg/dl)** | **Vit. E (mg/dl)** |
| **Group 1** | **0.33 ± 0.01$^{abc}$** | **0.54 ± 0.01$^{b}$** |
| **Group 2** | **0.15 ± 0.22$^{ab}$** | **0.45 ± 0.11$^{ab}$** |
| **Group 3** | **0.26 ± 0.05$^{a}$** | **1.38 ± 0.04$^{a}$** |
| **Group 4** | **1.00 ± 0.02$^{cd}$** | **0.59 ± 0.03$^{a}$** |
| **Group 5** | **10.20 ± 0.03$^{d}$** | **1.41± 0.07$^{a}$** |
| **Group 6** | **0.20± 0.03$^{cd}$** | **1.53 ± 0.03$^{b}$** |

**Results are expressed in Means ± SE (n = 6)**

Mean values with different letters as superscripts across the column are considered significant at

Group 1 = Normal control

Group 2 = Positive control (induced with Phenylhydrazin and untreated)

Group 3 = Standard control (induced with Phenylhydrazin and treated with hematic suspenion).

Group 4 = induced with Phenylhydrazin and treated with 200 mg/kg b.w. of the extract.

Group 5 = induced with Phenylhydrazin and treated with 400 mg/kg b.w. of the extract.

Group 6 = induced with Phenylhydrazin and treated with 600 mg/kg b.w. of the extract.

# CHAPTER FOUR
# DISCUSSION

## 4.1. Discussion

The effects of ethanol extract of *Justicia secunda* leaves on haematological indices and antioxidant status in phenylhydrazine induced rats were evaluated in this study. The extract was well tolerated by rats as neither death nor signs of toxicity were observed in the rats dosed with 5000mg/kg body weight of the ethanol extract of *Justicia secunda* during the period of observation and this records the same on the journal publications done by Onyeabo et al., 2017 on haematological and biochemical studies on justicia carnea which is of the same genus but different specie and zero toxicity was also recorded the work done and published by Samuel et al., 2017 antioxidant, anti-inflammatory and antinociceptive activities of methanolic extract of justicia secunda vahl leaf which indicates that Justicia secunda ethanolic extract is free from toxicity and healthy consumption. Phytochemical analysis of the extract revealed the presence of high amounts of flavonoids, terpenoids, alkaloids, saponins is relatively the same with the results gotten by Onyeabo et al., 2017 and in their some other publications indicating that the plant has high levels of important phytochemicals. The results from this research when compared with works done by Onyeabo et al., 2017, Paquel et al., 2012, and Anslem et al., 2017 etc agrees to each other and indicates more increase in the white blood cell count as the doses of the sample extracts are increased when administered on the grouped experimental animals. In this study significant increases in hematological parameters especially of red blood cells, packed cell volume and hemoglobin concentration were observed which was suggestive of polycythemia, explaining the ability of animals exposed to this extract to have increased oxygen carrying capacity to the tissues and carbon dioxide transport capacity from the tissues to the lungs. Increase in white blood cells count indicates stimulatory effects of the extract on leucocytosis

which explains the ability of ethanol extract of *Justicia secunda* leaves to improve immune related disease conditions. The presence of some important plant chemicals that improves hematological parameters have been reported in plants such as *viscum album* (mistletoe) and other commonly prescribed medicinal plants (Bendich, 1993; Al-mamary, 2002).This shows that the extract possesses antianemic property.The extract caused significant increase in antioxidant parameters when compared with the anemic control rats. Also the extract treated groups of the rats showed significant decrease in the concentration of lipid perioxidaation parameter (MDA) when the treated groups of rats were compared with the anemic group of rats (positive control group). This indicates that the extract possesses potential antioxidative property which may be attributed to some of the phytoconstituents of the extract such as the flavonoids. The mechanism of the in-vitro antioxidant effects of the extract may be through the scavenging of the reactive oxygen species (ROS) and in vivo may be via the inhibition of the reactive oxygen-generating oxidases, enhancement of the endogenous antioxidant and /or direct inhibition of enzyme that catalyzes oxidation of cellular components. The antioxidant effects of the extract may be responsible for its antianemic activities.

## 4.2 Conclusion

These results showed that the ethanol extract of *Justicia secunda* leavessignificantly reduced the anemic condition in the rats and restored some of the changes in biochemical parameters of the phenylhydrazine-induced anemic rats. Results obtained from the present study indicate that the leaf extract of *Justicia secunda* possesses antianaemic and antioxidant activity and this supports the therapeutic use of the plant in traditional medicine for the treatment of anemia.

### 4.3 Suggestions for further studies

The plant should be investigated for Bioactive compound(s) which should be purified and used for bioassay-directed experinments.

Chronic and subchronic toxicity studies should be carried out.

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# APPENDIX I

**APPENDIX II**

**GET**

**FILE='C:\Users\Documents\CHINEDU\CHINEDU SPSS PHYTOCHEMICAL INPUT.sav'.**

**DATASET NAME DataSet1 WINDOW=FRONT.**

**ONEWAY PHYTOCHEMISTRY BY GROUPS**

**/STATISTICS DESCRIPTIVES**

**/MISSING ANALYSIS**

**/POSTHOC=DUNCAN LSD ALPHA (0.05).**

**Oneway**

**Notes**

| | | |
|---|---|---|
| Output Created | | 20-SEP-2018 12:07:44 |
| Comments | | |
| Input | Data | C:\Users\CHINEDUM\Documents\CHINEDU\CHINEDU SPSS PHYTOCHEMICAL INPUT.sav |
| | Active Dataset | DataSet1 |
| | Filter | <none> |
| | Weight | <none> |
| | Split File | <none> |
| | N of Rows in Working Data File | 48 |
| Missing Value Handling | Definition of Missing | User-defined missing values are treated as missing. |
| | Cases Used | Statistics for each analysis are based on cases with no missing data for any variable in the analysis. |
| Syntax | | ONEWAY PHYTOCHEMISTRY BY GROUPS /STATISTICS DESCRIPTIVES /MISSING ANALYSIS /POSTHOC=DUNCAN LSD ALPHA(0.05). |

| Resources | Processor Time | 00:00:00.31 |
|---|---|---|
| | Elapsed Time | 00:00:00.42 |

[DataSet1] C:\Users\CHINEDUM\Documents\CHINEDU\CHINEDU SPSS PHYTOCHEMICAL INPUT.sav

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean |
|---|---|---|---|---|---|
| | | | | | Lower Bound |
| PHENOL | 3 | 435.6920 | 84.72846 | 48.91800 | 225.2148 |
| POLYPHENOL | 3 | 331.7600 | 65.62167 | 37.88669 | 168.7467 |
| TANINES | 3 | 186.8067 | 154.90808 | 89.43622 | -198.0063 |
| SAPONIN | 3 | 134640.0000 | 48647.97632 | 28086.92222 | 13791.7274 |
| FLAVONIODS | 3 | 13787.6493 | 141.91091 | 81.93230 | 13435.1231 |
| ALKALOIDES | 3 | 2400.3333 | 1806.91247 | 1043.22140 | -2088.2861 |
| CYANIDES | 3 | 19.3093 | 1.45222 | .83844 | 15.7018 |
| TERPENOIDE | 3 | 1383.8240 | 149.99944 | 86.60221 | 1011.2047 |
| STEROIDE | 3 | 146.1000 | 156.50439 | 90.35785 | -242.6785 |
| ANTHOCYANANCE | 3 | 35.4333 | 13.27916 | 7.66673 | 2.4461 |
| CERATENOID | 3 | 22.1240 | 3.68072 | 2.12506 | 12.9806 |
| GLYCOSIDES | 3 | 85.4133 | 14.53318 | 8.39074 | 49.3109 |
| Total | 48 | 9620.5716 | 34294.05544 | 4949.92053 | -337.3841 |

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean |
|---|---|---|---|---|---|
| | | | | | Lower Bound |
| PHENOL | 3 | 435.6920 | 84.72846 | 48.91800 | 225.2148 |
| POLYPHENOL | 3 | 331.7600 | 65.62167 | 37.88669 | 168.7467 |
| TANINES | 3 | 186.8067 | 154.90808 | 89.43622 | -198.0063 |
| SAPONIN | 3 | 134640.0000 | 48647.97632 | 28086.92222 | 13791.7274 |
| FLAVONIODS | 3 | 13787.6493 | 141.91091 | 81.93230 | 13435.1231 |
| ALKALOIDES | 3 | 2400.3333 | 1806.91247 | 1043.22140 | -2088.2861 |
| TERPENOIDE | 3 | 1383.8240 | 149.99944 | 86.60221 | 1011.2047 |
| STEROIDE | 3 | 146.1000 | 156.50439 | 90.35785 | -242.6785 |
| ANTHOCYANANCE | 3 | 35.4333 | 13.27916 | 7.66673 | 2.4461 |

| | | | | | |
|---|---|---|---|---|---|
| GLYCOSIDES | 3 | 85.4133 | 14.53318 | 8.39074 | 49.3109 |
| Total | 48 | 9620.5716 | 34294.05544 | 4949.92053 | -337.3841 |

**ANOVA**

PHYTOCHEMISTRY

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 50535842485.040 | 15 | 3369056165.669 | 22.745 | .000 |
| Within Groups | 4740022718.552 | 32 | 148125709.955 | | |
| Total | 55275865203.592 | 47 | | | |

**Homogeneous Subsets**

**PHYTOCHEMISTRY**

| | GROUPS | N | Subset for alpha = 0.05 | |
|---|---|---|---|---|
| | | | 1 | 2 |
| Duncan[a] | PROTEINS | 3 | 12.1667 | |
| | CYANIDES | 3 | 19.3093 | |
| | CERATENOID | 3 | 22.1240 | |
| | ANTHOCYANANCE | 3 | 35.4333 | |

| | | | |
|---|---|---|---|
| CARBOHYDRATES | 3 | 36.2667 | |
| GLYCOSIDES | 3 | 85.4133 | |
| TOTAL_LIPIDS | 3 | 125.0000 | |
| STEROIDE | 3 | 146.1000 | |
| TANINES | 3 | 186.8067 | |
| RESIN | 3 | 281.2667 | |
| POLYPHENOL | 3 | 331.7600 | |
| PHENOL | 3 | 435.6920 | |
| TERPENOIDE | 3 | 1383.8240 | |
| ALKALOIDES | 3 | 2400.3333 | |
| FLAVONIODS | 3 | 13787.6493 | |
| SAPONIN | 3 | | 134640.0000 |
| Sig. | | .252 | 1.000 |

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 3.000.

| CATALASE | GLUTHATIONE | GSH | GPX | PCV | RBC | WBC | HB | PLATELET | Na | K | Cl | VIT_C | VITE |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 23.41 | 31.30 | 26.82 | 24.38 | 38.00 | 6.00 | 8,600.00 | 13.76 | 142.00 | 155.27 | 0.33 | 0.39 | 0.27 | 1.00 |
| 26.67 | 33.09 | 25.95 | 30.76 | 36.00 | 5.80 | 8,400.00 | 23.07 | 152.00 | 166.81 | 0.44 | 0.47 | 0.29 | 2.00 |
| 21.42 | 32.01 | 27.37 | 29.38 | 36.00 | 5.60 | 8,800.00 | 19.54 | 144.00 | 189.36 | 0.55 | 0.42 | 0.37 | 2.00 |
| 24.12 | 33.27 | 24.93 | 27.57 | 43.00 | 6.20 | 8,000.00 | 16.52 | 140.00 | 147.85 | 0.43 | 0.64 | 0.25 | 1.00 |
| 23.89 | 33.27 | 24.32 | 30.66 | 41.00 | 6.60 | 9,600.00 | 20.61 | 131.00 | 159.89 | 0.42 | 0.59 | 0.29 | 1.00 |
| 20.65 | 31.48 | 27.97 | 31.51 | 35.00 | 6.30 | 10,200.00 | 22.41 | 147.00 | 160.88 | 0.44 | 0.47 | 0.51 | 1.00 |
| 20.01 | 21.76 | 16.28 | 22.67 | 30.00 | 4.60 | 5,600.00 | 15.68 | 110.00 | 131.16 | 0.21 | 0.33 | 0.13 | 2.00 |
| 14.21 | 23.92 | 17.30 | 23.42 | 26.00 | 5.80 | 8,200.00 | 15.05 | 100.00 | 120.70 | 0.31 | 0.34 | 0.19 | 1.00 |
| 16.25 | 21.04 | 22.10 | 21.82 | 29.00 | 5.40 | 7,600.00 | 20.15 | 128.00 | 133.71 | 0.23 | 0.41 | 0.14 | 2.00 |
| 16.24 | 22.12 | 19.53 | 21.39 | 30.00 | 5.20 | 7,600.00 | 20.76 | 112.00 | 119.80 | 0.20 | 0.38 | 0.15 | 0.00 |
| 13.33 | 21.22 | 17.70 | 22.67 | 30.00 | 4.60 | 6,000.00 | 22.15 | 109.00 | 137.80 | 0.31 | 0.33 | 0.13 | 1.00 |
| 13.80 | 20.32 | 20.41 | 23.10 | 28.00 | 4.80 | 7,800.00 | 17.70 | 113.00 | 140.68 | 0.24 | 0.22 | 0.14 | 1.00 |
| 20.66 | 26.80 | 17.50 | 26.61 | 32.00 | 6.40 | 11,200.00 | 24.91 | 144.00 | 160.80 | 0.41 | 0.42 | 0.31 | 2.00 |
| 22.36 | 30.04 | 21.35 | 25.44 | 30.00 | 5.40 | 9,200.00 | 13.06 | 150.00 | 158.71 | 0.40 | 0.52 | 0.30 | 2.00 |
| 21.37 | 30.58 | 22.64 | 25.97 | 35.00 | 5.90 | 10,000.00 | 20.46 | 152.00 | 161.72 | 0.43 | 0.47 | 0.22 | 3.00 |
| 23.26 | 29.68 | 21.62 | 25.01 | 35.00 | 4.90 | 8,200.00 | 19.73 | 128.00 | 149.86 | 0.35 | 0.39 | 0.27 | 2.00 |
| 22.14 | 25.72 | 23.18 | 24.16 | 37.00 | 5.00 | 8,200.0 | 12.5 | 148.00 | 155.96 | 0.44 | 0.42 | 0.30 | 1.00 |

| | | | | | | 0 | 1 | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 20.41 | 30.40 | 23.58 | 27.25 | 34.00 | 4.70 | 8,000.00 | 18.03 | 136.00 | 140.97 | 0.38 | 0.49 | 0.18 | 1.00 |
| 20.21 | 24.46 | 19.87 | 23.74 | 36.00 | 4.80 | 9,200.00 | 19.36 | 128.00 | 147.81 | 0.32 | 0.42 | 0.20 | 3.00 |
| 21.22 | 25.90 | 19.05 | 23.95 | 32.00 | 4.60 | 7,600.00 | 21.86 | 134.00 | 152.70 | 0.30 | 0.37 | 0.21 | 2.00 |
| 20.18 | 25.00 | 18.18 | 23.31 | 31.00 | 5.50 | 7,200.00 | 20.23 | 123.00 | 140.92 | 0.21 | 0.33 | 0.21 | 2.00 |
| 17.66 | 22.48 | 21.76 | 25.55 | 29.00 | 5.80 | 8,800.00 | 21.12 | 120.00 | 131.17 | 0.34 | 0.38 | 0.21 | 1.00 |
| 20.12 | 27.34 | 21.42 | 25.01 | 31.00 | 5.00 | 7,200.00 | 17.68 | 126.00 | 139.86 | 0.34 | 0.24 | 0.17 | 1.00 |
| 18.56 | 26.98 | 22.10 | 24.06 | 30.00 | 5.20 | 8,600.00 | 18.42 | 131.00 | 140.77 | 0.29 | 0.40 | 0.18 | 1.00 |
| 20.13 | 29.14 | 20.95 | 25.76 | 33.00 | 5.80 | 6,400.00 | 18.18 | 128.00 | 147.77 | 0.41 | 0.34 | 0.21 | 1.00 |
| 21.28 | 27.52 | 21.62 | 23.10 | 33.00 | 4.80 | 7,600.00 | 18.07 | 129.00 | 161.78 | 0.37 | 0.35 | 0.16 | 0.00 |
| 20.24 | 26.98 | 23.04 | 26.29 | 36.00 | 4.80 | 9,200.00 | 22.63 | 130.00 | 155.24 | 0.38 | 0.42 | 0.24 | 1.00 |
| 20.76 | 27.70 | 21.49 | 25.33 | 30.00 | 5.00 | 8,000.00 | 17.19 | 110.00 | 140.47 | 0.37 | 0.42 | 0.20 | 1.00 |
| 18.66 | 26.44 | 23.04 | 25.76 | 26.00 | 4.80 | 7,200.00 | 27.89 | 121.00 | 142.45 | 0.39 | 0.39 | 0.22 | 1.00 |
| 18.60 | 25.00 | 24.12 | 21.29 | 30.00 | 5.00 | 8,600.00 | 27.07 | 125.00 | 147.91 | 0.41 | 0.39 | 0.18 | 2.00 |
| 20.28 | 26.44 | 24.32 | 24.59 | 30.00 | 5.80 | 8,800.00 | 25.25 | 127.00 | 150.50 | 0.41 | 0.40 | 0.20 | 1.00 |
| 22.42 | 27.52 | 22.64 | 22.35 | 32.00 | 5.20 | 8,600.00 | 19.87 | 133.00 | 150.69 | 0.37 | 0.37 | 0.18 | 1.00 |
| 22.55 | 26.98 | 19.26 | 25.33 | 30.00 | 5.00 | 7,800.00 | 20.83 | 120.00 | 143.84 | 0.36 | 0.39 | 0.24 | 1.00 |
| 21.39 | 28.78 | 22.23 | 25.87 | 34.00 | 5.30 | 8,400.0 | 18.2 | 125.00 | 151.36 | 0.50 | 0.36 | 0.19 | 0.00 |

## APPENDIX III



| | | | | | | 0 | 5 | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 21.37 | 30.03 | 22.77 | 24.38 | 32.00 | 5.60 | 8,000.00 | 15.82 | 120.00 | 147.77 | 0.32 | 0.40 | 0.18 | 1.00 |
| 20.42 | 28.42 | 21.76 | 27.25 | 36.00 | 5.60 | 7,600.00 | 20.87 | 129.00 | 150.82 | 0.32 | 0.41 | 0.24 | 1.00 |

# APPENDIX IV