A STUDY ON EVALUATION OF OXIDATIVE STRESS WITH SPECIAL EMPHASIS TO XANTHINE OXIDASE ACTIVITY, ANTIOXIDANTS AND PLASMA ELASTASE ACTIVITY IN PRE-ECLAMPSIA

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by

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DECLARATION BY THE CANDIDATE

I **Mrs. Vanishree B**. hereby declare that this thesis entitled **"A study on evaluation of oxidative stress with special emphasis to xanthine oxidase activity, antioxidants and plasma elastase activity in pre-eclampsia"** is an original research work carried out by me for the award of **Doctor of Philosophy** in the subject Biochemistry under the guidance of **Dr. C.D. Dayanand**, Professor, Department of Biochemistry, Sri Devaraj Urs Medical College, a constituent Institute of SDUAHER and co-guidance of **Dr.S.R. Sheela** , Professor and Head, Department Obstetrics and Gynecology, SDUMC. No part of this thesis has formed the basis for the award of any degree or fellowship previously elsewhere.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES

LIST OF ABBREVIATIONS

1	NHBPEP	National high blood pressure education program
2	BP	Blood pressure
3	ACOG	American college of obstetricians and gynecologists
4	NHLBI	National heart lung and blood institute
5	MTHFR	Methylene tetra hydro folate reductase
6	HLA	Human leukocyte antigen
7	ACE	Angiotensin converting enzyme
8	PGI_2	Prostacyclin
9	PLA_2	Phospholipase A_2
10	ROS	Reactive oxygen species
11	DNA	Deoxy ribonucleic acid
12	O_2	Oxygen
13	$HO_2^{\cdot}$	Perhydroxy radical
14	$O_2^{\cdot}$	Super oxide anion radical
15	H_2O_2	Hydrogen peroxide
16	AGE	Advanced glycation end products
17	GSH	Glutathione reduced
18	SOD	Super oxide dismutase
19	GPx	Glutathione peroxidase
20	GR	Glutathione reductase
21	GST	Glutathione-s-transferase
22	G-6-PD	Glucose-6-phosphate dehydrogenase

LIST OF ABBREVIATIONS

23	MDA	Malondialdehyde
24	IL	Interleukin
25	TNF-α	Tumor necrosis factor-α
26	CRP	C-reactive protein
27	hs-CRP	High sensitive C - reactive protein
28	XO	Xanthine oxidase
29	XDH	Xanthine dehydrogenase
30	MPO	Myeloperoxidase
31	GDM	Gestational diabetes mellitus
32	NO	Nitric oxide
33	IMA	Ischemia modified albumin
34	GMCSF	Granulocyte macrophage colony stimulating factor
35	FRAP	Ferric Reducing Ability of Plasma
36	ROC	Receiver operating characteristics curve
37	AUC	Area under curve
38	IUGR	Intra uterine growth restriction
39	WHO	World health organization
40	TAS	Total antioxidant status
41	LHP	Lipid hydro peroxide
42	OSI	Oxidative stress index
43	VEGF	Vascular endothelial growth factor

LIST OF ABBREVIATIONS

44	PIGF	Placental induced growth factor
45	AST	Aspartate transaminase
46	ALT	Alanine transaminase
47	PON	Paraoxanase
48	β-HCG	Beta human chorionic gonadotropin
49	DAM-12	Disintegrin and metalloproteinase
50	kDa	Kilodalton
51	ADMA	Assymetric dimethyl arginine

CHAPTER-1

INTRODUCTION

1.0. INTRODUCTION

1.1. Background

Preeclampsia is a muiltifactorial disorder characterized by hypertension, proteinuria and edema after twenty weeks of gestation[1]. The symptoms of preeclampsia can range from mild to severe which includes persistent headache, blurred vision, vomiting and abdominal pain[2]**.** The complications of preeclampsia are a major cause of fetal uterine growth retardation, preterm delivery, maternal and prenatal morbidity and mortality[3,4]. It is a metabolic syndrome that involves endothelial dysfunction and activation, systemic inflammatory response, oxidative stress, insulin resistance and dyslipidemia. Potential causes and mechanisms behind preeclampsia remain unknown, but the involvement of maternal, immune, genetic factors and placenta have been implicated.

1.2. Epidemiology

Preeclampsia is a major cause of maternal and fetal morbidity and mortality worldwide, complicating up to 10% of the pregnancies. The mortality rate of preeclampsia in the developed and developing countries varies. Approximately 800 women die from pregnancy and child birth related complications around the world every day[5]. Women in developing countries expected to have seven fold chances of preeclampsia than developed countries. However, from this data nearly 10-25% of preeclampsia cases results in maternal death[6]. In Asia and Africa, nearly one tenth of maternal deaths are associated with hypertensive disorders of pregnancy.

In the hypertensive disorders, preeclampsia and eclampsia have huge impact on the maternal and fetal morbidity and mortality. In Indian scenario, preeclampsia and

eclampsia accounts for 24% of the maternal deaths [7]. The incidence of preeclampsia alone in India is reported to be 8-10% among the pregnant women. It usually occurs during the second half of the pregnancy. Pre-eclampsia is more common in primigravida women than the second or later pregnancies [8,9].

1.3. Criteria for the diagnosis of preeclampsia by World Health Oganization

According to the WHO 2011 report, onset of new episode of hypertension during pregnancy characterized by persistent hypertension with diastolic blood pressure ≥ 90 mm Hg and substantial proteinuria > 0.3g / 24hrs [10].

1.4. Definition of preeclampsia by American College of Obstetricians and Gynecologists (ACOG)

In a women with previously normal blood pressure (BP) having greater than or equal to 140 mmHg systolic or greater than or equal to 90 mm Hg diastolic on two occasions at least 4 hours apart after 20 weeks of gestation accompanied by significant proteinuria greater than or equal to 300 mg/ 24 hours urine collection or protein: creatinine ratio greater than or equal to 0.3. In the absence of proteinuria, thrombocytopenia with platelet count less than 1,00,000/ml, renal insufficiency with serum creatinine >1.1 mg/dl and impaired liver function with elevated liver transaminases to twice the normal concentration [11].

1.5. Revised definition of preeclampsia by ACOG

New American College of Obstetricians and Gynecologists guideline describes about the diagnosis of preeclampsia that does not require the detection of high amount of proteinuria. Since, the problem with kidney and liver can occur without the signs of proteinuria was evident and also the amount of protein in urine does not reflects severity and the progress of the disease. Preeclampsia is now to be diagnosed by persistent high

blood pressure that develops during pregnancy or during the postpartum period that is associated with a lot of protein in the urine or the new development of decreased blood platelets, trouble with the kidney or liver, fluid in the lungs, or signs of brain trouble such as seizures and/or visual disturbances[12].

1.6. Classification of Hypertensive disorders of pregnancy

According to the National high blood pressure education program of National heart lung and blood institute classified hypertensive disorder as below.

1. **Gestational Hypertension**:

   Charecterised by Systolic blood pressure ≥140mm Hg or diastolic BP ≥ 90 mmHg for the first time during pregnancy, no proteinuria, BP returns to normal before 12 weeks of postpartum.

2. **Preeclampsia**:

   Minimum criteria are BP ≥140/90 mmHg after 20 weeks of gestation, Proteinuria ≥ 300 mg/24 hours or ≥ 1+ dipstick. Increased certainty for preeclampsia is BP ≥160/110 mm Hg, proteinuria ≥ 2.0 g/24 hours or ≥ 2 + dipsticks, serum creatinine ≥1.2 mg/dl, platelets <1,00,000/μL, increased lactate dehydrogenase, elevated serum transaminases, persistent headache or other cerebral or visual disturbances along with persistent epigastric pain.

3. **Eclampsia**:

   Manifestation of convulsions or coma unrelated to other cerebral conditions with signs and symptoms of preeclampsia.

4. **Super imposed preeclampsia on chronic hypertension**:

New onset of proteinuria ≥300 mg/24 hours in hypertensive women but no proteinuria before 20 weeks of gestation but after that there is a sudden increase in proteinuria or BP or decrease in platelet count <1,00,000/µL .

5. **Chronic hypertension**:

Occurrence of BP ≥140/90 mmHg before pregnancy or before 20 weeks of gestation not attributable to gestational trophoblastic disease or hypertension first diagnosed after 20 weeks gestation and persistent after 12 weeks of postpartum[13].

1.7. Etiology

Preeclampsia commonly develops during second half of the pregnancy and remits after delivery which suggests that the placenta has a critical role. It is a two stage disorder, which comprises poor placentation characterized by faulty trophoblastic vascular remodeling of uterine arteries that causes placental hypoxia. In the later stage, placental oxidative stress releases placental factors in to maternal circulation that results in systemic inflammatory response and endothelial cell activation [14, 15.]

A number of mechanisms have been proposed to answer the exact cause of multifactorial disorder of pregnancy. Possible mechanisms may consist of abnormal trophoblastic invasion of uterine vessels during implantation, circulating angiogenic factors, immunological factors between feto-maternal interactions and endothelial cell activation, lipid peroxides, genetic and nutritional factors.

Normal placentation involves extensive implantation of spiral arterioles which were invaded by endovascular trophoblast that replaces vascular endothelial and muscular linings leading to the enlargement of the vessel diameter. Abnormal placentation involves incomplete trophoblastic shallow invasion which fails to replace

vascular endothelial cell and muscular linings. The defective trophoblastic invasion leads to the constriction of spiral arteries and diminish the vessel diameter compare to normal placentation. Thus, leads to the varied oxygenation in placenta generating free radicals and oxidative stress. As s compensatory mechanism to hypo perfusion and oxidative stress, placenta releases factors in to the circulation which may results in systemic alterations[16, 17].

Ischemic placenta contributes to the endothelial dysfunction by altering circulating levels of angiogenic and antiangiogenic factors. Endothelial cell dysfunction may be also due to activated leukocytes by cytokines which adds to the oxidative stress and destructs the endothelial cells, thus decreases nitric oxide availability [18].

In preeclampsia feto-maternal and paternal antigenic and antiangiogenic interactions causes immunological intolerance by releasing placental factors in to the circulation. Preeclampsia is a polygeneic disorder comprising seventy candidate genes. Seven of them are widely studied for their association with preeclampsia syndrome. Methylene tetrahydrofolatereductase (MTHFR) gene coding for methylene tetrahydrofolatereductase in association with vascular disease, FV (Leiden) gene coding for factor V leiden with thrombophilia, angiotensinogen (AGT) gene for angiotensinogen in association with hypertension, human leukocyte antigen gene (HLA) codes for human leukocyte antigens in immunity, nitric oxide synthase 3 gene coding for nitric oxide synthase in vascular endothelial function, F2 gene coding for prothrombin in coagulation and angiotensin converting enzyme (ACE) gene coding for angiotensin-converting enzymes in blood pressure regulation. The nutritional factors have small effects in lowering consequences of preeclampsia[19,20].

At present, the relation between placental hypoxia, oxidative stress and maternal vascular dysfunction remains elusive but speculated to be a reaction to either placental derived or humoral factors released in to the maternal circulation.The exact mechanism by which these humoral factors induce the maternal vascular endothelial dysfunction is indefinite and open ended.

1.8. Pathophysiology of preeclampsia

Although exact cause of preeclampsia is unknown, evidences on placenta playing a role in the pathophysiology have become significant. Shallow endovascular cytotrophoblast invasion in spiral arteries, inappropriate endothelial cell activation and an exaggerated inflammatory response are key features in the pathogenesis of preeclampsia. Pathological placental specimen in preeclampsia shown infarcts by ischemia, occlusion of spiral arteries and failure of vascular remodeling of spiral arteries by trophoblastic cells. This is life threatening for mother and fetus by vasospasm, endothelial dysfunction and ischemia. As a consequence of possible etiological factors, several disturbances in the mother is reason for preeclampsia, which includes changes in hemodynamic, cardiovascular, hematological, endocrine, liver, kidney functions, cerebrovascular, neurological and visual activity [21].

Placental unknown factors secreted into maternal circulation provoke endothelial activation and results in impaired endothelial function which leads to the clinical symptoms of preeclampsia. Intact endothelium has anticoagulant properties that prevent platelet aggregation by increasing bioavailability of nitric oxide and enhanced fibrinolysis by generation of tissue plasminogen activator. *In vivo* reactive oxygen species damages vascular endothelium which decreases the production of nitric oxide and promotes coagulation process and increases the sensitivity to vasopressors [22].

Phospholipase A_2 (PLA_2) mediates the synthesis of prostacyclin (PGI_2) in endothelial cells play major role in causing vasoconstriction in preeclampsia by decreased synthesis. There by PLA_2 activity is important for endothelial normal function[23].

Free radicals generated during oxidative stress implicated in promotion of maternal vascular malfunction by affecting endothelial cells. Oxidative stress is prominent when the balance between the reactive oxygen species (ROS) prevails the antioxidant capacity of the target cell. Thus, altered equilibrium leads to tissue injury and damages cellular biomolecules. Therefore, a homeostasis between oxidants and antioxidants is crucial in health and disease.

1.9. Oxidative stress

Oxidative stress results when the balance between the generations of reactive oxygen species (ROS) overrides the antioxidant capacity of the target cell. The disturbance in balance between the production of ROS and antioxidant defense may lead to tissue injury. The formation of oxidative stress may result in damage to critical cellular macromolecules including carbohydrates, lipids, proteins and DNA. In most cases free radicals are secondary to the disease process, but in some instances free radicals are causal [24]. Virtually all diseases involve free radicals, but there is a delicate balance between oxidants and antioxidants in health.

1.10. Free Radicals

Free radicals are chemical species capable of independent existence that contains an unpaired electron and highly reactive in nature [25]. They are continuously produced in the cells either by aerobic metabolism or as accidental by-products of metabolism,

detoxification process and phagocytosis. The most important active reactants include ground state oxygen (O_2), Super oxide anion radical ($O_2^{\cdot}$), hydroxy radical ($OH^{\cdot}$), perhydroxy radical ($HO_2^{\cdot}$) and hydrogen peroxide (H_2O_2) etc. Although, super oxide is a free radical which is not considered as damaging species, but at low pH value super oxide will promote formation of protonated free radical as perhydroxyl radical which is more reactive. The hydroxyl radical is an extremely non-diffusing reactive oxidizing radical that damages most biomolecules within a small radius. Other free radicals of importance are carbon centered radicals ($R^{\cdot}$) that arise from the attack of oxidizing radical ($OH^{\cdot}$) on biological molecule (RH) such as carbohydrate, lipid, protein and nucleic acids. The production of free radicals occur either by the loss of single electron from the molecule

Radical formation by electron transfer: $A + e^{-} A^{-} \longrightarrow \bullet$

or by homolytic cleavage of covalent bond of a normal molecule that retains each fragment with a single electron from the paired electrons

Radical formation by homolytic fission: $X{:}\ Y \longrightarrow X + Y$

Ion formation by heterolytic fission: $X{:}\ Y \longrightarrow X^{-\bullet} + Y^{+\bullet}$

In vivo, the formation of free radical takes place by any of the above mechanism under unusual circumstances by the influence of ionizing radiation and redox reactions of transition metal ions. Electron leakage from the mitochondrial respiratory chain, endoplasmic reticulum, flavin oxidases of peroxisomes, transition metal dependent autoxidation of L-ascorbic acid and sulphur containing compounds, flavin coenzymes and adrenalins etc which turn down the scavenging action of antioxidant. Detoxification of certain foreign toxic compounds such as carbon tetra chloride has overwhelming oxidative stress that supersedes the antioxidant defense which results in the oxidative

destruction of cellular membrane and thus finally results tissue damage which are implicated in many human diseases [26].

1.11. Reactive oxygen species

These are oxygen containing molecules that have a higher reactivity than the ground state oxygen. High amount and/or inadequate removal of reactive oxygen species (ROS) results in oxidative stress, which may cause damage to biological macro molecules[27]. There are many different sources by which the ROS are generated. Exogenous sources include exposure to cigarette smoke, emission from automobiles and industries, excess alcohol, asbestos, exposure to ionizing radiation and bacterial, fungal or viral infections. Endogenous sources of ROS includes as by-products of normal and essential metabolic reactions such as energy generation from mitochondria or the detoxification reactions involving the liver cytochrome P-450 enzyme system.

1.12. Mechanisms of the toxic influence of reactive oxygen species

Mechanisms of the toxic influence of ROS are causing damage to important biological structures through membrane lipid per oxidation of polyunsaturated fatty acids [28], covalent binding to proteins that alters intracellular free calcium homeostasis and DNA damage by chain break [29, 30]. DNA is an important target of ROS related to aging, inflammation and cell transformation etc. Similarly, oxidation by ROS results in modification of amino acid side groups in protein [31].

Damage to lipids and proteins from free radicals is of great clinical significance as it causes irreversible structural and functional damages to these molecules. This oxidative stress is attributed as a cause for several molecular and cellular damages in a spectrum of human disease. Generally, in living systems the damaging effects of the oxidative status is alleviated through effective antioxidant mechanisms[32]. However,

reduced antioxidant and over production of free radical could arise from prolonged malnutrition or due to the extended drug intake and other disease conditions. Carbonyl groups such as aldehydes and ketones were produced on protein side chains especially of proline, arginine, lysine, and threonine when they were oxidized. These moieties are chemically stable, which is useful for both their detection and storage [33].

1.13. Oxidative stress and preeclampsia

Placental trophoblasts and endothelial cells constitute the placental barrier which effectively separates the fetal and maternal circulation. Elevated oxidative stress causes tissue damage and inflammation which alters the barrier and causes leakage of fetal and placental derived factors in to the maternal circulation [34].

The effect of oxidative stress at cellular level described by the degree of placental insufficiency due to inadequate implantation of spiral arteries has been proposed to influence the extent of cellular oxidative and endoplasmic reticulum stress. Thus oxidative stress disturbs the normal redox state of the cell and brings toxic effects on the cellular components which results in apoptosis [35]. Due to high reactive nature of reactive species and its influence on endoplasmic reticulum triggers unfolded protein response pathway in placental cell. This results in accumulation of abnormally folded proteins with high turnover accounts to cell death and has become basis of pathogenesis of preeclampsia [36, 37].

The effect of oxidative stress at systemic level occurs by inflammation process, which is mediated through the localization of leukocytes, pro-inflammatory cytokines, adhesion molecules, chemokines and heme. Increased superoxide production in oxidative stress binds to nitric oxide and limits its availability, which results in an altered endothelial function and also inflammatory process. This elevated systemic oxidative

stress responsible for the release of substances from placenta into maternal circulation in preeclampsia [34].

1.14. Antioxidants

These are the substances which will significantly delay or inhibit oxidation of an oxidizable substrate[33]. They act as defense mechanisms against the free radicals. They may be extra cellular antioxidants such as albumin, uric acid, ferritin and ceruloplasmin etc and cellular antioxidants such as glutathione (GSH), L-ascorbic acid and α-tocopherol and enzyme antioxidants. Primary enzyme antioxidants include super oxide dismutase (SOD), catalase, and glutathione peroxidase (GPx). Secondary enzyme antioxidants include glutathione reductase (GR), glutathione-s-transferase, glucose-6-phosphate dehydrogenase (G-6-PD).

According to their mode of action, antioxidants are classified as scavenging antioxidants which prevent oxidative damage by scavenging radical as they form.

1. *Preventing antioxidants:* They are largely protein in nature, which acts by sequestering transition metal ions.
2. *Enzyme antioxidants:* These antioxidants act by catalyzing the oxidation of other molecules.

Membrane lipid peroxidation markers, protein markers and markers denoting nucleic acid damage are increased in plasma of preeclamptic women. Defensive role of nutrient antioxidants such as carotenoids, α-tochopherol, L- ascorbic acid and enzyme antioxidants minimize the oxidative damage by virtue of their capacity to scavenge free radicals generated during cellular metabolic process [38]. Karacy et al. reported the oxidative stress in terms of malondialdehyde (MDA) and antioxidants in preeclampsia[39].

In the present study, we measured xanthine oxidase enzyme activity and compared with other oxidative stress parameters and antioxidants in preeclampsia. The possibility of considering xanthine oxidase as an enzyme oxidant marker along with other oxidative stress markers in pre and post-delivery of preeclampsia, as well as to obtain the baseline data from normotensive pregnant in comparison with non pregnant control has become the requisite and purpose of the study [40].

1.15. Inflammation

Inflammation is the body's immediate response to the damage of its tissues and cells by pathogens, noxious stimuli or physical injury. Acute inflammation is short response which results in healing. Chronic inflammation is prolonged, deregulated active inflammation which involves tissue destruction and attempts tissue repair. Acute inflammation is triggered by infection or tissue injury that involves the liberation of plasma leucocytes to the sites of injury or infection. The initial recognition of infection is by macrophages and mast cells leading to the production of variety of inflammatory mediators including cytokines, chemokine's, vasoactive amines and products of photolytic cascade. Plasma proteins and leukocytes are normally restricted to blood vessels which gains access through post capillary venules to the extra vascular tissues at the site of infection or injury. The activated endothelium of blood vessels allows extravasations of neutrophils. Neutrophils attempt to kill the invading agents by releasing toxic contents which include reactive oxygen species, reactive nitrogen species, proteinases, cathepsins and elastase [41].

An inflammatory response is usually accompanied by increase in concentration of inflammatory cytokines, acute phase proteins which may involve leukocyte

activation. The activation of neutrophils may occur in the presence of cytokines. Elastase is the enzyme used to measure neutrophil activation [42].

Preeclampsia is influenced by several factors such as activated coagulation process, platelets, neutrophils and inflammatory cells. Thus inflammatory status was generally measured by cytokines such as interleukin-6 (IL-6), IL-1α, IL-1β, tumor Necrosis Factor- α (TNF-α) and C-reactive protein (CRP) etc [43].

Neutrophil elastase gene located on chromosome 19 at p13.3. It is a serine protease (E.C No.3.4.21.37) consisting of 218 amino acid residues with a molecular weight 29.5kDa. The biological role is degradation of collagen IV and elastin causing inflammation [44].

High sensitive C- reactive protein (hs-CRP) is a plasma globulin fraction synthesized in liver during acute inflammation phase as a response to pneumococci based infection where it binds to carbohydrate moiety of the capsule of bacteria. Circulating levels of CRP in the range of 0.5-10 mg/L is considered as hs-CRP. The concentration between 10-1000 mg/L is considered as CRP which is an indicator of inflammation. CRP is nonspecifically rises to wide variety of disease and inflammatory process considered as standard marker of inflammation. CRP is an α-globulin with a molecular weight of 11-14 kDa and composed of five identical subunits which were non-covalently assembled as a cyclic pentamer. It is synthesized in liver and normally present in the concentration of less than 0.3 mg/dl. Its increase in serum is nonspecific to particular disease [45].

Phospholipase A_2 gene located on chromosome number 1 at q31.1. It is heat stable, calcium dependent enzyme (E.C No.3.1.1.4) with Molecular weight 85 kDa.

Phospholipase A_2 enzyme cleaves arachidonic acid at Sn-2 position in phospholipids that has become the precursor of leukotrienes synthesis which serves as inflammatory mediators [46].

The nomenclature of phospholipases is based on the cleavage of specific bond in membrane phospholipids. They are phospholipase A_1 (PLA_1) which cleaves at Sn-1 position of acyl chain, phospholipase A_2 (PLA_2) which cleaves at Sn-2 position of acyl chain and releases arachidonic acid, Phospholipase B which cleaves Sn-1 and Sn-2 of acyl group, phospholipase C which cleaves the site before the phosphate bond and releasing diacylglycerol and a phosphate containing group and phospholipase D which cleaves the site after phosphate group and releases phosphatidic acid [47].

1.16. Xanthine oxidase

Xanthine oxidase (E.C 1.17.3.2) is an iron-molybdenum containing flavoprotein catalyzes the oxidation of xanthine/hypoxanthine into uric acid (2, 6, 8, trioxy purine) and hydrogen peroxide. Xanthine dehydrogenase (E.C.1.17.1.4) is NAD+ dependent and xanthine oxidase uses oxygen. However, limited proteolysis and oxidation of sulfhydryl groups converts irreversibly xanthine dehydrogenase in to xanthine oxidase [48]. Placental incompatibility in ischemic condition due to free radical formation and increased oxidative stress makes endothelial damage and cell death that leads to elevated activity of xanthine oxidase in circulation.

Inadequacy of placental perfusion might result in hypoxia. This hypoxic interface between maternal-fetus results in destruction of placental tissue which leads to increased xanthine oxidase activity [49]. Activated leukocytes produce cytokines which in turn

increases the xanthine oxidase activity and production of uric acid [50, 51]. Xanthine oxidase levels are very less in healthy individuals but have shown to be increased in the pathological conditions[52]. Pre-eclampsia occurs only in the presence of placenta and resolves after placental delivery. The main hypothesis depends on the decreased placental perfusion due to impaired remodeling of spiral arteries.

1.17. Screening of xanthine oxidase inhibition

Xanthine oxidase is associated with pathological conditions involving inflammation, metabolic disorders, reperfusion damage and hypertension etc. Hence, xanthine oxidase is well characterized as a drug target for the treatment and management of diseases involving high enzyme activity. At present, allopurinol is the mainly used inhibitor of xanthine oxidase [53]. Still, it includes rare adverse effects such as bone marrow depression, hepatotoxicity and Stevens-Jones syndrome characterized by skin rashes, which were collectively known as allopurinol hypersensitivity syndrome have been reported especially in patients with renal insufficiency. Therefore, there is a need to develop new effective, less toxic and more affordable inhibitors.

The potential of plants to yield new therapeutic agents has motivated extensive investigation in screening natural xanthine oxidase inhibitors. Among them, flavonoids is a kind of natural product have increasing attention due to their low toxicity and various activities including antioxidation,anticancer,antiallergic,inhibition of advanced glycation end products (AGE) and cardio-cerebrovascular protection [54].

Traditional system of medicine comprises variety of plants and their bioactive compounds. The expeditions in search of medicinal values of plants are continuous

process in a quest for detection of newer phytochemical biomolecules for the therapeutics. Plants exhibit rich source of pharmacologically active compounds such as phenolics, tannins, flavonoids, lignin etc [55]. These active components distributed in various parts of the plants possess health related effects like antimicrobial, anti-mutagenic, anti-carcinogenic, anti-inflammatory, vasodilatation and enzyme inhibitory activity [56]. Hence in the present study, we used flavonoid extracts from the Indian conventional plants such as *Pongamia pinnata Linn (Leguminoseae*), *Mangifera indica Linn (Anacardiaceae*), *Morinda citrifolia Linn (Rubiaceae*) and *Zingiber officinale Roscoe* (*Zingiberaceae*) which were reported to have anti-inflammatory and xanthine oxidase inhibitory activity.

Pongamia pinnata L is an angiosperm commonly known as Indian beach tree belongs to family *Fabaceae*. This plant is known to have anti plasmodium characteristics, anti-inflammatory, antiulcer, antidiarrheal and wound healing, hypoglycemic, antibacterial and anticonvulsant characteristics [57].

Morinda citrifolia L is also known as Indian mulberry or noni belongs to family *Rubiaceae*. Different parts of this plant used as antioxidant, anti-inflammatory, anti-hypertensive, anti-diarrheal, anti-thrombotic, xanthine oxidase inhibitory, hypoglycemic and anti-cancer agents etc [58, 59].

Mangifera indica L is known as mango belongs to family *Anacardiaceae*. The different parts of the plants used as remedial measure for infection, hypertension, insomnia, asthma, rheumatism, hemorrhage and anemia [60, 61].

Zingiber officinale Roscoe is commonly known as ginger belongs to family *Zingiberaceae*. The rhizome of this plant known to contains zingiberene and gingerols.

Precisely 6-gingerol is a constituent responsible for pungent taste. It is used as antipyretic, analgesic, hypotensive, anti-platelet aggregation and thromboxane synthesis[62].

Flavonoids are phytochemical compounds widely distributed in plants and permeate into the human diet through beverages, vegetables, fruits, grains, tea, and wine and other plant derived foods. Flavonoids are characterized by a basic C6−C3−C6 carbon skeleton. Due to their relationship with some health effects, flavonoid rich products have become increasingly popular. Consumption of these dietary supplements may result in higher supply of flavonoids than those ingested with a normal diet. However, whether the enhanced intake will bring beneficial effects on human health are to be determined.

Hence, this has become subject of active research to screen for natural ingredients from plants for therapeutic benefit. Therefore, flavonoids have attracted increasing the attention due to their less toxic property and beneficial actions.[63] The flavonoid family has been reported to possess a high potential for inhibition of Xanthine oxidase. Few studies showed that, the planar structure and the C2=C3 double bonds of flavonoids are advantageous for binding to Xanthine oxidase and its inhibition [64]. Therefore, selected plants were subjected for the isolation of flavonoids and to determine the extent of Xanthine oxidase inhibition using commercially available standard flavonoid quercetin.

A research report of Chappell LC et al. stated that risk of preeclampsia by reducing oxidative stress can be achieved by the supplementation of the α-tocopherol {(2R)-2, 5, 7, 8-tetramethyl -2-[(4R,8R)-(4,8,12-trimethyltrideacyl)-6-chromanol} and

L-ascorbic acid {2-oxo-L-threo-hexono-1,4-lactone-2,3-enediol}[65]. WHO 2011 report stated that supplementation of vitamin C and E has no role in decreasing preeclampsia complications[10]. Yet an attempt was made to find out *in vitro* Xanthine oxidase inhibition by using vitamins C & E in comparison with competitive inhibitor allopurinol {1,5-dihydro-4H Pyrazolo [3,4-d] pyrimidin - 4- one}.

1.18. Lacunae of the knowledge

Preeclampsia is a disease of placental origin and has become challenging to health care system. This is a leading disorder which has more impact on maternal and perinatal morbidity and mortality. The pathophysiology involves impaired placentation, inflammation and vascular endothelial damage. The early understanding of preeclampsia is much needed and important goal in obstetrics for the management of preeclampsia. More research is going on for improvement of maternal and fetal outcome.

Several biomarkers have been extensively studied for the prediction and diagnosis of preeclampsia which perhaps useful in disease understanding. General screening strategies for biomarkers consist of methods with sensitivity, specificity, cost effectiveness, patient acceptability and quality maintenance. The suggested markers for the prediction or detection of preeclampsia are fms-like tyrosine kinase-1, soluble endoglin, placental protein 13 , pregnancy-associated plasma protein A, inhibin A, P-selectin, activin A, pentraxin 3, cell-free fetal DNA, disintegrin and metalloproteinase 12 (DAM-12) , β human chorionic gonadotropin (β-HCG) , 2-Methoxyestradiol [66, 67]. But till date, single or combinations of biomarkers were not useful in the diagnosis of preeclampsia due to its low predictive value. Hence, large prospective studies are

required to understand the etiology of preeclampsia clearly. Therefore , current research study is prioritized to find out the relationship between oxidative stress, antioxidants and inflammation parameters in preeclampsia and also determining the same parameters during post-delivery conditions within 48 hours has become the newer aspect of the study.

Xanthine oxidase activity during before and after delivery in preeclampsia is not documented. Furthermore, increased uric acid may be due to elevated xanthine oxidase activity or decreased renal excretion has to be established. Xanthine oxidase is well characterized as a drug target for the treatment and management of diseases involving high enzyme activity. At present, allopurinol is the inhibitor of xanthine oxidase [55]. But due to its side effects there is a need to develop new inhibitor. However, flavonoids have been reported to possess a high potential for inhibition of xanthine oxidase and vitamin supplementation showed contradictory result in decreasing oxidative stress [66]. Therefore, i*n vitro* screening for xanthine oxidase inhibition using flavonoids extracted from selected Indian conventional plants and vitamin C, vitamin E might be useful therapeutics by reducing of oxidative stress in preeclampsia.

Placental oxidative stress, inflammation, endothelial dysfunction play a significant role. Although, considerable amount of research reports on inflammatory response [14], leukocyte activation [68], dyslipidemia [69], oxidative stress and intrauterine hypoxia[70]as potential cause for generation of free radicals and inflammatory mediators facilitating to understand the etiology. The inconclusive and contradictory findings about the inflammatory cytokines challenge the role of inflammation as the main cause of preeclampsia. Hence, considering plasma elastase activity may help to understand the

impact of this enzyme on host tissues and phospholipase A_2 activity in representing imbalance between biological actions of prostacyclin and thromboxane in preeclampsia. These two enzymes activities are going to be compared with hs-CRP a well-established inflammatory marker in clinical practice. In addition to these, obtaining information on oxidants, antioxidants and inflammatory parameters before and after delivery within 48 hours serves as research gap and need of the current research.

CHAPTER-2

REVIEW OF LITERATURE

2.0. REVIEW OF LITERATURE

2.1. Xanthine oxidase and uric acid in preeclampsia

In 1996, Many A and his co-workers reported in a comprehensive review about an association between preeclampsia and hyperuricemia. The exact reason for increased concentration of serum uric acid either due to reduced renal uric acid handling or by increased production was not illustrated. The study hypothized that oxidative stress induced trophoblastic cell destruction also contributes to hyperuricemia. However, the same study measured uric acid level but not documented xanthine oxidase activity [71].

In 2003, Yassaee F in a brief report presented measurement of serum uric acid as prognostic factor for determining the prenatal outcome of preeclampsia. The maternal and fetal complication of preeclampsia was high with respect to cases containing more than ≥6mg/dl. Even though, this report describe hyperuricemia is responsible for determining associated complications in preeclampsia but failed to explain exact mechanism for uric acid elevation[72].

In 2013, Anjum Sayyed and Alka Sontakke conducted a study on lipid peroxidation and antioxidants levels in preeclampsia. They reported serum levels of MDA, uric acid, vitamin C, E and super oxide dismutase in preeclampsia. Study showed that elevated uric acid is the reflection of placental debris which serves as rich sources of purines. The decreased vitamin C&E, in the report reflected to combat free radical stress. Though, the study limits in the assessment of endothelial dysfunction and xanthine oxidase activity[73].

In 2013, Masaharu Murata and co-workers conducted a study on oxidative stress and its effect on apoptosis of trophoblastic cell cultured by using chorionic membrane. They reported xanthine oxidase catalyzes the oxidation of xanthine and hypoxanthine into uric acid and hydrogen peroxide. The study supported increased trophoblastic cell destruction by reactive species and expression of xanthine oxidase activity is the underlying cause of pathophysiology of preeclampsia[74].

In 2014, Periera KN and his co-workers conducted a study to find an association between serum uric acid and severity of preeclampsia. They observed inverse relationship between uric acid, systolic blood pressure with weight of the new born. Study results were similar with other previous research reported relationship between hyperuricemia and pregnancy outcomes but lacks information on possible mechanism [75].

2.2. Free radicals and Antioxidants in Preeclampsia

In 1998, Morris JM et al. conducted a prospective study on plasma oxidative stress markers such as 8 epi-prostglandin F2-α, lipid hydro peroxides, malondialdehyde and vitamin E levels in normal pregnancy and preeclampsia. Study focused on search of new circulating plasma markers in better understanding of the disease at the early stages. However, in this study, evidences on placental biomarkers and vascular endothelial dysfunction are not emphasized [76].

In 1999, Riza Madazli and co-workers in a cross sectional study reported association of lipid peroxidation marker, vitamin C and E in preeclamptic women with diastolic blood pressure. Results of the study showed inverse relationship between oxidative stress and nutrient antioxidants. Study has not furnished any data that can

denote the direct involvement of placental pathophysiology and the activity of circulating enzyme antioxidants [77].

In 2001, Cuilin Zhang et al. conducted a case control study on plasma concentrations of carotenoids (α-carotene, β-carotene, lycopene, lutein, zeaxanthin, and β-cryptoxanthin), vitamin A and vitamin E in normal pregnancy and preeclampsia. They observed negative correlation between plasma retinol and risk of preeclampsia. Unlike other studies they showed positive relationship between vitamin E and preeclampsia which is in contradictory with other studies. However, the study lacks the information on oxidative stress, trophoblastic dysfunction, endothelial dysfunction markers and enzyme antioxidants in preeclampsia [78].

In 2004, Sheikh Nazrul Islam and his co-workers conducted a cross sectional case control study to find the serum concentrations of vitamin A, C & E in preeclampsia associated with blood pressure. They observed no difference of vitamin A and E in normal pregnancy and preeclampsia. But there was considerable increase in vitamin C level in preeclampsia but the reason for the same was not clear. Even though, this study has not described relation of vitamins with oxidative stress parameters and antioxidants enzymes [79].

In 2006, Sharma JB and his co-workers measured the levels of glutathione peroxidase, superoxide dismutase along with malondialdehyde in normal pregnancy and preeclampsia. They categorized SOD and GPx under oxidative stress markers which were in contradictory with various reports besides reporting the unaltered vitamin C and decreased lycopene under antioxidants [80].

In 2008, Mohd Suhail and his co-workers measured altered balance between oxidants and antioxidants along with its impact on osmotic fragility of erythrocytes. The oxidative stress markers in were MDA, oxidized glutathione and antioxidants such as GSH, uric acid, SOD, GPx, GR and catalase activity along with erythrocyte osmotic fragility in preeclamptic patients with normal controls. They observed decreased SOD, GPx, GR activities and elevated levels of MDA. Increased catalase activity may be a compensatory regulation in response to increased oxidative stress. The increased catalase activity was a futile effort to counter act the over production of ROS and providing relief to increased oxidative damage in preeclampsia. They concluded that there was increased oxidative stress in preeclampsia along with decreased glutathione and increased oxidative stress which were linked to osmotic fragility of erythrocytes. However, this study included less number of sample size, lack of follow up and no information was available after delivery [81].

In 2010, Vanitha Gowda and co-workers conducted a cross sectional study to determine the extent of oxidative stress in preeclampsia by lipid peroxidation marker MDA with ceruloplasmin, uric acid and blood pressure. They observed increased circulating levels of these parameters in antenatal and post natal period. But this study did not explain the exact category to which ceruloplasmin fit in to oxidants, antioxidants or inflammation [82].

In 2010, Ozlem Karacy et al. reported plasma total antioxidant status (TAS), lipid peroxidation marker (MDA), protein oxidation marker, advanced oxidation protein products (AOPP), myeloperoxidase (MPO) and lipid hydro peroxide(LHP) in preeclampsia, gestational diabetes mellitus (GDM) patients and compared with

non-complicated normotensive pregnancies between 24 and 36 weeks of gestation. They stated that increased oxidative stress and decreased antioxidant defense system may contribute to disease process both in gestational diabetes mellitus and preeclampsia. But this study included less number of samples and measured total antioxidant status [39].

In 2012, Hawkins T and co-workers in a cohort study reported a relationship between hyperuricemia with maternal and fetal outcomes in preeclampsia as well as gestational hypertension. This study was in accordance with several other research reports which presented hyperuricemia in women with hypertensive pregnancy. Although, study did not present the exact cause for elevated uric acid and its relationship with other oxidative stress markers. However, uric acid is a measure of renal tubular function but its diagnostic utility in the assessment of severity of preeclampsia needs to be ascertained [83].

In 2012, Miranda Guisado ML et al. conducted a study to establish the markers of lipid peroxidation and antioxidant defense in hypertensive disorders of pregnancy. They measured MDA, oxidized LDL, SOD, GPx, catalase, glutathione (reduced and oxidized) form and nitric oxide in gestational hypertension, chronic hypertension and preeclampsia in comparison with normal pregnant women. They observed decreased SOD, reduced glutathione and elevated catalase, GPx, GR and oxidized glutathione in gestational hypertension, chronic hypertension and preeclampsia when compared to normal pregnancy. Nitric oxide levels were higher in preeclampsia. They conclude that increase in oxidative stress coupled with decrease in antioxidant system was seen not only in preeclampsia but also hypertensive disorders of pregnancy [84].

In 2013, Malinova M and Paskaleva V measured selenium and glutathione peroxidase in normal pregnancies and preeclampsia. Decreased selenium and its association with decreased GPx activity observed in preeclampsia suggested the need of selenium for antioxidant function. Hence, they hypothesized that insufficient antioxidant defense may be a contributing factor to the pathophysiological mechanisms associated with oxidative stress and preeclampsia. However, this study has not covered information on oxidative stress parameters [85].

In 2013, Tortladze and co-workers conducted a case control study to determine the role of oxidative metabolism in non-pregnant, normal pregnancy and preeclampsia. They reported catalase, GR activity did not change significantly compared to non-pregnant but SOD activity increased slightly. However in preeclampsia, catalase activity was increased and SOD, GR activity decreased compared to normal pregnancy. They concluded that chronic oxidative stress in preeclampsia may be due to defect in reperfusion oxygenation of placenta tissue with decreased trophoblast invasion and angiogenesis. Nevertheless, this study did not cover nutrient antioxidant status in preeclampsia [86].

In 2014, Kiondo P and co-workers conducted a randomized controlled clinical trial and reported supplementation of vitamin C neither reduced the incidence of pre-eclampsia nor the adverse maternal or neonatal outcomes. Hence, the use of vitamin C supplementation in prevention of preeclampsia complications was not recommended[87].

In 2014, Atiba AS and co-workers in a case control study observed no change in plasma levels of membrane lipid oxidation product malondialdehyde, vitamins C and E as pregnancy advances into the third trimester of both normal and preeclamptic pregnancies. Therefore, this study does not recommend the supplementation of antioxidant vitamins in prevention of oxidative stress in preeclampsia [88].

In 2015, Nasrollahi and co-workers reported in their cross sectional study that patients over and under the age of 30 years there was no significant difference in antioxidants, catalase and superoxide dismutase between the normotensive pregnant and preeclampsia. However, glutathione peroxidase level was significantly higher in the normotensive group over the age of 30 years. Total antioxidant status was similar between preeclamptic and normotensive patients. This study revealed no importance with respect to total antioxidant status in preeclampsia. But gestational age was not taken in to the consideration which may had effect on antioxidant levels [89].

In 2015, Nikolic A and co-workers conducted prospective study to evaluate diagnostic value of ceruloplasmin together with other enzymatic and nonenzymatic antioxidants such as SOD, GPx and uric acid to find the status of oxidative stress in patients with preeclampsia in comparison to normal pregnancy. They observed higher levels of serum ceruloplasmin, uric acid and superoxide dismutase in the preeclampsia compared to the normotensive pregnancy. This study also showed a non-significant glutathione peroxidase between the case and control. This report also stated that serum ceruloplasmin and serum uric acid have the best diagnostic accuracy for oxidative stress in preeclampsia. Still, this study did not measure specific oxidative stress markers in preeclampsia [90].

In 2015, Cross CE et al. conducted a study to evaluate the role of oxidative stress on gene expression in first trimester trophoblast through the measurement of micro RNA (miRNA) and mRNA to understand the placental mechanism. They exposed trophoblastic cells to reactive oxygen species at different concentrations to study the expression of key genes which is involved in placental deregulation. They observed altered mi-RNA and mRNA expression in preeclampsia. Conversely, this study fails to explain long term effect of hydrogen peroxide and feasibility of extrapolating results to mimic *in vivo* condition [91].

In 2015, Turgut A et al. conducted a study to know the serum levels of adipokines (Serum insulin, leptin, nesfatin, ghrelin, and chemerin), free fatty acids, oxidative stress index and total antioxidant status between obese and non-obese preeclamptics. They observed high total antioxidant status and decreased oxidative stress index in normotensive group compared to preeclampsia but there was no difference between obese and non-obese preeclamptics. However, this study did not measure each antioxidant separately [92].

In 2015, Cohen JM et al. carried out case control study to determine an association of antioxidants level between mid-pregnancy and preeclampsia. The antioxidants studied were α-tocopherol, lycopene, lutein, and carotenoids (sum of α-carotene, β-carotene, anhydrolutein, α-cryptoxanthin, and β-cryptoxanthin). They suggested an inverse association between antioxidant levels and preeclampsia. The study findings suggested that lutein was significantly associated with overall preeclampsia risk and also most of the antioxidants were shown strong association with early onset of preeclampsia. This indicated the role of oxidative stress in pathophysiology of early

onset of preeclampsia. Study evinced carotenoid like compound lutein from plant origin may be serves as promising nutrient in preeclampsia prevention. But, this observation has to be scientifically validated by means of clinical trials [93].

In 2015, Rumbold A and Crowther CA in a Cochrane review analyzed the effects of vitamin C and E supplementation either alone or in combination with other supplements on pregnancy outcomes, adverse events, side effects and use of health resources. They reported that data do not support routine vitamin C supplementation alone or in combination with other supplements for the prevention of fetal or neonatal death, poor fetal growth, preterm birth or preeclampsia. There was no convincing evidence on the benefit or harm with the vitamin C supplementation alone or in combination with other supplements. This observation was in agreement with the recommendation put forward by WHO 2012 report [94].

2.3. Protein Carbonyls in Preeclampsia

The term protein carbonyl applied to denote the extent of protein modification in tissues and body fluids by reactive oxygen species. The following are the review of literature pertaining to the protein carbonyls in preeclampsia.

In 2000, Petra LM Zusterzeel and co-workers conducted a study to determine the plasma protein carbonyls in non-pregnant, normal pregnant and preeclamptic women. They observed preeclamptic women had higher plasma protein carbonyl levels compared to normal pregnant, similarly normal pregnancy when compared to non-pregnant. They reported that plasma protein carbonyl may serve as a biomarker for

oxidative stress. This study lacks establishing diagnostic utility of protein carbonyl in all trimesters of pre-eclampsia [95].

In 2008, Tsukimori K and co-workers conducted a case control study to examine the correlation of serum uric acid, plasma hydrogen peroxide and protein carbonyl content to explore the superoxide generation and oxidative stress in preeclampsia. They concluded that plasma protein carbonyls along with uric acid and hydrogen peroxide levels as an indicator of oxidative stress in preeclampsia. Though, uric acid has to be considered either as an antioxidant or oxidative stress marker which is controversial. Nevertheless, large sample size is required to denote protein carbonyl as an oxidative stress marker [96].

In 2013, Asha Rani and Naidu conducted an age and trimester matched case control study in preeclamptic and normal pregnant women where they reported serum MDA, protein carbonyl and vitamin E levels. They observed increased MDA and decreased Vitamin E levels in preeclampsia when compared to normal pregnant represent increased oxidative stress and reduced membrane antioxidants. However, this study did not mention complete picture of oxidative stress and antioxidant status in postpartum of preeclampsia [97].

2.4. Ischemia Modified Albumin in preeclampsia

In 2008, Aris T Papageorghiou and co-workers conducted a prospective study in women with singleton pregnancy undergoing nuchal translucency assessment at 11-14 weeks to determine whether serum IMA levels were further elevated with defective tropoblast development in the first trimester. They observed elevated maternal serum

IMA levels early in pregnancy before the onset of clinically evident preeclampsia which mandates further prospective evaluation of IMA as a potential biomarker for abnormal placental development related to miscarriage and preeclampsia. This study creates a gap to explore the status of IMA in pre and postpartum of preeclampsia [98].

In 2008, Van Rijn BB et al. conducted study on patient with preeclampsia, normal pregnant and non-pregnant control to assess the levels of serum IMA and albumin concentrations reported that serum IMA concentration was elevated in normal pregnancy. They found no significant relationship between IMA levels and preeclampsia. This observation was contradictory in comparison to other similar studies that need to be ascertained [99].

In 2010, Gafsou B and co-workers conducted a case control study to determine whether a maternal serum ischemia modified albumin as a biomarker to distinguish between normal pregnant and preeclampsia cases. They compared the concentration of serum IMA to serum albumin ratio between non-pregnant, normal pregnant and preeclampsia cases. They reported IMA to albumin ratio in non-pregnant 2.10±0.22kU/g, normotensive pregnant 2.60±0.38kU/g and preeclampsia 116.9±12.3kU/g respectively. Even though, IMA has been considered as significant parameter in preeclampsia but its diagnostic utility needs to be tested [100].

In 2011, Osmanagaoglu MA et al. conducted a case control study to investigate serum levels of total carnitine, arginine, asymmetric dimethyl arginine (ADMA) and IMA levels in preeclampsia and normotensive pregnancy. They observed significant increase in total carnitine, asymmetric dimethyl arginine, IMA and decreased L-arginine

in the preeclamptic group. They reported that the sensitivity of asymmetric dimethyl arginine levels was found to be higher in individual measurements. Although, there is still no independent placental ischemia factor which can predict preeclampsia [101].

In 2011, Ustun Y and co-workers conducted a case control study to examine the serum IMA levels in normal pregnant and preeclamptic women and its correlation with the severity of preeclampsia. They observed high IMA levels in the mild and severe preeclamptic group than controls. They concluded that serum IMA levels correlated with the severity of preeclampsia. However, IMA levels in postpartum samples of preeclampsia have to be established [102].

In 2013, Alberto Rossi et al. conducted a prospective study on IMA in pregnancy. They measured pregnancy associated plasma protein A (PAPP-A) and IMA in pregnant women during first, second trimester and post-partum period. Study consisted of two groups based on the outcome such as mothers bearing appropriate for gestational age fetuses (AGA) and mothers bearing small for gestational age fetuses (SGA).The control groups were non pregnant women. Study reported serum levels of IMA as an oxidative stress marker at two different stages of pregnancy and in the immediate post-partum period. IMA levels were elevated in pregnancy compared to the control population which is in similar with the previous study. Increase of serum IMA level found in women with AGA and SGA fetuses affirm the possible physiological oxidative stress onset in pregnancy. Study suggested that fetuses exposed to higher degree of oxidative stress are more likely to become SGA. The study outcome suggests an early assessment of IMA and PAPP-A levels in the first trimester helps to

differentiate SGA fetuses. But study demands further validation with large size cohort studies to confirm theses markers potentially in prediction of fetal growth restriction [103].

In 2015, Sapna Vyakaranam and co-workers conducted a case control pilot study in normotensive pregnant, pregnancy induced hypertension and preeclampsia. Study demonstrated IMA as oxidative stress marker and its level helps in differentiating hypertensive disorders of pregnancy from preeclampsia in comparison with normal pregnancy. But the diagnostic utility of the IMA has to be established along with other oxidative stress markers [104].

2.5. Endothelial dysfunction marker in preeclampsia

In 1998, Irina A Buhimschi and co-workers in a review report stated about the nitric oxide pathway and its pathophysiological implication in preeclampsia. It comprises pathophysiology by the involvement of placenta and hypoxia evidences oxidative stress in preeclampsia. Their report also stated general mechanism of action of nitric oxide responsible for vasodilatation triggered by acetyl choline. Endothelium derived relaxing factor nitric oxide generated during conversion of L- arginine to L citruline by nitric oxide synthase (neuronal, cytokine inducible and endothelial). Endothelial nitric oxide synthase associated with endothelial cell membrane due to the presence of myristoylation and palmitoylation site. Increased activity of endothelial nitric oxide synthase produces nitric oxide at endothelial lining. The nitric oxides thus produce in response to increased blood flow diffuses towards muscular layer of vessel and activates guanylate cyclase which inturn relaxes blood vessel. There by nitric oxide regulates vessel diameter as per the need of the tissue.

Report also envisaged the interaction of nitric oxide in oxidative stress with super oxide combined to form peroxy nitrite anion. This complex acts as powerful long acting oxidants have deleterious effects on biomolecules. Thus oxidative stress regulates bioavailability of nitric oxide. Report also stated that cytokine inducible nitric oxide synthase (iNOS) produces abundant nitric oxide production in normal pregnancy to impart relaxing effect during gestation period. Report concluded that high production of nitric oxide in the maternal reproductive and vascular system by the action of isoforms of nitric oxide synthase (iNOS in the uterus and placenta and eNOS in vessels in pregnancy). However, there are contradictions regarding the unchanged, excess or decreased nitric oxide in preeclampsia [105].

In 2002, Jong Weon Choi and co-workers conducted a study on nitric oxide and serum iron markers (serum iron and ferritin) at all trimesters in pre-eclampsia and normal pregnancy. They reported high nitric oxide concentration in first trimester of normal pregnancy which consistently remained same. This study did not specify onset of oxidative stress in relation with nitric oxide production and its bioavailability [106].

In 2004, Hatice Pasaoglu and co-workers conducted a case control study to assess nitric oxide, lipid peroxide and uric acid level in normotensive pregnant, preeclampsia and eclampsia cases. Reports suggested gradual increase of nitric oxide, MDA (plasma and erythrocytes) and uric acid from normotensive pregnant, preeclampsia and eclampsia cases. Study suggests measurement of plasma nitric oxide, MDA and uric acid had a gradual increase in oxidative stress [107].

In 2007, Meher S and Duley L presented a review using randomized trials to Cochrane data base on nitric oxide in preventing preeclampsia and its complications.

The objective of the review was to assess the effectiveness and safety of nitric oxide donors in management of preeclampsia. Amongst the studies reviewed, authors found paucity of good intervention trials that used glyceryl trinitrate or precursors L-arginine. Due to insufficient evidences study fails to draw any conclusion [108].

In 2009, Ebru Eerdemoglu and co-workers conducted a case control study to investigate association between nitric oxide level and homocysteine. Homocysteine levels were significantly increased but there was no difference in nitric oxide level in preeclampsia when compared to normal pregnancy. This study has no correlation between homocysteine and nitric oxide level. The study failed to demonstrate any difference in serum nitric oxide level which is contradictory to other studies which showed change in nitric oxide level in preeclampsia. Hyperhomocystenemia is well known in association with vascular diseases such as hypertension, cerebro-vascular accidents, peripheral vascular disease as well in early pregnancy loss and placental abruption. Generally homocysteine level was reported to be decreased in pregnancy and higher in preeclampsia. Even though observation of increased homocysteine has more relevance clinically in preeclampsia condition study has major limitation by virtue of very less number of patients in preeclampsia and ecclampsia groups. Increased homocysteine has an impact on reduction of plasma nitric oxide by inhibiting endothelial nitric oxide synthase [109].

In 2011, Suchanda Sahu and co-workers conducted a case control study to estimate serum and urine uric acid, plasma nitric oxide, urine pH, urine creatinine in normal pregnancy and preeclampsia at third trimester. They concluded that there was negative correlation between systolic blood pressure and nitric oxide as well as there

was increase in uric acid to urine creatinine ratio with decrease in nitric oxide level. However, studies with larger sample size needed to validate the sensitivity and specificity of nitric oxide [110].

In 2013, Saha T and co-workers conducted a study to know the role of nitric oxide(NO), angiogenic factors and other biochemical analysis in preeclampsia. They selected angiogenic parameters such as vascular endothelial growth factor (VEGF) and placenta induced growth factor (PIGF) to know the development of blood vessels and placenta. Besides they also measured other biochemical parameters such as urea, uric acid, triacylglyceride, AST and ALT. Serum concentration of nitric oxide found to be decreased in preeclampsia compared to normal pregnancy which indicated involvement of the nitric oxide role in vasodilatation process during angiogenesis. The study showed decreased NO, VEGF and PIGF. The exact mechanism behind decreased production of these factors was not described in the study[111].

In 2013, Sandra T and coworkers conducted a study on urinary and plasma nitric oxide metabolites in women with preeclampsia and normal pregnant. They measured plasma and urine nitric oxide metabolite and found reduced production of nitric oxide in women with preeclampsia when compared to normotensive pregnant [112].

In 2013, JT Sertorio et al. conducted a genetics based case control study to find the role of hapatoglobin polymorphism that affects the bioavailability of nitric oxide in preeclampsia. They reported in addition to altered circulating levels of angiogenic factors elevated arginase expression, oxidative stress and inhibition of eNOS by asymmetric dimethyl arginine (ADMA), genetic variations and increase in circulating cell free hemoglobin which contribute to impaired nitric oxide bioavailability. Cell free

hemoglobin scavenges nitric oxide through formation of meth hemoglobin and nitrate when nitric oxide reacted with oxy hemoglobin. The serum hapatoglobin (Hp) binds with Hb to form Hp-Hb complex which is removed from the circulation. The hapatoglobin gene is polymorphic contains two common alleles Hp^1& Hp^2 encoding three different proteins such as Hp 1-1, Hp2-1 & Hp2-2. Study also presented that there was no difference in genotype of Hp1-1 therefore it has protective role in preeclampsia by reducing nitric oxide scavenging. However Hp2-1 &Hp2-2 involved in increased intensity of preeclampsia by reducing the bioavailability of nitric oxide. However, Hp genotype in normal pregnancy has no effect on altering bioavailability of nitric oxide. Limitation of the study was that there is no direct influence of cell free hemoglobin on hypertension in preeclampsia [113].

In 2014, Swati D and co-workers conducted a case control study to investigate the association of human monocytic paraoxonase 2 and serum nitric oxide levels in preeclampsia patient. They measured paraoxonase 2 isoenzyme from family of paraxonases (PON1, PON2, PON3) expressed from gene located on chromosome 7 q21-22. The activity of this enzyme also compared with lipid profile and nitric oxide in control and preeclampsia group. They concluded that decreased PON 2 lactonase activity, abnormal lipid profile and decreased nitric oxide may have role in pathogenesis of preeclampsia. The possible cause for decreased PON2 activity was elucidated [114].

In 2015, Keiichi Matsubara and co-workers wrote a review on role of nitric oxide and reactive oxygen species in the pathogenesis of preeclampsia. They reported pathogenesis of preeclampsia is mediated by an altered bioavailability of nitric oxide and tissue damage caused by increased levels of reactive oxygen species. Superoxide

quickly inactivates nitric oxide to form peroxynitrite which in turn affects the placental function. Reactive oxygen species also stimulates platelet adhesion and aggregations leading to intravascular coagulopathy which affects the placental blood flow which may lead to intra uterine growth restriction. Enzyme antioxidants protect the vasculature from ROS. But placental ischemia alters antioxidants enzyme activity and more oxidative stress which leads to pathophysiology of preeclampsia [115].

2.6. Inflammatory markers

In 2005, E Teran and co-workers conducted a prospective study to investigate C-reactive protein in normal pregnancy and preeclampsia. They measured variations of CRP in non-pregnant and preeclamptic women. They recruited pregnant women from 16 weeks of gestation and followed up to 36 weeks, between this period samples were collected at every 4 week up to 36 week to determine the levels of CRP. After 36 weeks samples were drawn at every 2 weeks up to delivery state. Amongst 207 women participated and completed the study. They reported 24 cases developed with preeclampsia (11.6%).The study demonstrated that women with onset of preeclampsia has increased CRP from 24th week onwards up to 5.9mg/L. However, it remains elevated up to delivery. Whereas in normal pregnant CRP level moderately elevated (2.9mg/L) from week 20 and significantly elevated (4.3mg/L) after 36 weeks. Therefore measurement of CRP is not a good predictor of preeclampsia at early stage (week 16). However, marked increase indicated at 32 weeks as a predictor of preeclampsia [116].

In 2005, J Tavakkol Afshari et al. performed a cross sectional study to determine interleukin-6 and tumor necrosis factor-α concentrations in preeclamptic women of

Iranian population.The study objective was to determine role of IL-6 and TNF-α as marker of immune activation and endothelial dysfunction in patients with preeclampsia. They reported increased IL-6 as a pro-inflammatory cytokine and unchanged TNF-α in preeclampsia. This study reported about the immunological interaction and inflammation cause in development of preeclampsia. Study opens further scope to perform longitudinal study before onset of preeclampsia to elucidate the role of IL-6 in pathogenesis of preeclampsia [117].

In 2006, Gupta AK and co-workers conducted a retrospective study to evaluate the plasma elastase activity in patients with early (<34 weeks of gestation) and late onset preeclampsia (>34 weeks of gestation) in comparison with with eight cases and an equal number of gestational age matched normotensive pregnant. A plasma elastase concentration was significantly elevated in preeclampsia study group when compared to the normotensive control group. But due to less sample size, the study could not find out the association between serum elastase and preeclampsia particularly in relation to inflammation. Therefore, it is worthwhile to design study on elastase activity and its impact on host tissue in preeclampsia [118].

In 2007, Hossein Ayatollahi and co-workers conducted a case control study to examine the hs-CRP levels in mild and severe preeclampsia in comparison with normal pregnant. They reported high serum hs-CRP level in preeclampsia compared to normal pregnancy. There were also significant hs-CRP levels in mild and severe preeclampsia. They concluded that hs-CRP may be useful in prediction and diagnosis of preeclampsia. However, they did not check the diagnostic utility and validity of hs-CRP before onset of preeclampsia [119].

In 2009, Luis Belo et al. conducted a cross sectional study to investigate neutrophil activation and C-reactive protein concentration in preeclampsia. They also measured leukocyte count, plasma lactoferrin, granulocyte macrophage colony stimulating factor (GM-CSF) and uric acid. They observed higher levels of CRP, elastase activity and uric acid in preeclampsia when compared to normal pregnant in the third trimester. However, no differences were found in total leukocyte count, lactoferrin and plasma GM-CSF. The study concluded that prominent inflammation is seen in preeclampsia by neutrophil activation and which correlates with the severity of preeclampsia. But the study has not covered the cause of neutrophil activation in terms of ROS and its association with inflammation in preeclampsia [120].

In 2010, Dan Mihu et al. conducted a transversal study to evaluate the leukocytes and neutrophils in preeclampsia and compared these values to normal pregnancy. They reported that leukocyte and neutrophil count in peripheral blood was significantly increased in last trimester of pregnancy in preeclampsia compared to normal pregnancy. They concluded that leukocyte and neutrophil values can be considered as markers of the inflammatory syndrome present in preeclampsia. Even though, this study not reported any information on neutrophil elastase activity with respect to inflammation [121].

In 2011, Samira BG and co-workers conducted a prospective cohort study to find out the association between serum hs-CRP in normal pregnant women between 14 to 20 weeks, the subjects were followed up to delivery. During this period, based on the clinical condition subjects were divided in to normal pregnant, mild and severe preeclampsia groups. They reported relationship between hs-CRP levels in mild and severe preeclampsia. They also stated high percentage of mild (47 %) and severe (45%)

preeclampsia was noticed between >29 to <35 BMI. However, 47 % of pregnant women were normal with BMI <25kg/m^2. However, the study showed hs-CRP levels >4.5mg/L and >5mg/L could predict mild and severe preeclampsia respectively as an evidence of inflammation [122].

In 2013, Maryam K and co-workers conducted a prospective cohort study to evaluate diagnostic importance of hs-CRP level in the prediction of preeclampsia at first trimester. They included 394 women, amongst 42 cases (10.7%) were identified with preeclampsia complication, further from them 23 women recorded with severe preeclampsia (56.1%).They were compared with normal pregnant group. The hs-CRP level was higher (7.06mg/L) in preeclampsia than control group (3.6 mg/L). As per the ROC characteristics hs-CRP has diagnostic utility and importance. Study concluded that measurement of hs-CRP during first trimester of pregnancy were useful in the prediction of preeclampsia. However, the successive trimester hs-CRP value up to delivery might be useful in better understanding the onset of preeclampsia [123].

In 2014, Tayal D and co-workers conducted a case control study to evaluate association of MDA, nitric oxide, IL-6 and TNF-α in non-pregnant, normal pregnant and preeclampsia. Nitric oxide and MDA levels were high in preeclampsia when compared to other two groups. They concluded that TNF-α as a better proinflammatory marker in preeclampsia [124].

In 2015, Mihu D and co-workers carried out a case control study to evaluate maternal systemic inflammatory response in preeclampsia. They measured leukocytes, neutrophils, tumor necrosis factor-α, interleukin-6, C-reactive protein along with

oxidative stress. They observed higher systemic inflammation in preeclampsia when compared to normal pregnancy [125].

In 2016, Kanak KM and co-workers conducted a case control study to find the level of hs-CRP in preeclampsia and normal pregnancy in the third trimester. They distributed case and control according to the religions viz. Hindu, Muslims and Christian. The study showed that 65.4% cases were Hindus, 23.1% Muslims and 11.5% Christian's respectively in preeclampsia. They reported that serum hs-CRP level as an indirect risk factor for placental vasculature in preeclampsia. However, systemic contribution to elevated CRP concentration by proinflammatory cytokines was not elucidated in the study [126].

In 2016, Stubert J et al. in a case control study presented predictive value of serum amyloid A, progranulin, transthyretin, C-reactive protein and interleukin-6 in preeclampsia at second trimester. Results of the study expressed the probable onset of inflammation in the third trimester than second trimester during development of preeclampsia. They also reported acute-phase proteins are not predictive markers for preeclampsia in a high-risk population without clinical symptoms during the second trimester. However, study has limitation by virtue of not screening the above parameters in third trimester and after delivery within 48 hours. Study lacks the possibility of determining high sensitive CRP to ascertain inflammation [127].

CHAPTER-3

AIM AND OBJECTIVES

3.0. AIM AND OBJECTIVES

3.1. Aim

Determination of oxidative stress parameters with xanthine oxidase along with antioxidants, endothelial dysfunction marker and inflammatory parameters in preeclampsia before and after delivery within 48 hours and also xanthine oxidase inhibitors.

3.2. Objectives of the study

1. To determine the levels of oxidative stress markers such as malondialdehyde, ischemia modified albumin, protein carbonyl and xanthine oxidase activity in non-pregnant, normotensive pregnant and preeclampsia during before and after delivery within 48 hours.
2. To assess the endothelial dysfunction by nitric oxide in non-pregnant, normotensive pregnant and preeclampsia during before and after delivery within 48 hours.
3. To estimate the levels of vitamin C, vitamin E, uric acid, ferric reducing ability of plasma along with enzyme antioxidants (glutathione peroxidase, glutathione reductase, catalase, super oxide dismutase) in non-pregnant, normotensive pregnant and preeclampsia during before and after delivery within 48 hours.

4. To determine the high sensitive-C-reactive protein level in non-pregnant, normal pregnant and preeclampsia during before and after delivery within 48 hours.

5. To determine the plasma elastase, phospholipase A_2 activity and hs-CRP level in non-pregnant, normotensive pregnant and preeclampsia during before and after delivery within 48 hours.

6. To find out the correlation and diagnostic importance of the above measured parameters in preeclampsia.

7. To screen for an *in vitro* xanthine oxidase inhibitory activity by vitamin C and E.

8. To isolate flavonoids from medicinal plants such as *Pongamia pinnata L, Morinda citrifolia L, Mangifera indica L, Zingiber officinale Roscoe* and screen for *in-vitro* xanthine oxidase inhibitory activity using quercetin as internal standard.

9. To determine the percentage of inhibition, IC_{50} and K_i of flavonoid extract from plants, vitamin C & vitamin E using allopurinol as standard inhibitor.

CHAPTER-4

MATERIALS & METHODS

4.0. RESEARCH METHODOLOGY

4.1. MATERIALS

Study designed was observational prospective case control which was confined to R L Jalappa Hospital and Research Centre, Tamaka, Kolar. The duration of the study was three years (August 2013 to August 2016). Ethical Clearance was obtained from Institutional Ethics Committee of Sri Devaraj Urs Medical College with vide number DMC/KLR/UDOME/IEC/38 dated 9-12-2013. The study population enrollment was commenced after obtaining patient written informed consent. The study was conducted by the Department of Biochemistry in collaboration with Department of Obstetrics and Gynecology.

Sample size was calculated for the study parameters such as oxidative stress, antioxidant and inflammatory markers based on the mean difference and standard deviation levels of the research reports by adapting the following power formula.

$$n = \frac{2[z\alpha + z\beta]\ 2s^2}{(d)\ 2}$$

At 90% power (zβ), α=0.05%,

Where, d= difference in means, s^2= combined variance, zα = confidence interval,

Zβ=Power =90%, at 95% confidance intervalzα= 1.96, zβ=1.28

As per this formula, sample size was fifty seven in each group. Dropout rate of 10%was considered during the follow up (6 samples/group).There by arrived final sample size of 63 per group.The study population comprised of three groups. Group 1(n=63) non pregnant women, Group 2 (n=63) normotensive pregnant women and Group 3 (n=63) clinically diagnosed preeclamptic women. Group 2 and 3 were followed

after delivery within 48 hours. The non-pregnant control populations were age matched. Group 2 & 3 were in 30-39 weeks of gestation.

Four ml of blood was collected and aliquoted from the non pregnant, normotensive pregnant and preeclamptic women visited department of obstetrics and gynecology. Blood samples were collected and centrifuged at 3000 rpm for 10 minutes to obtain the clear plasma and serum. Thus obtained clear sera and plasma were stored at -80˚C until analysis.

4.1.1. Inclusion Criteria

All pregnant women after 20 weeks of gestation with preeclampsia diagnosed as per National High Blood Pressure Education Programme working group (NHBPEP) classification admitted with singleton pregnancy, no fetal anatomical anomaly, nonsmokers were included in the study at R L Jalappa Hospital and Research Center. Pre-eclampsia was diagnosed with blood pressure of ≥140/90 mm of Hg noted for the first time during pregnancy on ≥2 occasions at least 4 hours apart, after 20 weeks of gestation with proteinuria of ≥300 mg/24 hours or ≥ 1+ by dipstick method in a random urine sample.

4.1.2. Exclusion Criteria

History of renal disease, history of thyroid disorder, history of chronic hypertension, history of gestational diabetics, history of epilepsy, history of hypertensive encephalopathy, history of cardio vascular disease and multigravida were excluded.

4.2. METHODS

4.2.1. Malondialdehyde

Method: Plasma Malondialdehyde (MDA) level determined as thiobarbituric acid reactive substances according to the method of Sinnhuber [128].

Principle: Free malondialdehyde, as a measure of lipid peroxidation, measured spectrophotometrically as thiobarbituric acid reactive substances (TBARS) after precipitating the proteins with trichloroacetic acid.

Reagents

1. Tri chloro acetic acid (24%)
2. Thiobarbituric acid (TBA) reagent (0.37%)
3. Hydrochloric acid (0.25N)
4. Tetra methoxy propane (30µmol/L)

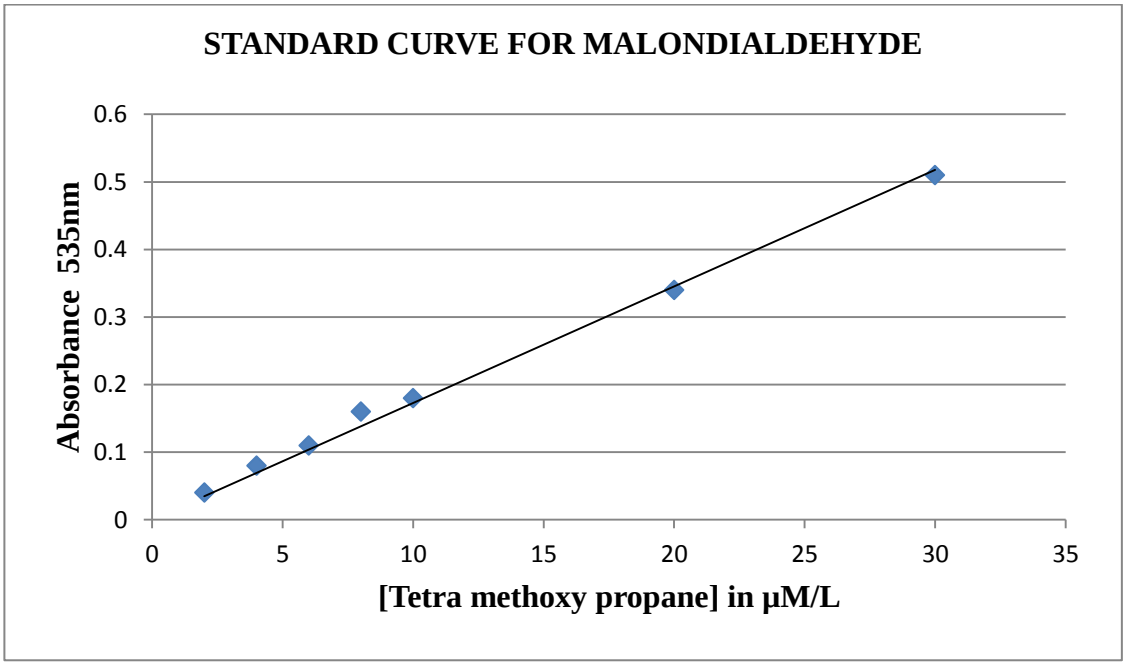
Procedure: 0.5ml of plasma sample was mixed well with 1.5ml of 24% trichloro acetic acid and centrifuged the protein precipitated at 1000 rpm for 10 minutes. To 1.0ml of the clear protein free supernatant, added 0.5 ml 0.25N HCl and 0.5ml 0.37% of thiobarbituric acid and kept in boiling water bath for 15 minutes. Then the tubes were removed and cooled under tap water. Absorbtion was read at 535 nm in spectrophotometer (Perkin Elmer Lamda 1.2).

Calculation:

$$[\text{MDA in } \mu M/L] = \frac{\text{OD of Test} - \text{OD of Blank}}{\text{OD of standard} - \text{OD of Balnk}} \times \text{Concentration of standard}$$

Preparation of standard curve for malondialdehyde

Standard	Concentration (µM/L)	Absorbance (535nm)
Standard 1	2	0.04
Standard 2	4	0.08
Standard 3	6	0.11
Standard 4	8	0.16
Standard 5	10	0.18
Standard 6	20	0.34
Standard 7	30	0.51



4.2.2. Protein Carbonyl Content

Method: Plasma protein carbonyl determined according to the method of Levine [129].

Principle: Carbonyl groups in proteins react with 2, 4 di nitro phenyl hydrazine (DNPH) to produce hydrazones which can be quantified at 370 nm in spectrophotometer (Perkin Elmer Lamda 1.2).

Reagents

1. 2, 4-DNPH (10mM)
2. Hydrochloric acid (2.5M)
3. Trichloroacetic acid solution (20%)
4. Ethanol: ethyl acetate mixture (1:1 v/v)
5. Guanidine hydrochloride (6M)

Procedure: 0.25ml of plasma added in to test and control tubes. 1.0 ml of 2, 4, DNPH added to test and 1.0 ml of 2.5M HCl to control. The contents of the tubes were mixed thoroughly and incubated in the dark at room temperature for 1 hour. The tubes were shaken intermittently at every 15 minutes during incubation. 1.0ml of 20% trichloroacetic acid added to each tube and vortexed. Tubes were kept on ice for 10 minutes, centrifuged at 3500 rpm for 20 minutes to obtain the protein pellet. The supernatant obtained was discarded and protein pellet resuspended using 5ml of 1:1 ethanol: ethyl acetate mixture. The pellet was washed three times to remove any unreacted DNPH and lipid remnants. To the final washed pellet, 2.0ml of 6M guanidine hydrochloride added and the tubes were incubated at 37˚C for 10 minutes. Thus formed hydrazones in test was measured against the control at 370 nm. The carbonyl content

calculated using an absorption coefficient of 22,000 M^{-1} Cm^{-1}. The protein carbonyl content was expressed as nmol/ml.

Calculation:

Corrected absorbance (CA) = Absorbance of test –Absorbance of control

$$\text{Protein carbonyl content (nmol/ml)} = \frac{\text{Corrected absorbance}}{\text{Absorption coefficient}} \times \frac{\text{G.HCl volume}}{\text{Plasma volume}}$$

Preparation bovine serum albumin standard using oxidized and reduced BSA

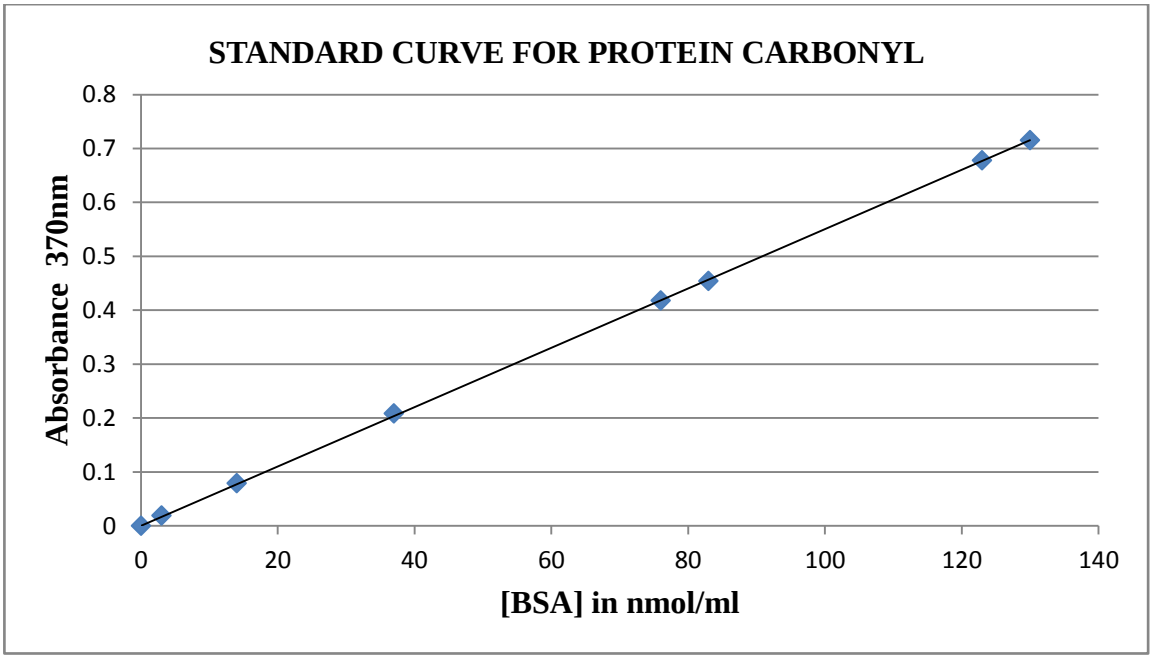
Protein carbonyls a marker of protein oxidation was measured in plasma by spectrophotometrically using internal BSA standards by mixing oxidized and reduced BSA.

Oxidized BSA containing additional carbonyl groups was prepared by reacting BSA (50mg/ml in phosphate buffer saline) with hypochlorous acid to final concentration of 5mM.

Reduced BSA was prepared by the reaction of existing carbonyl groups of BSA (0.5g in 100ml) with 0.1g solid sodium tetra borohydroborate for 30 minutes. The solution thus obtained brought to neutral pH by slow addition of 2M HCl. Since, this reaction produces hydrogen it was carried out in fume hood. The solution later subjected for ultafiltration using millipore membrane and then ultra-centrifuged for 10 minutes under 5000rpm and filtrate was collected. Reduced and oxidized BSA obtained from the above step was brought to final concentration of 4mg/ml protein as shown in table given below.

Preparation of standard curve for protein carbonyl using reduced and oxidized bovine serum albumin

Standard	Oxidized BSA (µl)	Reduced BSA (µl)	Absorbance (370nm)	Protein Carbonyl (nmol/ml)
Standard 1	0	400	0	0
Standard 2	20	380	0.019	3
Standard 3	40	360	0.079	14
Standard 4	80	320	0.208	37
Standard 5	160	240	0.418	76
Standard 6	240	160	0.454	83
Standard 7	320	80	0.678	123
Standard 8	400	0	0.715	130



4.2.3. Xanthine Oxidase

Method: Assay of plasma xanthine oxidase by the method of Bergmeyer [130].

Principle: Xanthine oxidase (EC 1.1.3.22) catalyzes the conversion of xanthine to uric acid and hydrogen peroxide. The increase in absorbance (ΔA) was measured at 290nm against control in spectrophotometer (Perkin Elmer Lamda 1.2).

Reagents

1. Potassium phosphate buffer at pH 7.5(50 mM)
2. Xanthine substrate solution (0.15 mM)
3. Xanthine oxidase solution (0.1 - 0.2 unit/ml)

Procedure: Pipetted the following reagents into suitable quartz cuvettes. To blank, added 1.90ml of 50mM phosphate buffer at pH 7.5, 1.0ml of 0.15mM xanthine solution and 0.10 ml deionized water. To test added 1.90 ml of 50mM phosphate buffer at pH 7.5, 1.0ml of 0.15mM xanthine solution. Mixed the contents by inversion and equilibrated to 25°C. Monitored the absorbance at 290nm until constant. Then added 0.10ml of plasma, mixed the contents and recorded the increase in absorbance at 290nm for approximately 5 minutes against the control. Absorbance change at 290nm/minute was calculated. Enzyme activity curve was prepared using different concentration of substrate under assay condition.

Calculation:

$$\text{XO activity (Units/ml)} = \frac{(\Delta A\ 290nm/\ \min \text{Test} - \Delta A290nm/\min \text{control})(3.0)(DF)}{(12.2)(0.1)}$$

3= Total volume (ml)

DF= Dilution factor

12.2= Millimolar extinction coefficient of uric acid

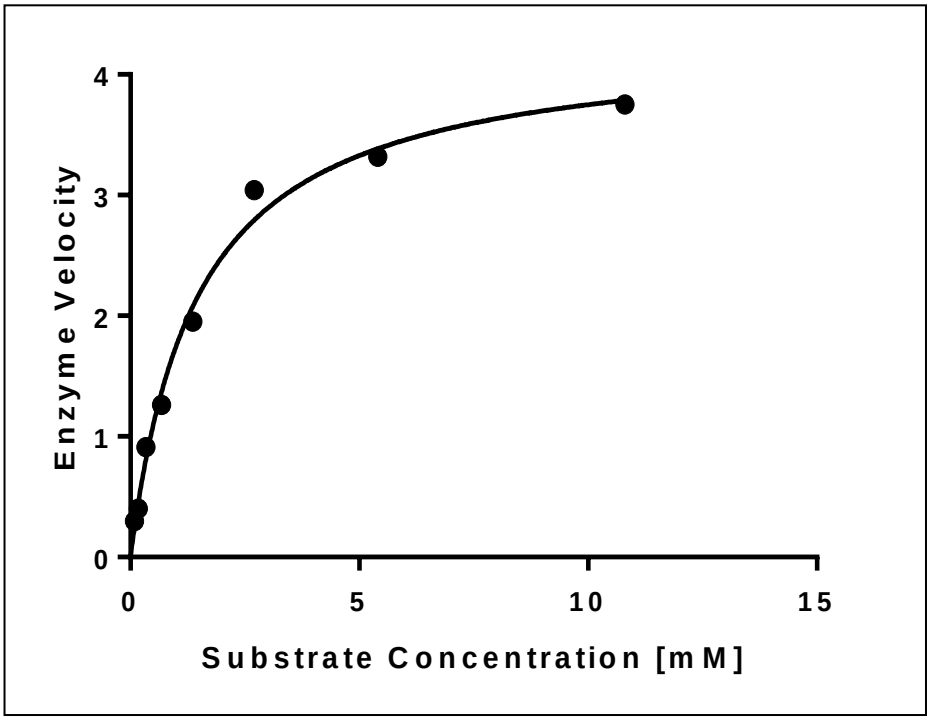
0.1= Volume of enzyme used (ml)

Unit of enzyme activity: One unit of enzyme activity defined as, the amount of enzyme that will convert 1.0 µmole of xanthine to uric acid per minute at pH 7.5 at 25°C.

Preparation of xanthine oxidase enzyme activity curve

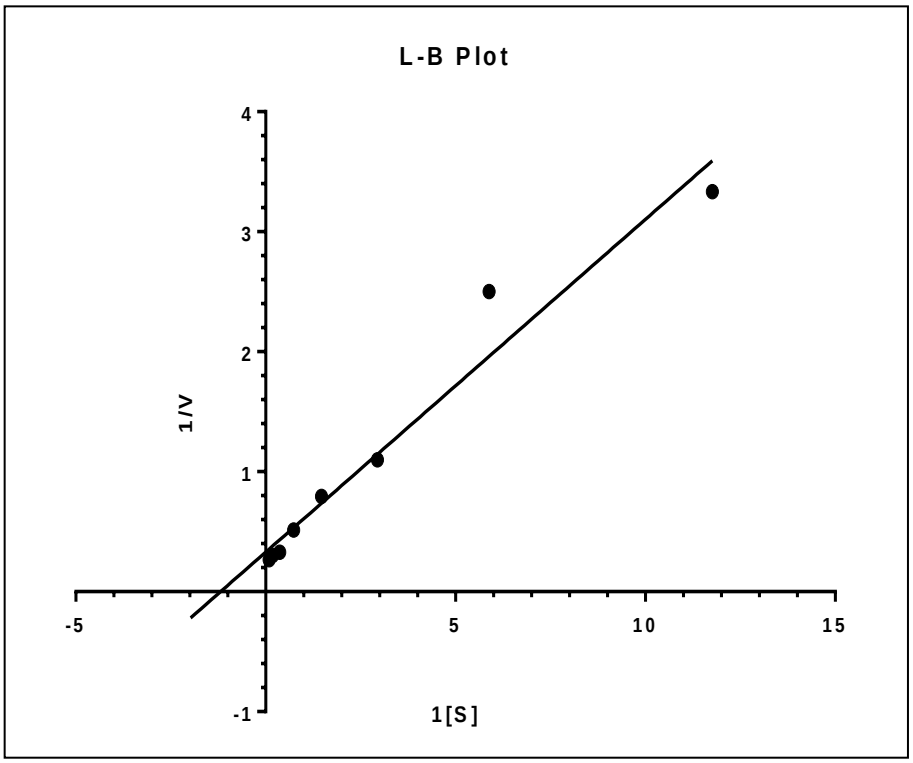
Substrate	Substrate (mM)	Velocity (290 nm)
1	0.085	0.3
2	0.17	0.4
3	0.34	0.91
4	0.68	1.26
5	1.36	1.95
6	2.7	3.04
7	5.4	3.32
8	10.8	3.75

Effect of substrate on xanthine oxidase activity



V_{max} = 4.295, K_m =1.453mM

Effect of substrate on xanthine oxidase activity-Linewaver Burk plot



Slope	0.2773 ± 0.02363
Y-intercept	0.3293 ± 0.1135
X-intercept	-1.187
1/slope	3.606

4.2.4. Ischemia Modified Albumin

Method: Ischemia modified albumin determined as per the method of Bar Or D[131].

Principle: Normal human serum albumin has exogenous cobalt binding sites. Ischemia condition modifies HSA and reduces $cobalt^{2+}$ binding sites. The concentration of ischemia modified albumin determined by addition of known amount of cobalt to serum specimen and unbound $cobalt^{2+}$ by reacting with dithiothretol quantified in spectrophotometer (Perkin Elmer Lamda 1.2) at 470nm. An inverse relationship exist between the cobalt bound IMA and intensity of the colour.

Reagents

1) Cobalt chloride (10%)
2) Dithiothreitol (15%)
3) Sodium chloride (0.9 %)

Procedure: Added 0.2ml of serum, 0.05ml cobalt chloride and 0.05ml DTT in to test and 0.25ml of demineralized water and 0.05ml cobalt chloride to blank. The content of the tubes were vortexed & incubated for 2 minutes at room temperature. Then added 1ml of 0.9% NaCl to both the tubes. Absorbance was measured at 470 nm in spectrophotometer (Perkin Elmer Lamda 1.2).

Calculation:

IMA value is estimated in absorbance units (ABU)

IMA value = absorbance of test – absorbance of blank.

4.2.5. Uric Acid

Method: Plasma uric acid measured by uricase method as per the kit procedure supplied by Lab-care diagnostics India private limited based on Trivedi RC[132].

Principle: Uric acid is oxidised to allantoin by uricase with the production of H_2O_2. The peroxide reacts with 4-aminoantipyrine and 3, 5-dichloro 2-hydroxy benzene sulfonic acid in the presence of peroxidase to yield a red-violet quinoneimine compound. The absorbance was measured at 505 nm which is proportional to uric acid concentration.

Reagents

1) Reagent 1: Uric acid reagent containing piperazine –N,N'-bis (2-ethane sulfonic acid), 3,5-dichloro 2-hydroxy benzene sulfonic acid, Uricase enzyme, Peroxidase enzyme and 4-Aminoantipyrine.
2) Reagent 2: Uric acid standard (5mg/dl)

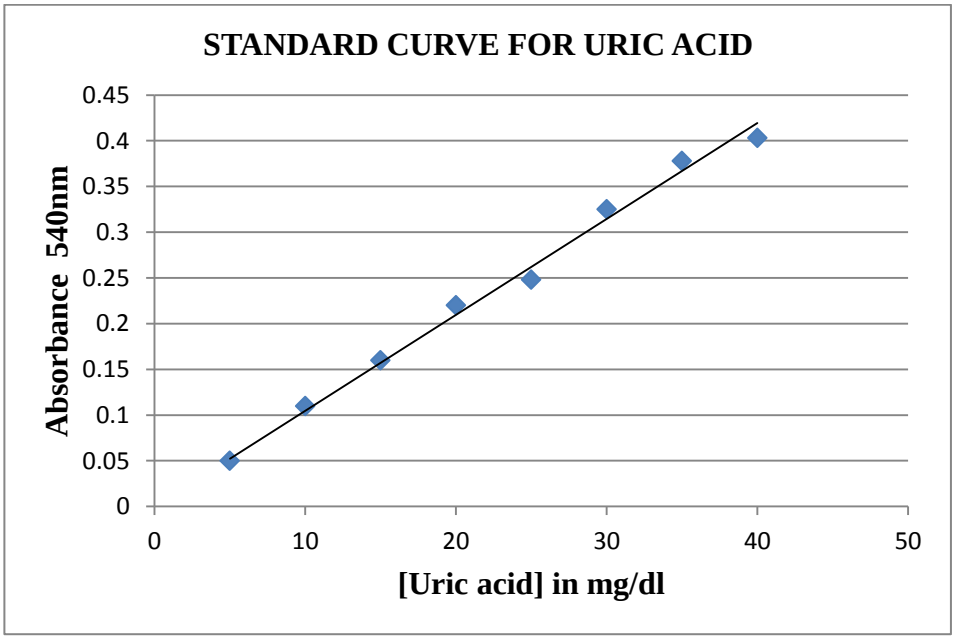
Procedure: Three tubes were labeled as reagent blank, standard and test.To all the tubes 1.0ml of uric acid reagent-1 was added. The standard tube received 0.025ml of reagent-2 and 0.025 ml of serum sample was added to test. Contents in the tubes mixed, incubated for 10 minutes at 37˚C. The absorbance of test and standard were measured against blank at 505nm in spectrophotometer (Perkin Elmer Lamda 1.2).

Calculation:

$$\text{Uric acid (mg/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{Concentration of standard}$$

Preparation of standard curve for uric acid

Standard	Concentration (mg/dl)	Absorbance (505nm)
Standard 1	5	0.05
Standard 2	10	0.11
Standard 3	15	0.16
Standard 4	20	0.22
Standard 5	25	0.248
Standard 6	30	0.325
Standard 7	35	0.378
Standard 8	40	0.403



4.2.6. Superoxide Dismutase

Method: Modified spectrophotometric assay of superoxide dismutase according to the method of P Kakkar [133].

Principle: Superoxide dismutase (SOD) activity measured based on the inhibition of the formation of Phenazenemethosulphate-Nitrobluetetrazolium formazon complex by superoxide. The formation of colour complex at the end of reaction can be extracted in to butanol and measured at 560nm.

Reagents

1. Phenazenemethosulphate (186 µM)
2. Nitro blue tetrazolium (300 µM)
3. NADH (780µM)
4. Sodium pyrophosphate buffer (0.052M) pH 8.3
5. Glacial Acetic acid

Procedure: To the test, added 1.2 ml sodium pyrophosphate buffer, 0.1 ml of phenazene methosulphate, 0.3ml of nitro blue tetrazolium, 0.1 ml of plasma, 0.2ml of NADH. Mixtures in the tube incubated for 90 seconds at 30˚C and then stopped the reaction by adding 1.0 ml of acetic acid. Finally, added 4.0 ml of n-butanol and the reaction mixture was vigorously shaken to extract chromogen to butanol. The mixture was allowed to stand for 10 minutes in dark at RT, centrifuged, the chromogen complex extracted in to butanol layer was sepearted. The colour intensity of the chromogen in butanol layer was measured at 560nm in spectrophotometer (Perkin Elmer Lamda 1.2) against butanol. The reaction mixture in the tube without plasma served as control.

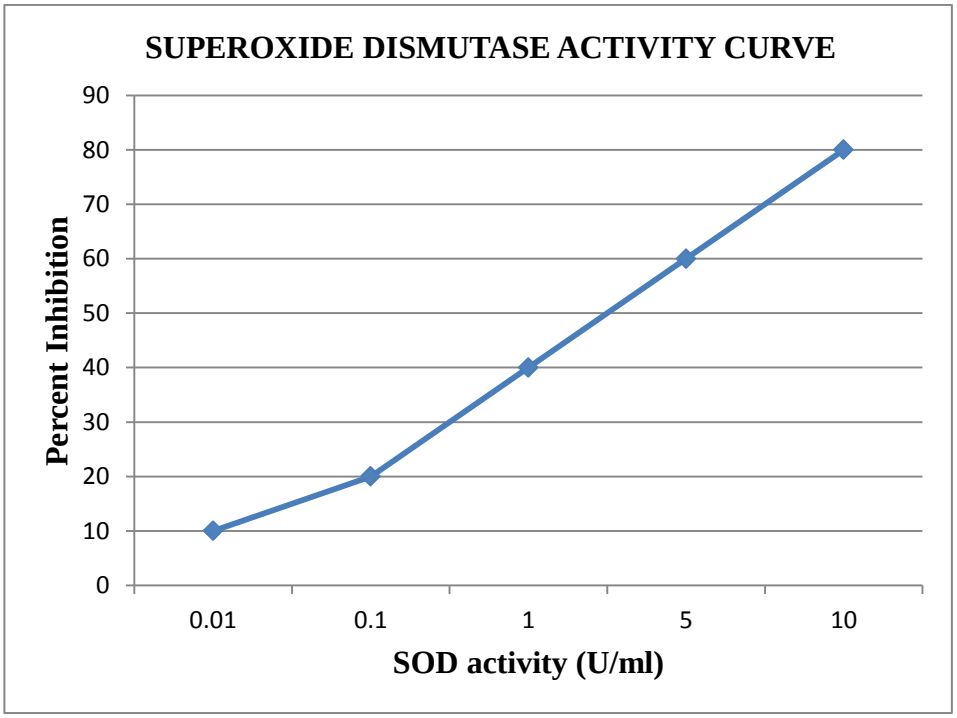
Calculation:

$$\text{Enzyme Units/ml} = \frac{\text{Maximum Inhibition}}{(50\%\ \text{inhibition})(\text{volume of the sample used})}$$

Unit of enzyme activity: One unit is defined as that amount of enzyme required to cause 50% of the maximum inhibition of nitro blue tetrazolium reduction in one minute.

Preparation of superoxide dismutase (SOD) activity curve

Standard	SOD Activity (U/ml)	Percent Inhibition
Standard 1	0.01	10
Standard 2	0.1	20
Standard 3	1	40
Standard 4	5	60
Standard 5	10	80



4.2.7. Glutathione Peroxidase

Method: Glutathione peroxidase measured by continuous spectrophotometric rate determination method according to Wendel [134].

Principle: Glutathione peroxidase reduces hydrogen peroxide in to water in a coupled reaction with glutathione reductase using reduced glutathione. The oxidized form of glutathione convert to reduced form in the presence of NADPH. The decrease in absorbance was measured at 340nm.

Reagents

1. Sodium phosphate buffer pH 7 (100mM)
2. Sodium azide solution (2 mM)
3. β-Nicotinamide adenine dinucleotide phosphate, reduced form (2 mM)
4. Glutathione reductase(2.4U/ml)
5. Reduced glutathione (10mM)
6. Hydrogen peroxide (1.5mM)

Procedure: Added 0.55ml of phosphate buffer, 0.05ml of sodium azide, 0.05ml sample, 0.1ml glutathione reductase, 0.1ml glutathione and 0.1ml NADPH in to a test. Mixed the contents and equilibrated to 25˚C. Monitored the absorbance until constant.Then added 0.05ml of H_2O_2. Immediately mixed the contents and recorded the decrease in the absorbance per minute (ΔA) for 5 minutes at 340nm in spectrophotometer (Perkin Elmer Lamda 1.2).

Calculation:

$$\text{Enzyme Units/ml} = \frac{(\Delta A \text{ of Test} - \Delta A \text{ of Blank}) \times 2.0 \times 3.1}{6.22 \times 0.05}$$

2.0 =2µmoles of GSH produced per 1.0 µmole of NADPH oxidized

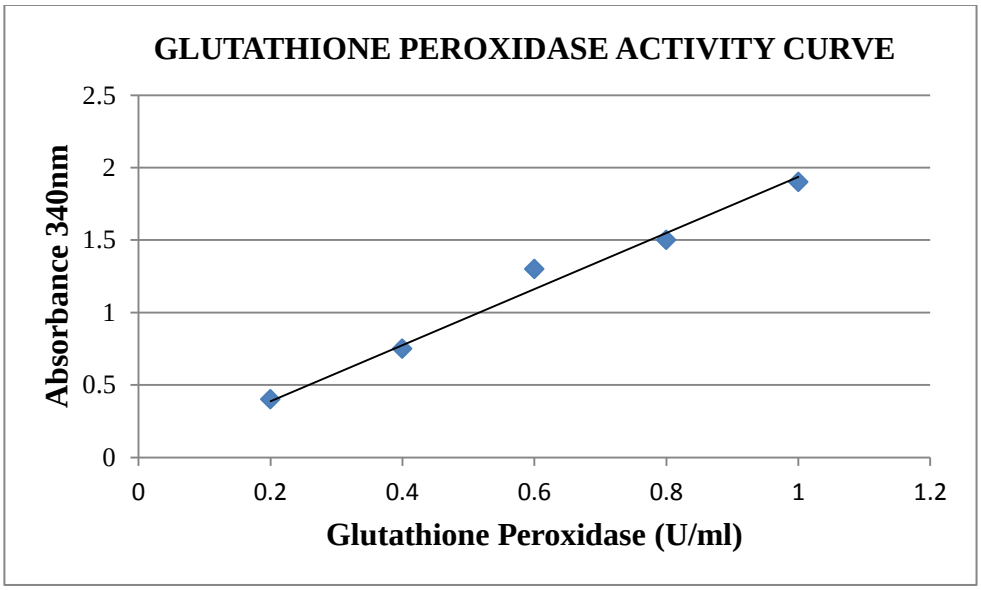
3.1ml=Total volume, 0.05ml=Volume of enzyme

6.22=Molar extinction coefficient of NADPH at 340nm

Unit of enzyme activity: One unit will catalyze the oxidation by hydrogen peroxide of one micromole of reduced glutathione to oxidized glutathione per minute at pH 7.0 at 25 ˚C.

Preparation of glutathione peroxidase activity curve

S.NO	Glutathione Peroxidase (Units/ml)	Absorbance (340nm)
1	20	0.4
2	40	0.75
3	60	1.3
4	80	1.5
5	100	1.9



4.2.8. Glutathione Reductase

Method: Glutathione reductase measured by continuous spectrophotometric rate determination method according to Mavis RD [135].

Principle: Reduced NADPH reacts with oxidized glutathione in the presence of glutathione reductase to form oxidized NADP and reduced glutathione.The utilization of NADPH in a reaction measured as decrease in absorbnce.

Reagents

1) Sodium phosphate buffer pH 7 (100mM) with (3.4 mM EDTA)
2) Oxidized glutathione (30mM)
3) Reduced β-nicotinamide adenine dinucleotide phosphate (0.8 mM)
4) Glutathione reductase(0.30-0.60 U/ml)
5) Bovine serum albumin (1%)

Procedure: Added 0.650 ml distilled water, 1.5 ml buffer, 0.1ml oxidized glutathione, 0.350ml NADPH and 0.3ml BSA to the test. Mixed the contents and equilibrated to 25˚C. Monitor the absorbance until constant and then add 0.1ml of sample. Immediately mix and record the decrease in absorbance for approximately 5 minutes in spectrophotometer (Perkin Elmer Lamda 1.2) at 340nm. Obtain the ΔA at 340 nm/min.

Calculation:

$$\text{Enzyme Units/ml} = \frac{(\Delta A \text{ of Test} - \Delta A \text{ of Blank}) \times 3}{6.22 \times 0.1}$$

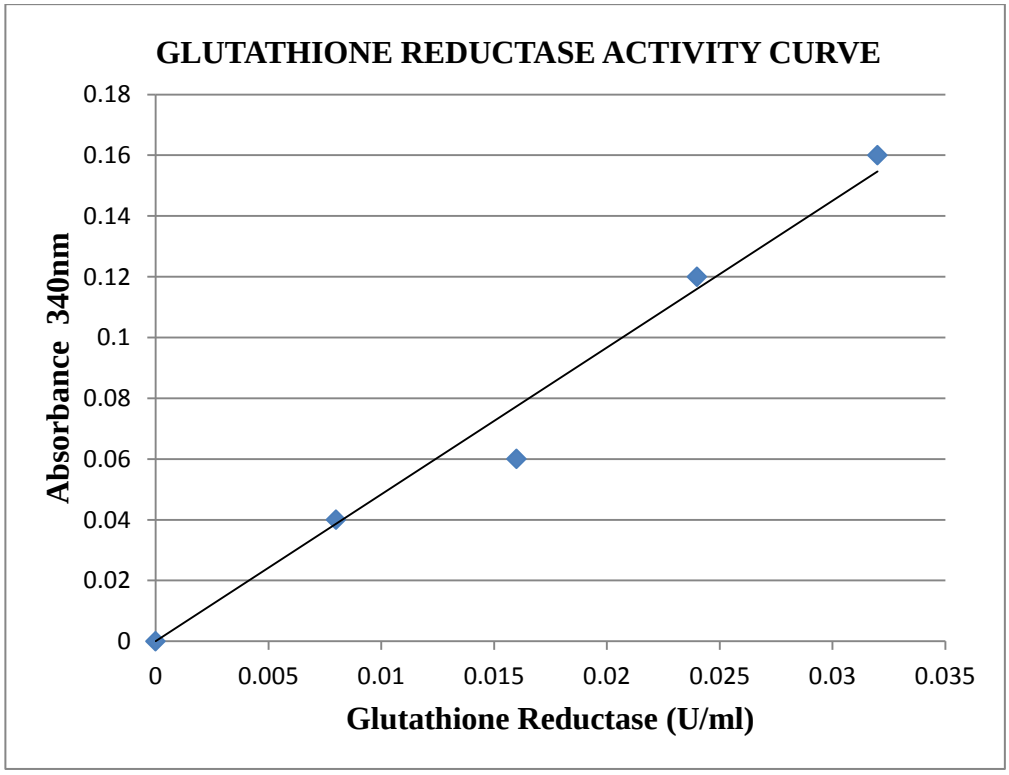
3.1ml= Total volume, 6.22= mMolar extinction coefficient of NADPH at 340nm

0.1ml= Volume of enzyme

Unit of enzyme activity: One unit will reduce 1 µmole of oxidized glutathione per minute at pH 7.6 at 25˚C.

Preparation of glutathione reductase activity curve

S.NO	Glutathione Reductase (Units/ml)	Absorbance (340nm)
1	0.008	0.04
2	0.016	0.06
3	0.024	0.12
4	0.032	0.16



4.2.8. Catalase

Method: Catalase measured by continuous spectrophotometric rate determination method according to Aebi H [136].

Principle: The catalae enzyme catalyses decomposition of hydrogen peroxide to ground state oxygen and water.The rate of decomposition of hydrogen peroxide by derease in the absorbance measured spectrophotometrically at 240nm.

Reagents

1) Phosphate buffer pH 7.0 (50mM)
2) Hydrogen peroxide (30mM)

Procedure: Added 0.5ml phosphate buffer, 0.5ml of hydrogen peroxide, 0.01ml of 1:10 diluted plasma in to a test. The decrease in absorbance was recorded for 1 minute at 240 nm in spectrophotometer (Perkin Elmer Lamda 1.2).

Calculation:

$$\text{Catalase activity} = \frac{\Delta\ \text{Absorbance / min}}{\text{Extinction coefficient}} \times \frac{\text{Total volume}}{\text{Sample volume}}$$

Extinction coefficient= 0.0436

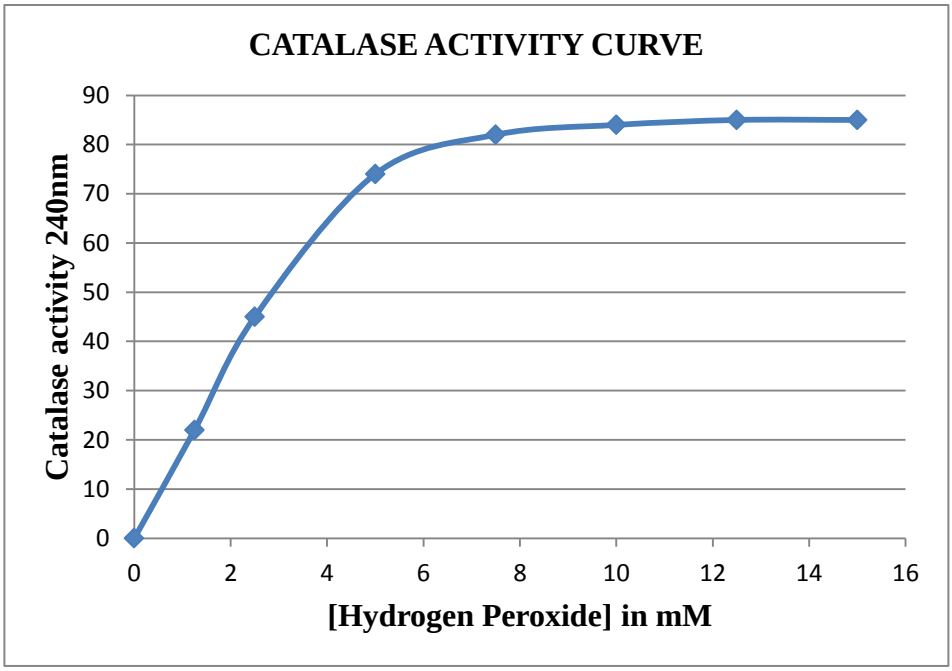
Total volume =1010 µl

Sample volume= 10 µl

Unit of enzyme activity: One unit of catalase will decompose 1.0µmole of H_2O_2 per minute at pH 7.0.

Preparation of catalase activity curve

S NO	Substrate (mM)	Catalase activity
1	1.25	22
2	2.5	45
3	5	74
4	7.5	82
5	10	84
6	12.5	85
7	15	85



4.2.9. Vitamin C (L-Ascorbic acid)

Method: L-ascorbic acid determined according to the method of ROE J.H. [137].

Principle: Ascorbic acid in plasma is oxidized by Cu 2^+ to form dehydroascorbic acid, which reacts with acidic 2,4-DNPH to form red bis-hydrazone, which is measured at 520nm.

Reagents:

1. Metaphosphoric acid (6%)
2. Sulphuric acid (4.5mol/L)
3. Sulphuric acid (12mol/L)
4. 2,4-DNPH (2%)
5. Thiourea (5%)
6. Copper sulphate solution (0.6%)
7. DNPH-thiourea-coppersulphate (DTCS) reagent
8. Ascorbic acid standard (50mg/dl)

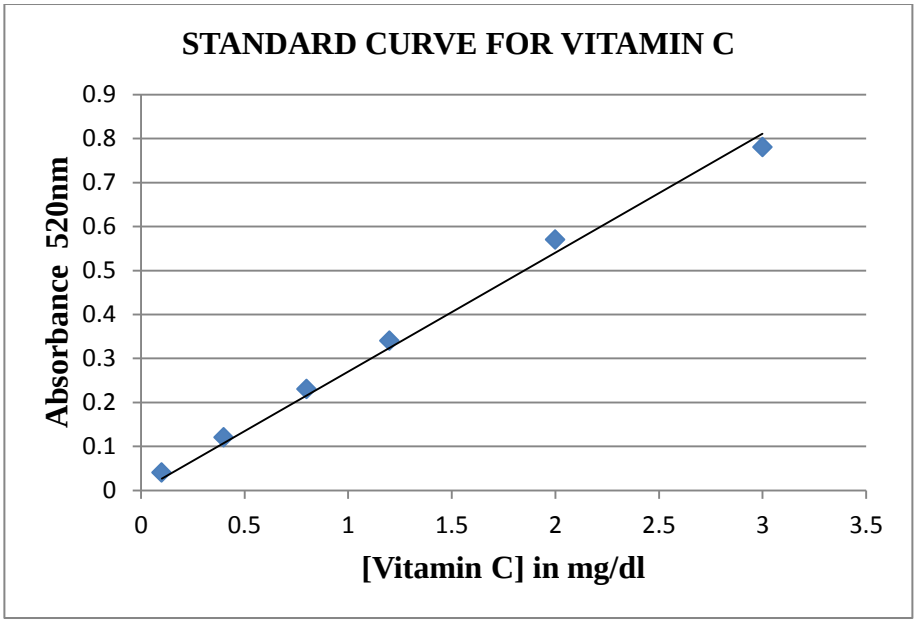
Procedure: 0.5 ml of plasma was added to 2.0 ml of freshly prepared metaphosphoric acid, vortexed and centrifuged for 10 minutes at 2500rpm. 1.2ml of the clear supernatant was taken in a tube. 1.2 ml of working ascorbic standards was taken in another tube. 1.2 ml of metaphophoric acid was taken as blank. 0.4ml DTCS reagent was added to all the tubes mixed and incubated at 37 ˚C in a water bath for 3 hours. After incubation the test tubes were chilled for 10 minutes on ice bath. 2.0 ml of cold sulphuric acid (12 mol/L) was added with mixing and absorbance was read at 520nm in spectrophotometer (Perkin Elmer Lamda 1.2).

Calculation:

$$[\text{Ascorbic acid}]\ \text{mg/dl} = \frac{\text{Absorbance of Test}}{\text{Absorbance of standard}} \times \text{Conc. of standard (5mg/dl)}$$

Preparation of standard curve for vitamin C

Standard	Concentration (mg/dl)	Absorbance (520nm)
Standard 1	0.1	0.04
Standard 2	0.4	0.12
Standard 3	0.8	0.23
Standard 4	1.2	0.34
Standard 5	2	0.57
Standard 6	3	0.78



4.2.10. Vitamin E

Method: Vitamin E measured according to the method of Martinek RG [138].

Principle: Plasma α-tocopherol reduces ferric form of iron to ferrous state, which forms a red complex on reaction with α, α^1-dipyridyl. Tocopherols and carotenes were first extracted in to petroleum ether and the absorbance was read at 460 nm to measure the carotenes. A correction was made for this after adding ferric chloride and read at 520 nm to measure tocopherol.

Reagents:

1. Petroleum ether
2. Absolute ethanol
3. α, α^1-dipyridyl (0.2%)
4. Ferric chloride (0.1%)
5. Chloroform
6. Vitamin E stock standard (1mg/ml ethanol)

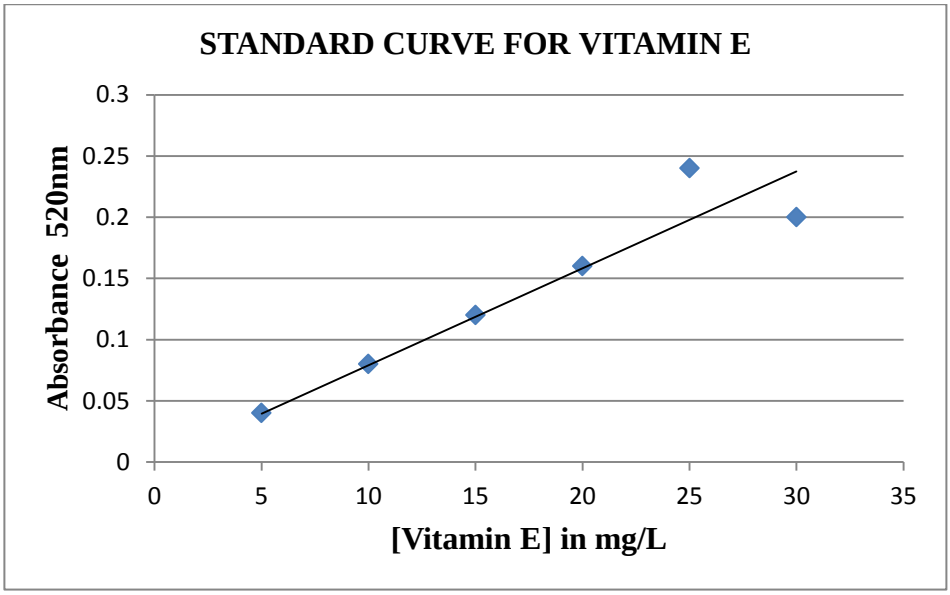
Procedure: Samples for vitamin E assay was stored in screw capped vial wrapped with aluminium foil to minimize vitamin E loss on exposure to light. Added 0.5 ml of stored plasma and 0.5ml of absolute ethanol to a test, mixed and added 1.5ml of petroleum ether, vortexed for 3 minutes, centrifuged and collected 1.5ml of clear supernatant. The absorbance was measured at 460 nm for carotenes and petroleum ether was subjected for evaporation at 50˚C. A residue obtained was dissolved in 1.0ml of chloroform. To this added1.0 ml of ethanol, 1.0ml α,α^1-dipyridyl and 0.1 ml of ferric chloride solution. Incubated 1 minute in dark and absorbance was measured at 520nm in spectrophotometer (Perkin Elmer Lamda 1.2).

Calculation:

$$\text{Vitamin E (mg/L)} = \frac{(\text{A of Test at 520 nm} - \text{A of Test at 460nm} \times 0.29)}{(\text{A of std at 520 nm})} \times \text{Conc. of standard}$$

Preparation of standard curve for vitamin E

Standard	Concentration (mg/L)	Absorbance (560nm)
Standard 1	5	0.04
Standard 2	10	0.08
Standard 3	15	0.12
Standard 4	20	0.16
Standard 5	25	0.24
Standard 6	30	0.2



4.2.11. Ferric reducing ability of plasma (FRAP)

Method: The FRAP measured as per the method of Benzi IF [139].

Principle: Plasma reducing ability at low pH reduces reduces ferric tripyridyltriazine complex to ferrous form that produses an intense blue colour measured spectrophotometrically at 593 nm.

Reagents:

1. Acetate buffer pH 3.6 (300mmol/L)
2. $FeCl_3.6H_2O$ (20mmol/L)
3. HCl (40mmol/L)
4. 2,4,6-Tripyridyl -triazine (10mmol/L)

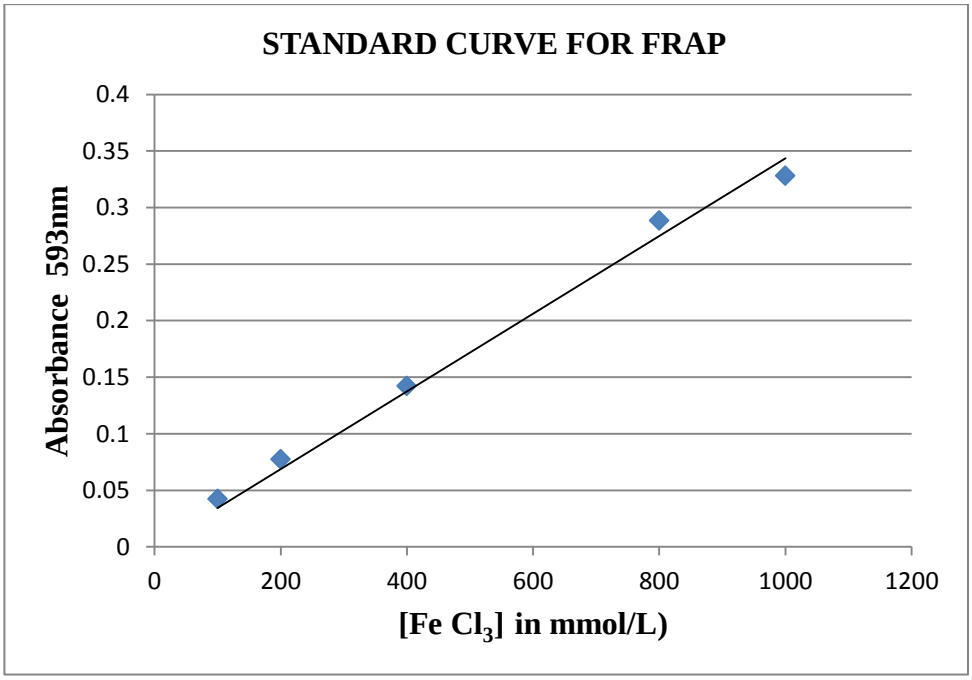
Procedure: Added 0.4ml of acetate buffer, 0.044ml of ferric chloride to test and 0.44ml of acetate buffer to control. Then 0.04ml of 2, 4, 6-tripyridyl-triazine was added to both test and control tubes, 0.06ml of the plasma to test and the tubes were incubated at 37˚C for 30 minutes. Then added 0.06ml of plasma to control tube after incubation. Reagent blank contained everything except sample. The intensity of the blue colour produced was measured at 593 nm against blank in spectrophotometer (Perkin Elmer Lamda 1.2).

Calculation:

$$\text{Total antioxidant capacity (µmol/L)} = \frac{\text{OD of Test} - \text{OD of Control}}{\text{OD of Standard} - \text{OD of Blank}} \times 1000$$

Preparation of standard graph for ferric reducing ability of plasma

Standard	Concentration (mmol/L)	Absorbance (593nm)
Standard 1	100	0.0425
Standard 2	200	0.0775
Standard 3	400	0.1422
Standard 4	800	0.2884
Standard 5	1000	0.3281



4.2.12. Nitrate

Method: Nitrate measured according to the method of Cortas NK [140].

Principle: Nitrate, the stable product of nitric oxide is reduced to nitrite by cadmium reduction after deproteinization. The nitrite produced is determined by diazotization of sulphanilamide and coupling with napthyl ethylene diamine.

Reagents

1. Glycine-NaOH buffer pH 9.7 (0.5M)
2. Sulphanilamide (1%)
3. N-Naphthylethylenediamine (0.2%)
4. Sodium nitrite stock standard (100mmol/L)
5. Working standard (10 µmol/L)
6. $ZnSO_4$ solution (75mmol/L)
7. NaOH solution (55mmol/L)
8. H_2SO_4 solution (0.1mol/L)
9. $CuSO_4$ solution (5mmol/L)

Procedure:

A) Deproteinization: In a clean, dry centrifuge tube 0.5ml of plasma was taken and 2.0 ml of 75mmol/L $ZnSO_4$ solution was added. After mixing 2.5 ml of 55mmol/L NaOH was added, contents was mixed again and centrifuged for 10 minutes at 3000rpm.

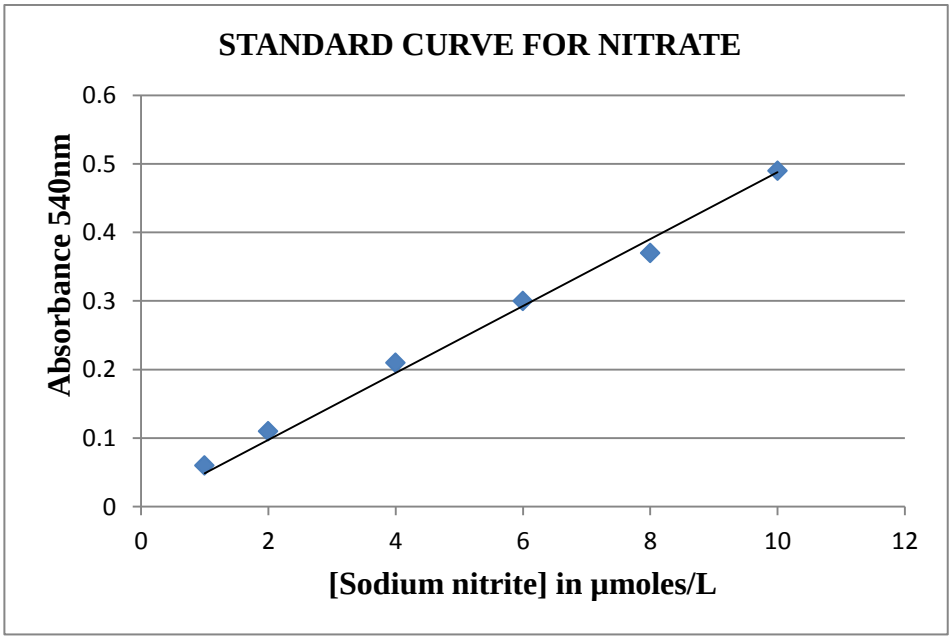
B) Activation of cadmium granules: Cadmium granules were stored in 0.1 mol/L H_2SO_4 solution. At the time of assay, the acid from granules was rinsed three times with deionized water. Then the granules were swirled in 5mmol/L $CuSO_4$ solution for 1-2 minutes.These copper coated cadmium granules were drained & washed with glycine

NaOH buffer.These activated granules were used within 10 minutes after activation.The granules were washed after use with deionised water and stored in 0.1mol/L H_2SO_4 .

C**)** Nitrite Assay: Three Erlenmeyer flasks were taken and labelled as test, standard and blank. To each of the flasks 1.0 ml glycine-NaOH buffer was added. To the flasks labeled as blank, test and standard; 1.0ml of deionised water, deproteinized sample and working standard solution (10µmol/L) was added respectively.With a spatula, 2.5 to 3.0 gm of freshly activated cadmium granules was added to each flask. All the flasks were stirred for swirling the granules. After 90 minutes, the mixture in all the three flasks was diluted to 4 ml with deionised water. 2.0 ml of this above diluted solution was pipetted out in clean, dry test tubes labeled as blank, test and standard respectively.1.0 ml of sulphanilamide followed by 1.0ml of N-naphthyl ethylene diamine solution was added to each tube, mixed well. The intense pink colour produced measured after 20 minutes against blank at 540 nm in spectrophotometer (Perkin Elmer Lamda 1.2).

Preparation standard curve for nitrate

Standard	Concentration (µmoles/L)	Absorbance (540nm)
Standard 1 (S1)	1	0.06
Standard 2 (S2)	2	0.11
Standard 3 (S3)	4	0.21
Standard 4 (S4)	6	0.3
Standard 5 (S5)	8	0.37
Standard 6 (S6)	10	0.49



Calculation:

$$\text{Concentration of nitrate (µmoles/L)} = \frac{\text{OD of T} - \text{OD of Control}}{\text{OD of Std} - \text{OD of Blank}} \times 10 \text{ µmoles/L}$$

4.2.13. Elastase activity

Method: Elastase measured by continuous spectrophotometric rate determination according to the method of Bieth J [141].

Principle: Elastase (EC 3.4.21.36) hydrolyses substrate N-succinyl-ala-ala-ala-p-nitroanilide to products N-succinyl-ala-ala-ala and p-nitroanilide. The absorbance of p-nitroanilide was measured in a spectrophotometer at 410 nm.

Reagents

1. Tris (hydroxymethyl) amino methane (Tris) buffer pH 8.0 (100 mM)
2. N-succinyl-Ala-Ala-Ala-*p*-nitroanilide (4.4mM)
3. Elastase (0.2–0.5 unit/ml)

Procedure: 2.7ml Tris buffer, 0.2ml N-succinyl-ala-ala-ala-p-nitroanilide was added to test tube. Mixed and then added 0.1ml of plasma, mixed well and recorded the increase in absorbance at 410nm for 5 minutes to obtain change in absorbance per minute in spectrophotometer (Perkin Elmer Lamda 1.2).

Calculation:

$$\text{Enzyme Units/ml} = \frac{(\Delta A410\text{nm Test} - \Delta A410\text{nm/min Blank})(3.00)}{8.8 \times 0.1}$$

3.00=Total volume

8.8= Millimolar extinction coefficient of p-Nitroaniline at 410nm at pH 8.0

0.1=Volume of enzyme

Unit of enzyme activity: One unit of elastase will hydrolyze 1.0 µmole of N-succinyl-L-ala-ala-ala-p-nitroanilide per minute at pH 8.0 at 25 ˚C.

4.2.14. High sensitive-C-reactive protein

Method: Quantitative turbidimetric method using euro diagnostic kit

Principle: The CRP-ultrasensitive is a quantitative test for the measurement of low levels of C- reactive protein (CRP) in human serum or plasma. Latex particles coated with specific anti- human CRP are agglutinated when mixed with samples containing CRP. The agglutination causes an absorbance change, dependent upon the CRP contents of the patient sample that can be quantified by comparison from a calibrator of known CRP concentration.

Reagents

1. Diluent R1: Containing tris buffer (20 mmol/L) and sodium azide pH 8.2 (0.95g/L)
2. Latex R2: Containing latex particle coated with goat IgG anti human CRP and pH 7.3 sodium azide (0.95g/L)

Procedure: Added 0.8ml R1diluent, 0.2ml R2 latex and 0.010ml of serum sample. Mixed the contents and incubated for 4 minutes. Then absorbance was read at 540nm in spectrophotometer (Perkin Elmer Lamda 1.2).

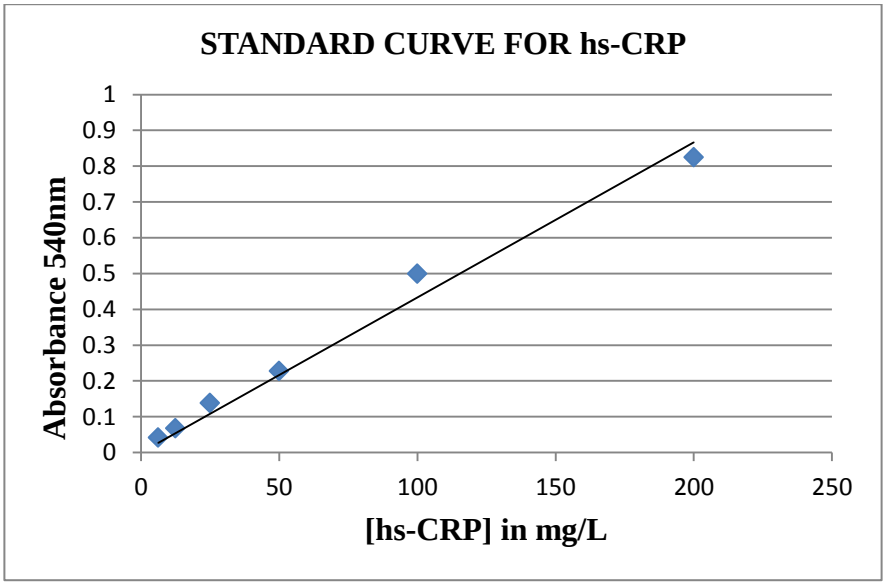
Calculation:

The concentration of the hs-CRP in the sample (mg/L) was calculated by interpolation of its absorbance (T-B) in the calibration curve.

Linearity limit= Up to 5 mg/L
Detection limit=<0.05mg/L
Reference value below 3mg/L considered as normal.

Preparation of standard curve for high sensitive-C- reactive protein

Calibrators	Concentration mg/L	Absorbance (540nm)
Calibrator1	6.25	0.042
Calibrator2	12.5	0.068
Calibrator 3	25	0.138
Calibrator 4	50	0.228
Calibrator 5	100	0.499
Calibrator 6	200	0.825



4.2.15. Phospholipase A_2

Method: Phospholipase A_2 measured according to the method described by Price JA[142].

Principle: Phospholipase A_2 hydrolyses the 2-fattyacyl ester bond of phospho glycerides liberating free fatty acids and lysophospholipids. Based on the absorbance change of bromothymol blue indicator with the concentration of hydrogen ion released from enzyme catalyzed reaction was measured at 620 nm.

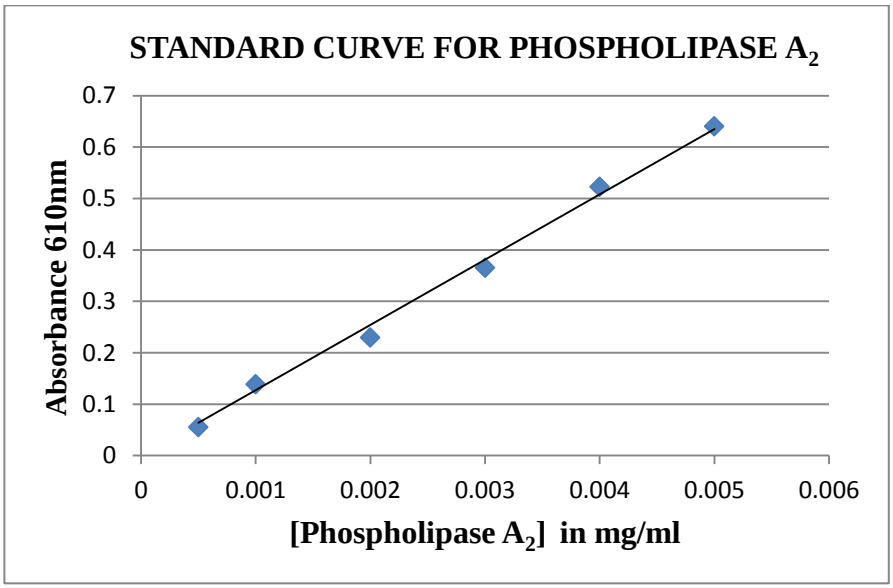
Reagents

1) 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (2 mM) HEPES buffer pH 7.5
2) TritonX-100 (5 mM)
3) Phosphatidylcholine (5 mM)
4) Calcium chloride (10 mM)
5) W/W. Bromothymol blue dye pH 7.5(0.124%)

Procedure: 0.180 ml of an assay mixture obtained by mixing equal volume of 5 mM Triton X-100, 5 mM phosphatidylcholine, 2 mM HEPES buffer, 10 mM calcium chloride and 0.124% bromothymol blue dye in water at pH 7.5 at 37 ˚C was added to test. Then 0.020 ml of the sample was added and mixed. Rate of change in absorbance at one minute interval was measured at 620nm against blank containing buffer in spectrophotometer (Perkin Elmer Lamda 1.2).

Preparation of standard graph for Phospholipase A_2

Standard	Concentration (mg/ml)	Absorbance (610nm)
Standard 1	0.0005	0.0551
Standard 2	0.001	0.139
Standard 3	0.002	0.2298
Standard 4	0.003	0.3654
Standard 5	0.004	0.5225
Standard 6	0.005	0.6402



Calculation:

Phospholipase A_2 (mg/ml):

Y = $mx + c$, x= $\frac{y-c}{m}$, x = $\frac{y-(-0.005)}{128.9}$

X = $\frac{y+0.005}{128.9}$

X = [mg/ml enzyme]

Enzyme Units/ml = $\frac{\Delta A/\min}{M \times 0.5 \times [\text{mg/ml enzyme}] \times 0.8}$

M=Slope of standard curve

0.5=Conversion factor (lysolecithin formation has one half the absorbance of lecithin)

0.8= Conversion factor of substrate from mg to µM substrate

4.2.16. Xanthine Oxidase Inhibition

4.2.16.1. Collection of plant material:

Screening of xanthine oxidase inhibition was carried out by using Indian conventional plants having medicinal property. They are *Pongamia pinnata L (seeds), Morinda citrifolia L (fruit), Mangifera indica L (bark), Zingiber officinale Roscoe (root) were* collected and authenticated from the Horticulture College, Tamaka, Kolar.

4.2.16.2. Preparation of plant extract:

The extraction of *Pongamia pinnata L (seeds), Morinda citrifolia L (fruit), Mangifera indica L (bark), Zingiber officinale Roscoe (root)* were carried out by the following procedure. The plant materials were cleaned, air dried at room temperature in dark, ground to fine powder using pestle and mortar.The powder was stored in amber coloured bottle and kept in dark at room temperature until extraction.

Five grams of powder obtained was dissolved in 50 ml absolute methanol and subjected for filtration by using Whatman No.1 filter paper. The filtrate was concentrated in vacuum evaporator under reduced pressure and air dried. Thus obtained powder was stored in sterile bottles at 4°C until further use[143].

4.2.16.3. Isolation and Purification of Flavonoids from the plant extracts by column chromatography: A glass column measuring 50 cm × 2 cm dimension developed using methanol with silica gel adsorbent on glass wool and allowed to settle by gravity flow. Column was allowed to equilibrate with suitable methanol as elution solvent. The even surface of the silica gel in the column protected by placing whatman

no.1 filter paper disc. 1.0 gm/ml of processed crude extract was applied for separation. All the eluted fractions were tested for flavonoid content, the active fractions were pooled and air dried under sterile conditions. The concentrated dried powder subjected for qualitative confirmation of flavonoids using dimethyl sulfoxide as a dissolving solvent and quercetin as internal standard [144].

4.2.16.4. Qualitative Detection of Flavonoids:

Two ml of above extract was treated with few drops of 20% sodium hydroxide which produced intense yellow color and on further addition of dilute hydrochloric acid becomes colorless confirming the presence of flavonoids[145].

Xanthine oxidase inhibition assay was done using pure form of commercially obtained vitamin C, vitamin E. Flavonoid extract obtained from *Pongamia pinnata L* (seeds), *Morinda citrifolia L* (fruit), *Mangifera indica L* (bark) *and Zingiber officinale Rosc* (root) were also tested for xanthine oxidase inhibition and were compared with quercetin as standard flavonoid and allopurinol as reference inhibitor.The concentration of isolated flavonoids ranges from 6 to 800 µg / ml were tested to detect inhibitory action on xanthine oxidase activity. Percent of inhibition, IC_{50} and inhibitory constant were calculated for the above inhibitors.

4.2.16.5. Determination of Percent of inhibition of Xanthine oxidase

Flavonoids of the above plants, vitamin E and C along with allopurinol were tested for xanthine oxidase inhibition.

Percentage of inhibition was calculated using below formula,

$$\% \text{ of inhibition} = \frac{(\text{Total activity of XO without Inhibitor}) - (\text{Total activity of XO with Inhibitor})}{\text{Total activity without Inhibitor}} \times 100$$

5.2.16.6. Determination of IC_{50} and inhibitory constant (K_i) of Xanthine oxidase

IC_{50} and K_i are measures of inhibitors ability to decrease enzyme activity [56, 146]. IC_{50} denotes the amount of an inhibitor that reduces the rate of an enzymatic reaction by 50%, it was calculated using graph prepared by plotting concentration of inhibitor on ordinate and percentage of inhibition on abscissa.

Inhibitory constant (K_i) is a true equilibrium constant and pure measure of constant enzyme-inhibitor complex; it was calculated using Cheng-prussoff equation.

$$K_i = \frac{IC\ 50}{1+[S]/Km}$$

4.2.17. Statistical analysis

The results were expressed as mean ± standard error mean and analyzed using one way ANOVA test with post-hoc Bonferroni analysis to compare the values between the three groups. Paired‘t’ test was used to compare before and after delivery status between the same group. Pearson correlation analysis was used to find the correlation between the various parameters. Receiver operating characteristics (ROC) curve analysis was done to assess diagnostic utility of a parameter in the study. A probability *p* value of <0.05 was considered as statistically significant. Statistical analysis was performed with the licensed version of SPSS 20.

CHAPTER-5

RESULTS AND DISCUSSION

5.0. RESULTS AND DISCUSSION

5.1. RESULTS

The study population was divided into 3 groups. Group 1 (n = 57) comprising of non-pregnant as control population; group 2 (n = 57) as normotensive pregnant and group 3 (n = 57) as preeclampsia cases. Normotensive pregnant and preeclampsia were in 30-39 weeks of gestation before delivery and same subjects followed after delivery within 48 hours. Normotensive pregnant and preeclampsia subjects were clinically diagnosed from the Department of Obstetrics and Gynecology. The age matched control populations (G1) were randomly recruited from the volunteers of Sri Devaraj Urs Medical College and were in 20-30 years.

The demographic characteristics such as age distribution of the normotensive pregnant and preeclampsia were depicted in percentage. 25% of the normal pregnant population were in the 18-20 age groups and 24% had preeclampsia. In 21-25 age group 56% were normotensive pregnant and 51% were in preeclampsia group. In 26-30 age group 17% were normotensive and 16% were in preeclampsia. The age group of more than 30 years 2% were in normotensive and 9% had preeclampsia. The total number preeclampsia cases of group 3 showed about 55 % of the cases were in primigravida.

The distribution of gestational age for the subjects of normotensive pregnant and preeclampsia were represented as percentage. 4% of the normal pregnant and 16% in preeclamptic group population was in the 28-33 gestational week. In 34-37 gestational week 14% were normotensive and 25% were preeclamptic. Gestational week between 38-40 had 82% normotensive and 59% preeclampsia.

Table 1: Demographic and haematological characteristics of the normotensive pregnant and preeclampticwomen

Parameters	Normotensive (Mean±SEM) (n=57)	Preeclampsia (Mean±SEM) (n=57)	p value
Gestational age	38.77±0.23	36.50±0.35	<0.001**
Systolic blood pressure (mm/Hg)	116.64±1.9	155.80±2.3	<0.001**
Diastolic blood pressure (mm/Hg)	80.58±1.4	100.80±1.7	<0.001**
Hemoglobin (gm/dl)	10.95±0.24	10.62±0.26	>0.05
Packed Cell Volume (%)	33.30±0.77	31.71±0.76	>0.05
Red Blood Cells (million cells/cu mm)	4.16±0.09	4.39±0.21	>0.05
White Blood Cells (cells/cu mm)	45,136±0.6	79,000±0.5	<0.001**
Platelets (lakhs/cu mm)	2.77±0.08	2.33±0.12	<0.001**
RBS (mg/dl)	63.06±4.2	75.41±1.8	>0.05

** p value <0.001

The demographic, haematological and biochemical characteristics of normotensive pregnant and preeclampsia cases were illustrated in Table 1.These investigations were screened during patient visit to Department of Obstetrics and Gynecology, for antenatal check-up. Significance was noticed for gestational age, systolic blood pressure, diastolic blood pressure, WBC and Platelet had p value <0.001 between normotensive pregnant and preeclampsia.

Table 2: Hematological characteristics of the mild and severe preeclamptic women

Parameters	Mild preeclampsia (Mean±SEM), (n=20)	Severe preeclampsia (Mean±SEM),(n=37)
Gestational age	36.47±0.71	36.50±0.40
Haemoglobin (gm/dl)	10.7±0.62	10.53±0.44
Packed Cell Volume (%)	31.44±1.2	31.7±0.97
Red Blood Cells (million cells/cu mm)	4.61±0.59	4.27±0.1
White Blood Cells (cells/cu mm)	51,000±2.04	27,719±1.10
Platelets (lakhs/cu mm)	3.64±1.21	2.6±0.15

** Indicates p value <0.001

Table 2 shows the values of the haematological parameters between mild and severe preeclampsia.These haematological parameters were not useful in defining the severity of preeclampsia. These investigations were screened as in preeclampsia patient during their patient visit to Department of Obstetrics and Gynecology for antenatal check-up.

Table 3: Biochemical characteristics of the mild and severe preeclamptic women

Parameters	Mild preeclampsia (Mean±SEM), (n=20)	Severe preeclampsia (Mean±SEM),(n=37)
Random blood sugar (mg/dl)	80±3.2	72.9±2.04
Total Bilirubin (mg/dl)	0.68±0.05	0.65±0.04
Direct Bilirubin (mg/dl)	0.40±0.15	0.21±0.02
AST (IU/L)	30±2.4	31.6±5.2
ALT (IU/L)	25.3±2.1	23.06±1.4
Alkaline phosphatase (IU/L)	256.1±21.1	271±13.6
GGT (IU/L)	26.6±3.1	27.31±2.3
LDH (IU/L)	508±65.4	526±42.0
Total protein (g/dl)	7.5±1.3	6.8±9.99
Albumin (g/dl)	2.8±0.09	2.9±0.08
Albumin/Globulin ratio	0.96±0.07	1.0±0.06

** Indicates p value <0.001

Table 3 shows the values of the liver function tests between mild and severe preeclampsia. The liver function parameters were not useful in defining the severity of preeclampsia. This observation is supported by 2013 ACOG guidelines where the term mild and severe preeclampsia is replaced. These investigations were screened in preeclampsia patient during their visit to Department of Obstetrics and Gynecology for antenatal check-up.

Table 4: Comparison of oxidative stress parameters between the study groups

Parameters	Non pregnant Mean±SEM	Normotensive pregnant Mean±SEM	Preeclampsia Mean±SEM
Malondialdehyde (µmoles/L)	4.53±0.29	6.8±0.72	15.4±1.3
Protein carbonyl (nmol/L)	98.95±5.3	137.21±7.3	153.9±14.8
Ischemia Modified Albumin IMA (ABU)	0.55±0.04	0.77 ± 0.03	1.24±0.04
Xanthine Oxidase (U/L)	3.04±0.03	37.07±6.8	193.6±25.3
Uric acid (mg/dl)	3.8±0.16	4.04±0.20	6.5±0.29

Table 4 shows biochemical parameters under evaluation of oxidative stress by measuring malondialdehyde, ischemia modified albumin, protein carbonyl, xanthine oxidase activity and uric acid in non-pregnant, normal pregnant and preeclampsia cases. Oxidative stress parameters such as malondialdehyde, ischemia modified albumin, protein carbonyl, xanthine oxidase activity and uric acid were significantly increased in preeclampsia when compared to normal pregnant.

Table 5: Comparison of antioxidant parameters between the study groups

Parameters	Non pregnant Mean±SEM	Normotensive pregnant Mean±SEM	Preeclampsia Mean±SEM
Catalase (U/ml)	114.2±8.5	46.5±7.6	84.7±7.6
Glutathione Peroxidase (U/L)	546.7±51.1	606.32±55.4	493.8±56.7
Glutathione Reductase (U/L)	30.5±3.2	12.1±1.0	22.5±3.09
Superoxide dismutase (U/ml)	7.7±0.62	11.6±0.37	8.5±0.61
Vitamin C (mg/dl)	1.31±0.05	0.6±0.04	0.5±0.03
Vitamin E (mg/L)	10.1±0.28	13.5±1.2	9.3±0.63
FRAP (µmoles/ml)	1953±84.08	1203±165.2	685.6±86.3

Table 5 represents nutrient antioxidant status (vitamin C, vitamin E) and enzyme antioxidants (superoxide dismutase, glutathione peroxidase, glutathione reductase, and catalase) were measured. Besides the antioxidant power of plasma denoted by FRAP was also determined. Antioxidants such as vitamin C, vitamin E, superoxide dismutase, glutathione peroxidase and ferric reducing ability of plasma were decreased in preeclampsia when compared to normal pregnancy. However increase in glutathione reductase and catalase was noticed in preeclampsia when compared to normal pregnancy. Results indicated inverse relation between oxidative stress and antioxidant status (FRAP) in preeclampsia patients.

Table 6: Comparison of inflammatory and endothelial dysfunction parameters between the study groups

Parameters	Non pregnant Mean±SEM	Normotensive pregnant Mean±SEM	Preeclampsia Mean±SEM
Elastase (U/ml)	4.01±0.17	5.8±0.40	26.85±10.5
Phospholipase A_2 (U/ml)	68.7±3.02	70.7±2.4	79.02±3.6
hs-CRP (mg/L)	2.9±0.21	8.6±1.9	11.5±1.9
Nitric Oxide (µmoles/L)	8.46 ± 3.02	7.3 ±2.4	6.3 ± 3.6

Table 6 depicting the mean ± SEM of elastase, phospholipase A_2 and hs-CRP as an inflammatory indicator. Considerable increase in elastase (5.8 U/ml ±0.40) Phospholipase A_2 (70.7 U/ml ±2.4) and hs-CRP (8.6 mg/L ±1.9) in normal pregnancy when compared to non pregnant control and further increase in these parameters (26.85 ± 10.5, 79.02 ± 3.6, 11.5 ±1.9) respectively in preeclampsia when compared to normal pregnancy. Results analysis showed that the marked rise in elastase, phospholipase A_2

enzyme activity in preeclampsia in comparison with the well-known inflammatory marker hs-CRP. In preeclampsia, there was 6.7 fold increase of elastase enzyme and 4.0 fold increase of hs-CRP observed when compared to non pregnant.

Table 7: Comparisons of biochemical parameters showing significance between groups in study population by Post-hoc Bonferroni test

Parameters	Non pregnant vs. Normal pregnant	Non pregnant vs. Preeclampsia	Normal pregnant vs. Preeclampsia
Malondialdehyde (µmoles/L)	0.180	0.001	0.001
Protein carbonyl (nmol/L)	0.023	0.001	0.723
IMA (ABU)	0.001	0.001	0.001
Xanthine oxidase (U/L)	0.266	0.001	0.001
Uric acid (mg/dl)	1.000	0.001	0.001
Glutathione peroxidase (U/L)	1.000	1.000	0.602
Glutathione reductase (U/L)	0.001	0.098	0.018
Superoxide dismutase (U//ml)	0.001	0.894	0.001
Catalase (U/ml)	0.001	0.023	0.003
Vitamin C (mg/dl)	0.001	0.001	0.156
Vitamin E (mg/L)	0.008	1.000	0.001
FRAP (µmoles/ml)	0.001	0.000	0.007
Nitric oxide (µmoles/L)	0.337	0.007	0.426
Elastase (U/ml)	1.000	0.026	0.046
Phospholipase A_2 (U/ml)	1.000	0.058	0.175
hs-CRP (mg/L)	0.027	0.001	0.584

$p<0.05$ considered as statistically significant

Table 7 highlights the significance of the study parameters between the groups such as non pregnant vs. normotensive pregnant, non pregnant vs. preeclampsia and normotensive pregnant vs. preeclampsia. Malondialdehyde, ischemia modified albumin, xanthine oxidase, uric acid, glutathione reductase, catalase, elastase was significantly increased and superoxide dismutase, ferric reducing ability of plasma was significantly decreased in preeclampsia when compared to normal pregnancy.

Table 8: Circulating oxidative stress parameters during before and after delivery in normal pregnant women

	Normal pregnant(G_2)		**p value**
Parameters	**Pre- delivery**	**Post- delivery**	
Malondialdehyde (µmoles/L)	6.85±0.72	5.4±0.66	0.001
Protein carbonyl (nmol/L)	137.21±7.3	127.1±6.4	0.125
IMA (ABU)	0.77±0.02	0.75±0.02	0.914
Xanthine oxidase (U/L)	37.07±6.8	17.1±1.8	0.005
Uric acid (mg/dl)	4.0±0.20	4.16±0.30	0.692

$p<0.05$ considered as statistically significant, $p<0.005$considered as statistically highly significant

Table 8 depicts malondialdehyde, ischemia modified albumin, protein carbonyl, xanthine oxidase and uric acid levels in normal pregnant women. All the oxidative stress parameters were decreased in post-delivery within 48 hours. But the statistical significance was observed for malondialdehyde and xanthine oxidase

Table 9: Circulating enzymatic antioxidant parameters during before and after delivery in normal pregnant women

	Normal pregnant(G_2)		p value
Parameters	**Pre- delivery**	**Post- delivery**	
Glutathione Peroxidase (U/L)	606.3±56.7	493±64.4	0.001
Glutathione Reductase (U/L)	12.1±1.0	7.8±0.49	0.001
Superoxide dismutase(U/L)	11.6±0.37	9.9±0.48	0.005
Catalase (U/L)	46.46±7.56	42.11±5.0	0.623

p<0.05 considered as statistically significant, p<0.005considered as statistically highly significant

Table 9 shows the enzymatic antioxidants in pre and post delivery of normal pregnant. Significanty decreased glutathione peroxidase, glutathione reductase and superoxide dismutase was noticed in post delivery of normal pregnancy. But catalase did not show much difference in after delivery.

Table 10: Circulating non enzymatic antioxidant parameters during before and after delivery in normal pregnant women

	Normal pregnant(G_2)		p value
Parameters	**Pre- delivery**	**Post- delivery**	
Vitamin C (mg/dl)	0.58±0.04	0.67±0.08	0.319
Vitamin E (mg/L)	13.5±1.2	13.96±1.2	0.655
FRAP (µmoles/ml)	1203±165.2	1123.5±135	0.474

p<0.05 considered as statistically significant, p<0.005considered as statistically highly significant

Table 10 depicts the non enzymatic antioxidants such as vitamin C, vitamin E and ferric reducing ability of plasma in pre and post delivery of normal pregnant. But these parameters did not show significant change in after delivery of normal pregnant.

Table 11: Circulating inflammatory parameters during before and after delivery in normal pregnant women

	Normal pregnant(G_2)		p value
Parameters	**Pre- delivery**	**Post- delivery**	
Nitric Oxide (µmoles/L)	7.3±0.42	6.1±0.39	0.005
Elastase (U/ml)	5.80±0.4	4.6±0.21	0.017
Phospholipase A_2 (U/ml)	70.72±2.4	70.03±1.9	0.823
hs-CRP (mg/L)	8.6±1.9	6.6±2.0	0.462

p<0.05 considered as statistically significant, p<0.005considered as statistically highly significant

Table 11 depicts elastase, phospholipase A_2, hs-CRP and nitric oxide levels in normal pregnant. However from these parameters, nitric oxide and elastase activity decreased significantly during after delivery in normal pregnancy but decrease in hs-CRP was not significant.

Table 12: Circulating oxidative stress parameters during before and after delivery in preeclamptic women

	Preeclampsia(G_3)		p value
Parameters	**Pre-delivery**	**Post-delivery**	
Malondialdehyde (µmoles/L)	15.42±1.3	10.5±1.3	0.001
Protein carbonyl (nmol/L)	153.9±14.8	98.6±5.8	0.001
IMA (ABU)	1.24±0.04	1.03±0.02	0.001
Xanthine oxidase(U/L)	193.6±25.3	99.99±18.4	0.001
Uric acid (mg/dl)	6.4±0.29	4.7±0.26	0.001

p<0.05 considered as statistically significant, p<0.005considered as statistically highly significant

Table 12 showed significantly higher (p<0.001) malondialdehyde, ischemia modified albumin, protein carbonyl, xanthine oxidase and uric acid in preeclampsia, whereas the same parameters significantly decreased in post-delivery within 48 hours.

Table 13: Circulating enzymatic antioxidant parameters during before and after delivery in normal pregnant and preeclamptic women

	Preeclampsia(G_3)		p value
Parameters	**Pre-delivery**	**Post-delivery**	
Glutathione Peroxidase (U/L)	493.8±56.0	803.7±74.7	0.093
Glutathione Reductase (U/L)	22.51±3.1	10.1±0.4	0.169
Superoxide dismutase (U/L)	8.5±0.6	7.5±0.6	0.005
Catalase (U/L)	84.7±7.5	88.8±7.5	0.642

$p<0.05$ considered as statistically significant, $p<0.005$considered as statistically highly significant

Table 13 shows the decreased glutathione peroxidase in preeclamptic cases and increased post-delivery. Superoxide dismutase was decreased in preeclamptic cases further decreased after delivery ($p<0.05$) when compared to normal pregnancy.

Table 14: Circulating non enzymatic antioxidant parameters during before and after delivery in preeclamptic women

	Preeclampsia(G_3)		p value
Parameters	**Pre-delivery**	**Post-delivery**	
Vitamin C (mg/dl)	0.47±0.03	0.59±0.08	0.109
Vitamin E (mg/L)	9.3±0.63	9.99±0.6	0.276
FRAP (µmoles/ml)	685.5±86.3	748.9±56	0.391

$p<0.05$ considered as statistically significant, $p<0.005$considered as statistically highly significant

Tables 14 depict vitamin C, vitamin E, and FRAP levels in preeclampsia. However vitamin C, E and FRAP were non significantly increased during after delivery in preeclampsia.

Table 15: Circulating inflammatory parameters during before and after delivery in preeclamptic women

	Preeclampsia(G_3)		p value
Parameters	**Pre-delivery**	**Post-delivery**	
Nitric Oxide (µmoles/L)	6.31±0.53	6.93±1.2	0.617
Elastase (U/ml)	26.85±10.5	12.91±1.03	0.200
Phospholipase A_2 (U/ml)	79.01±3.6	73.30±3.4	0.271
hs-CRP (mg/L)	11.54±1.9	10.3±2.7	0.462

$p<0.05$ considered as statistically significant, $p<0.005$considered as statistically highly significant

Table 15 depicts elastase, phospholipase A_2 and hs-CRP levels were increased in preeclampsia prominently compared to normal pregnant. However, from the same parameters, elastase activity during after delivery in preeclampsia decreased by 2.07 fold respectively. Whereas, phospholipase A_2 and hs-CRP found to be non-significantly decreased ($p >0.05$) in post-delivery.

Table 16: Receiver operating characteristics curve analysis of the oxidative stress, antioxidant and inflammatory parameters in preeclampsia

Parameters	Sensitivity	Specificity	AUC	95% CI
Malondialdehyde (µmoles/L)	84.21	68.42	0.805	0.720-0.873
Protein carbonyl (nmol/L)	47.37	71.93	0.517	0.422-0.612
Xanthine oxidase (U/L)	61.40	89.47	0.753	0.663-0.829
IMA (ABU)	87.72	91.23	0.920	0.854-0.962
Catalase (Unit/ml enzyme)	96.49	84.21	0.876	0.801-0.930
FRAP (µmoles/ml)	80.36	56.14	0.650	0.555-0.738
Glutathione Peroxidase (U/L)	85.96	39.29	0.564	0.467-0.657
Glutathione Reductase (U/L)	28.07	94.74	0.630	0.534-0.718
Superoxide dismutase (U/ml)	52.63	89.47	0.695	0.602-0.778
Uric acid (mg/dl)	71.93	89.47	0.841	0.761-0.903
Vitamin C (mg/dl)	70.18	49.12	0.618	0.522-0.707
Vitamin E (mg/L)	89.47	36.84	0.637	0.542-0.725
Elastase (U/ml)	64.3	86	0.758	0.669 - 0.834
Phospholipase A_2 (U/ml)	35.71	85.96	0.580	0.484-0.672
hs-CRP (mg/L)	57.1	73.7	0.708	0.615- 0.790
Nitric Oxide (µmoles/L)	56.14	75.44	0.616	0.521-0.706

Table 16 illustrates the sensitivity, specificity and area under curve of the parameters analyzed. The data showed good area under curve for malondialdehyde, ischemia modified albumin, xanthine oxidase, uric acid, catalase, elastase and hs-CRP in preeclampsia.

Table17: Correlation of oxidative stress parameters with nutrient antioxidants and total antioxidant status in preeclampsia

Parameters		Vitamin C	Vitamin E	FRAP
Malondialdehyde (µmoles/L)	r	-0.162	0.043	0.155
	p	0.229	0.752	0.253
Protein carbonyl (nmol/L)	r	-0.059	-0.014	0.175
	p	0.662	0.920	0.198
Xanthine oxidase (U/L)	r	-0.105	-0.069	0.155
	p	0.435	0.610	0.255
Ischemia Modified Albumin (ABU)	r	0.131	-0.107	-0.146
	p	0.331	0.429	0.282
Uric acid (mg/dl)	r	-0.012	0.099	-0.117
	p	0.928	0.463	0.390

r = correlation coefficient, p= probability value

Table 17 shows correlation coefficient and probability value of oxidative stress parameters with nutrient antioxidants and FRAP. Results did not show significant correlation between parameters.

Table 18: Correlation of oxidative stress parameters with enzymatic antioxidants in Preeclampsia

Parameters		Glutathione Peroxidase U/L	Glutathione Reductase U/L	Superoxide Dismutase (U/ml)	Catalase (U/ml)
Malondialdehyde (µmoles/L)	r	-0.041	-0.016	0.070	-0.068
	p	0.762	0.908	0.603	0.617
Protein carbonyl (nmol/L)	r	-0.034	-0.180	0.017	0.102
	p	0.803	0.179	0.902	0.448
XanthineOxidase (Units/L enzyme)	r	0.139	0.064	-0.209	0.084
	p	0.303	0.637	0.119	0.535
Ischemia Modified Albumin (ABU)	r	-0.409	-0.023	0.057	-0.004
	p	0.002	0.862	0.672	0.977
Uric acid (mg/dl)	r	-0.147	0.011	0.001	-0.129
	p	0.275	0.937	0.996	0.338

r = correlation coefficient, p= probability value

Table 18 shows correlation coefficient and probability value of oxidative stress parameters with enzyme antioxidants. IMA showed significant negative correlation with glutathione peroxidase.

Table 19: Correlation of oxidative stress parameters with inflammatory and endothelial dysfunction parameters in preeclampsia

Parameters		Elastase (Unit/ml)	PLA_2 (Unit/ml)	hs-CRP (mg/L)	Nitric Oxide (µmoles/L)
Malondialdehyde (µmoles/L)	r	0.170	0.021	0.118	-0.219
	p	0.206	0.879	0.383	0.102
Protein carbonyl (nmol/L)	r	0.029	0.186	0.023	-0.264
	p	0.828	0.166	0.867	0.047
XanthineOxidase (Units/L)	r	0.036	0.040	-0.154	-0.246
	p	0.788	0.767	0.252	0.065
Ischemia modified albumin (ABU)	r	-0.213	0.131	0.062	-0.145
	p	0.117	0.332	0.647	0.283
Uric acid (mg/dl)	r	0.049	0.012	0.094	-0.081
	p	0.720	0.931	0.488	0.550

r = correlation coefficient, p= probability value

Table 19 illustrated correlation coefficient and probability value of oxidative stress parameters with inflammatory and endothelial dysfunction parameters. Protein carbonyl showed significant negative correlation with endothelial dysfunction marker nitric oxide.

Table 20: Correlation of Enzymatic antioxidants parameters with inflammatory and endothelial dysfunction parameters in preeclampsia

Parameters		Elastase (Unit/ml)	PLA_2 (Unit/ml)	hs-CRP (mg/L)	Nitric Oxide (µmoles/L)
Glutathione Peroxidase (U/L)	r	0.110	-0.058	-0.121	-0.420
	p	0.415	0.668	0.369	0.001
Glutathione reductase (U/L)	r	0.003	0.320	0.076	-0.130
	p	0.981	0.015	0.573	0.335
Superoxide dismutase (U/ml)	r	0.059	-0.184	0.102	0.143
	p	0.665	0.171	0.450	0.290
Catalase (U/ml)	r	-0.011	-0.163	0.113	0.061
	p	0.933	0.227	0.403	0.650

r = correlation coefficient, p= probability value

Table 20 illustrated correlation coefficient and probability value of enzyme antioxidants with inflammatory and endothelial dysfunction parameters. Among enzymatic antioxidants Gpx showed significant negative correlation with endothelial dysfunction marker and GR showed significant positive correlation with phospholipase A_2.

Table 21: Correlation of nutrient antioxidants parameters with inflammatory and endothelial dysfunction parameters in preeclampsia

Parameter		Elastase (U/ml)	PLA_2 (U/ml)	hs-CRP (mg/L)	NO (µmoles/L)
Vitamin C (mg/dl)	r	0.001	-0.126	-0.088	-0.083
	p	0.994	0.351	0.513	0.539
Vitamin C (mg/dl)	r	0.084	0.109	-0.100	0.018
	p	0.536	0.418	0.461	0.893
FRAP (µmoles/ml)	r	0.243	0.098	0.034	-0.177
	p	0.071	0.472	0.805	0.192

r = correlation coefficient, p= probability value

Table 21 illustrated correlation coefficient and probability value of nutrient antioxidants and total antioxidant status with inflammatory and endothelial dysfunction parameters. There was no significant correlation was observed between nutrient antioxidants and inflammatory as well as endothelial dysfunction marker in preeclampsia.

Figure 1 to 16 depicts the mean ± SEM of oxidative stress parameters, antioxidants, inflammatory markers and endothelial dysfunction markers in non pregnant, before and after delivery of normotensive pregnant and preeclamptic women.

Figure 17 to 23 shows the receiver operating characteristics curve with specificity, sensitivity and area under curve for significant parameters such as malondialdehyde, ischemia modified albumin, xanthine oxidase, uric acid, catalase, elastase and hs-CRP in preeclampsia for the better diagnostic utility.

Receiver operating characteristics curve analysis of the studied parameters indicated ischemia modified albumin as a better ischemic marker with sensitivity (87.7%) and specificity (91.2%). As well as plasma elastase as good diagnostic marker for inflammation with respect to sensitivity (64.3%) and specificity (86%) even though hs-CRP has better clinical utility. Phospholipase A_2 did not show any appreciable diagnostic importance.

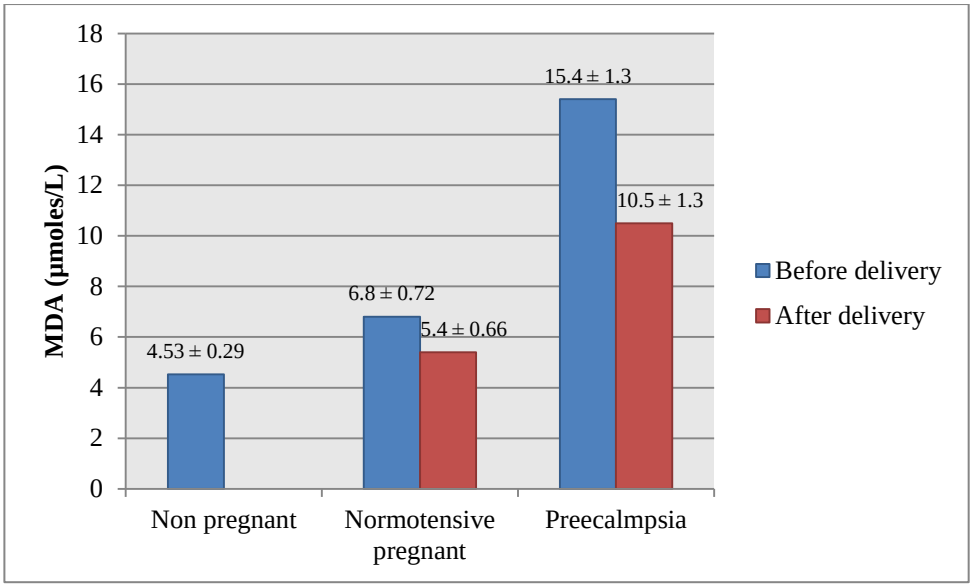


Figure 1: Depicting the malondialdehyde level in non pregnant, before and after delivery of normal pregnant and preeclampsia

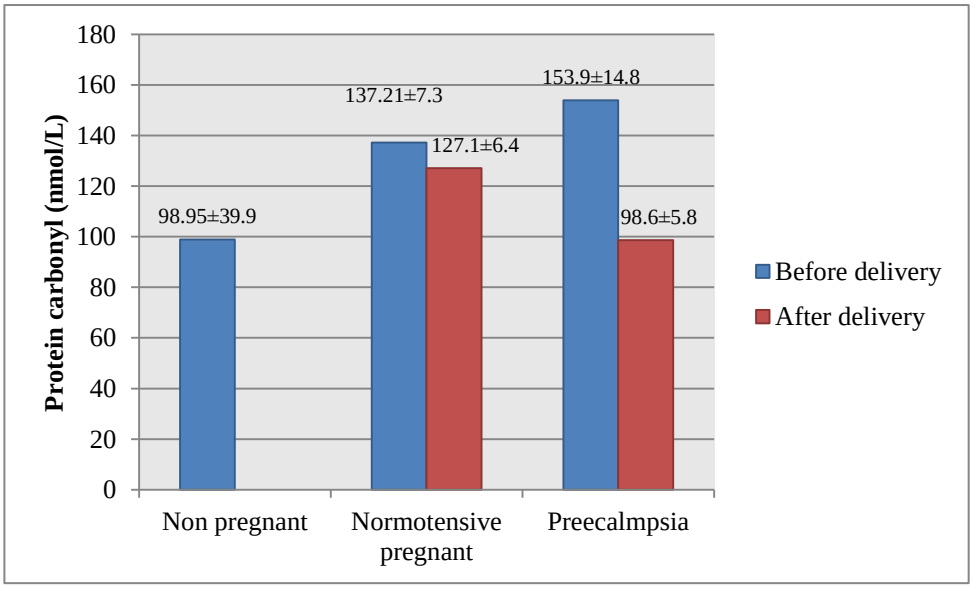


Figure 2: Depicting the protein carbonyl content in non pregnant, before and after delivery of normal pregnant and preeclampsia

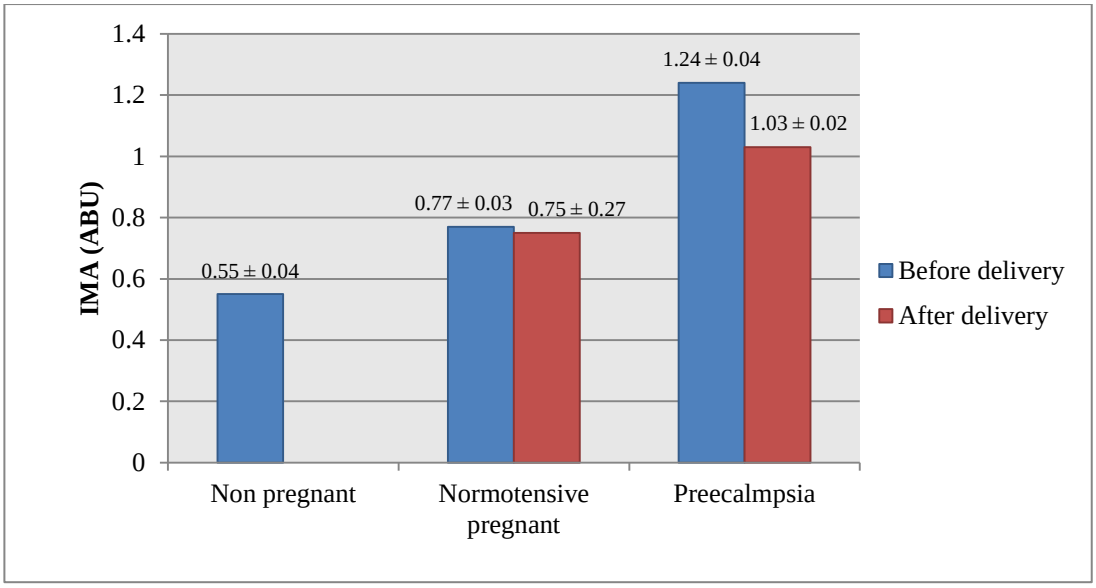


Figure 3: Depicting the ischemia modified albumin in non pregnant, before and after delivery of normal pregnant and preeclampsia

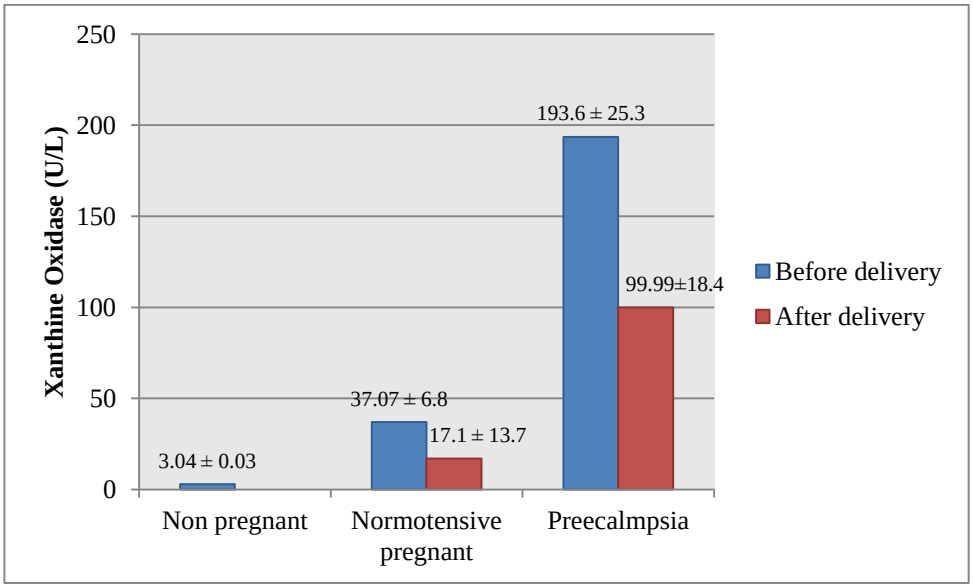


Figure 4: Depicting the xanthine oxidase activity in non pregnant, before and after delivery of normal pregnant and preeclampsia

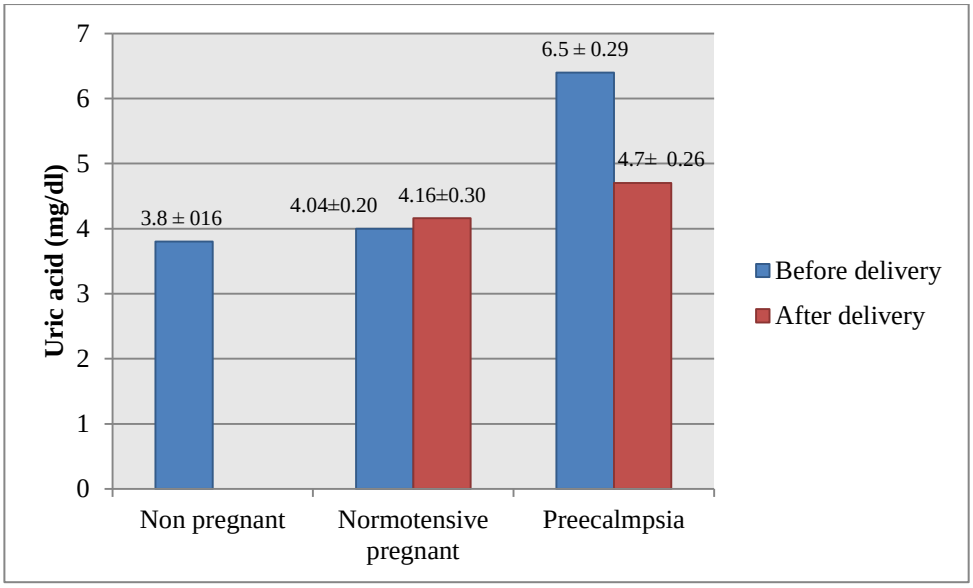


Figure 5: Depicting the uric acid levels in non pregnant, before and after delivery of normal pregnant and preeclampsia

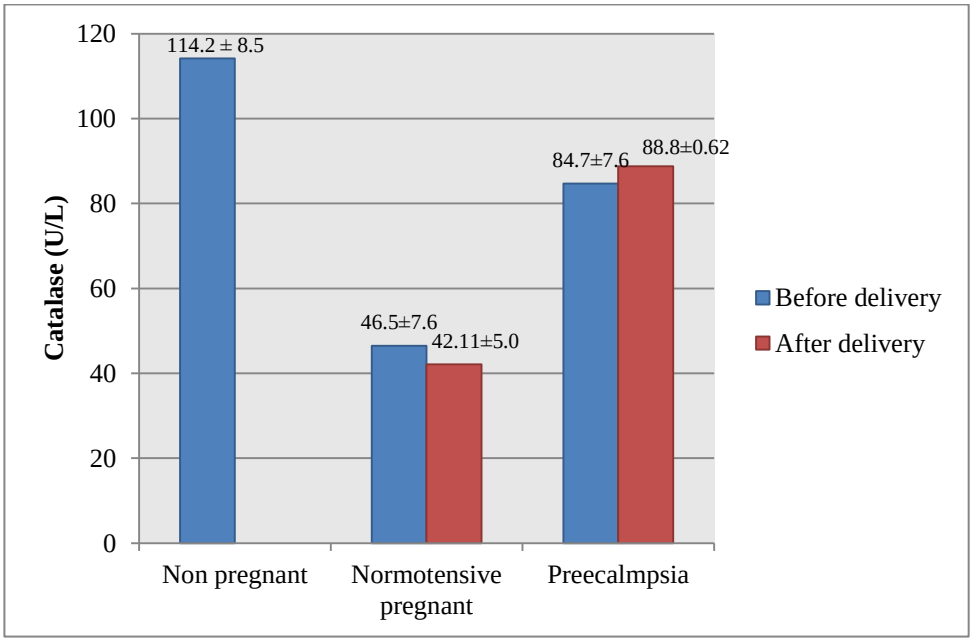


Figure 6: Depicting the catalase activity in non pregnant, before and after delivery of normal pregnant and preeclampsia

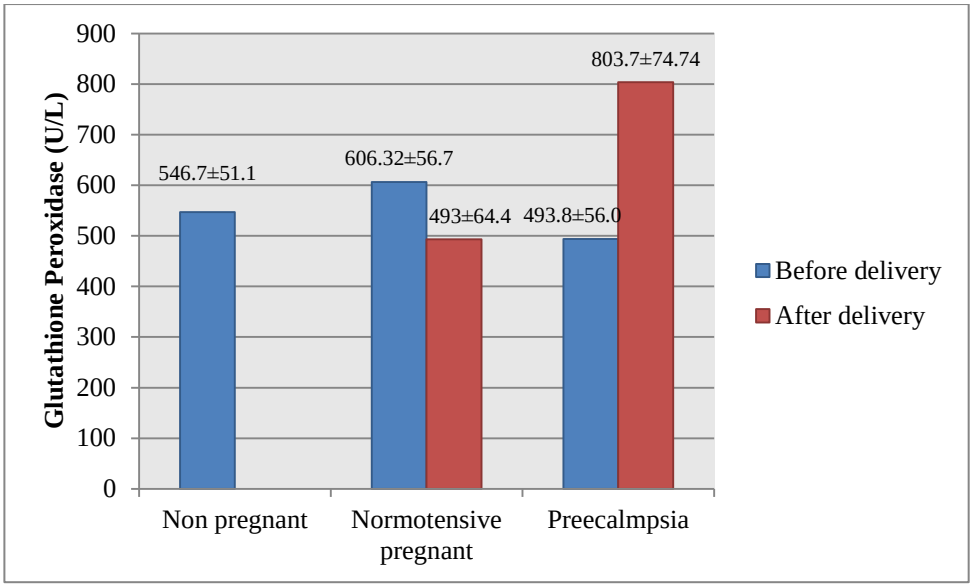


Figure 7: Depicting the glutathione peroxidase activity in non pregnant, before and after delivery of normal pregnant and preeclampsia

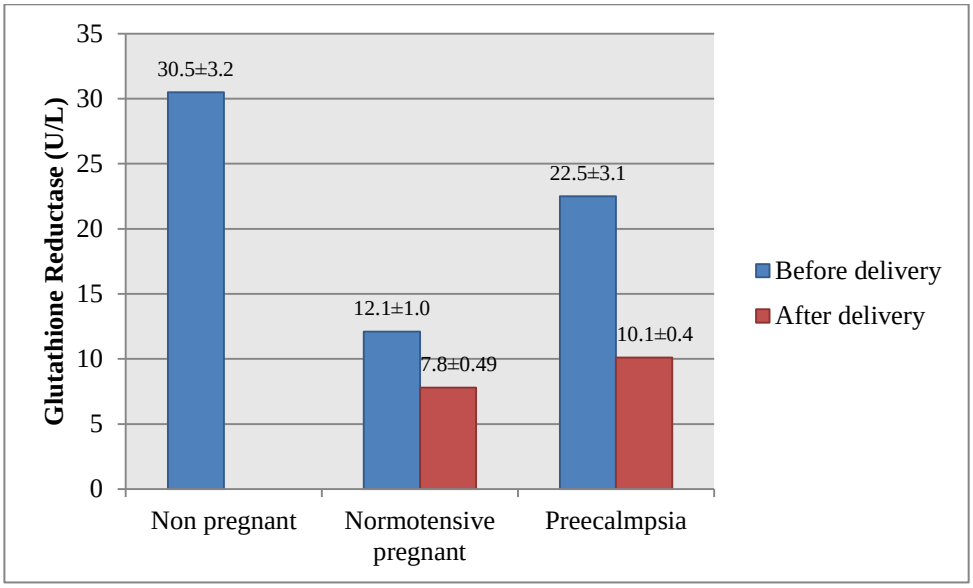


Figure 8: Depicting the glutathione reductase activity in non pregnant, before and after delivery of normal pregnant and preeclampsia

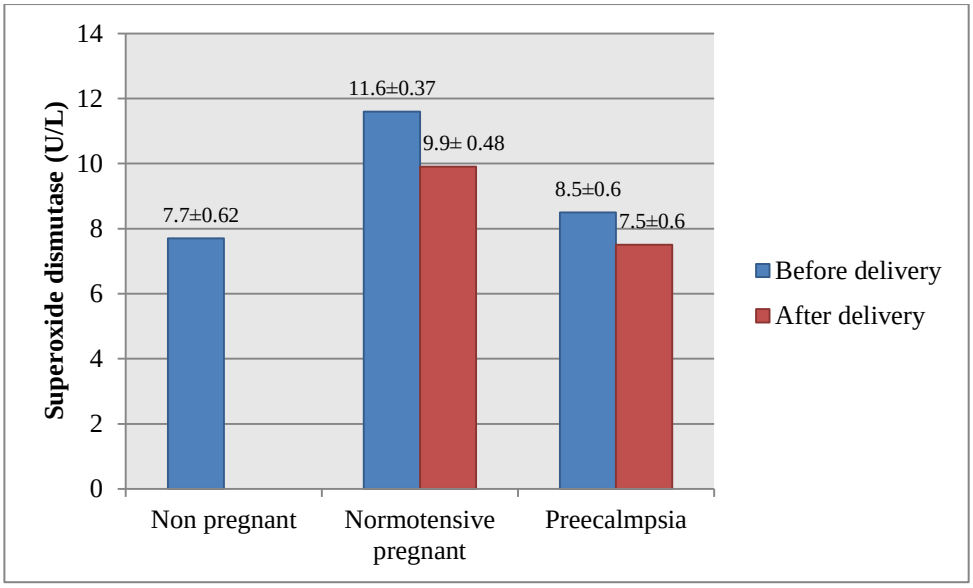


Figure 9: Depicting the superoxide diamutase activity in non pregnant, before and after delivery of normal pregnant and preeclampsia

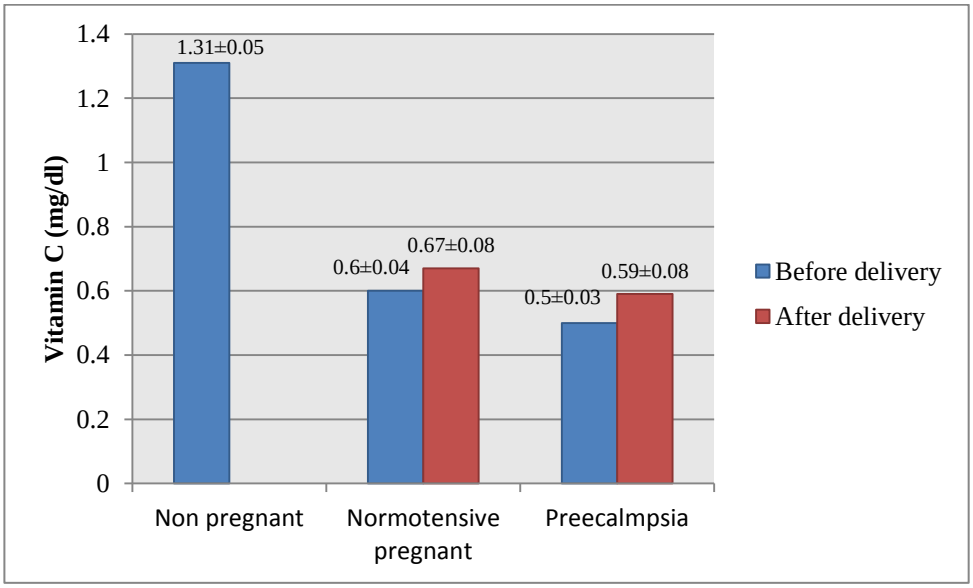


Figure 10: Depicting the vitamin C levels in non pregnant, before and after delivery of normal pregnant and preeclampsia

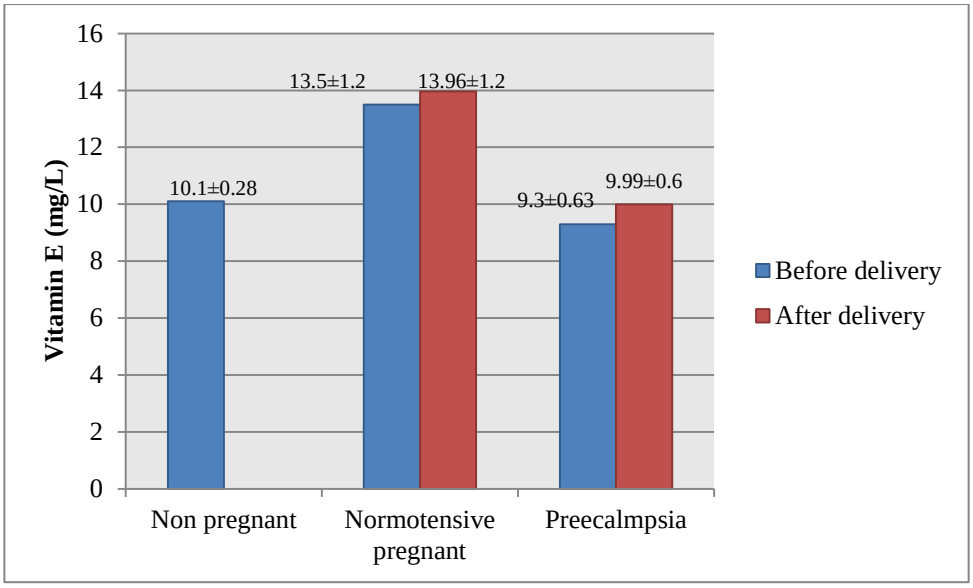


Figure 11: Depicting the vitamin E levels in non pregnant, before and after delivery of normal pregnant and preeclampsia

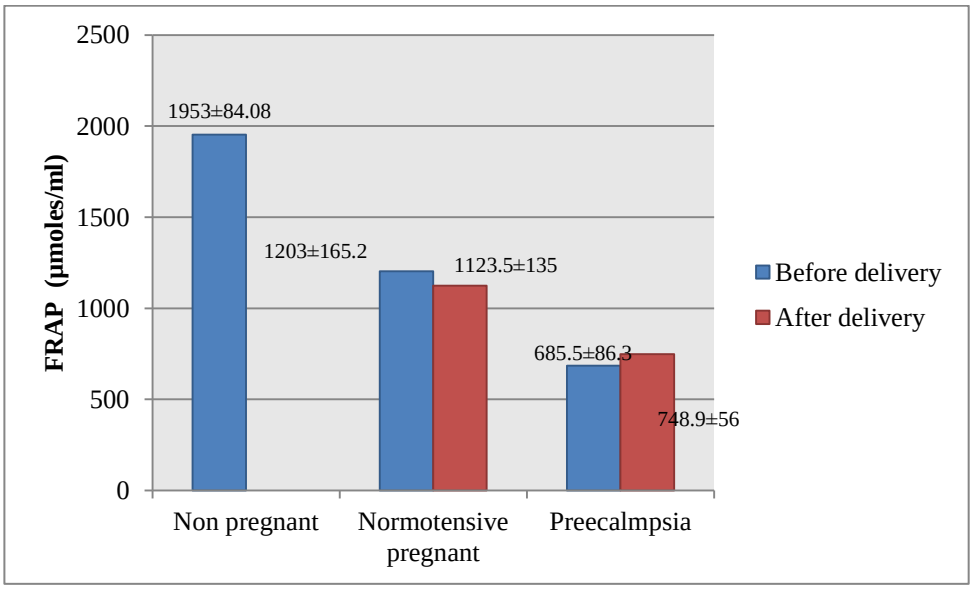


Figure 12: Depicting the total antioxidant levels in non pregnant, before and after delivery of normal pregnant and preeclampsia

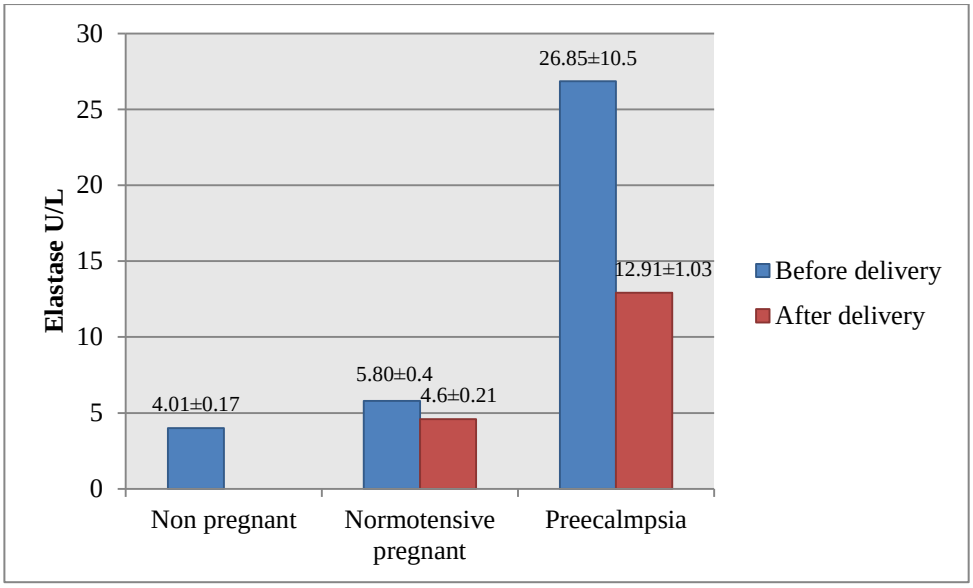


Figure 13: Depicting the plasma elastase activity in non pregnant, before and after delivery of normal pregnant and preeclampsia

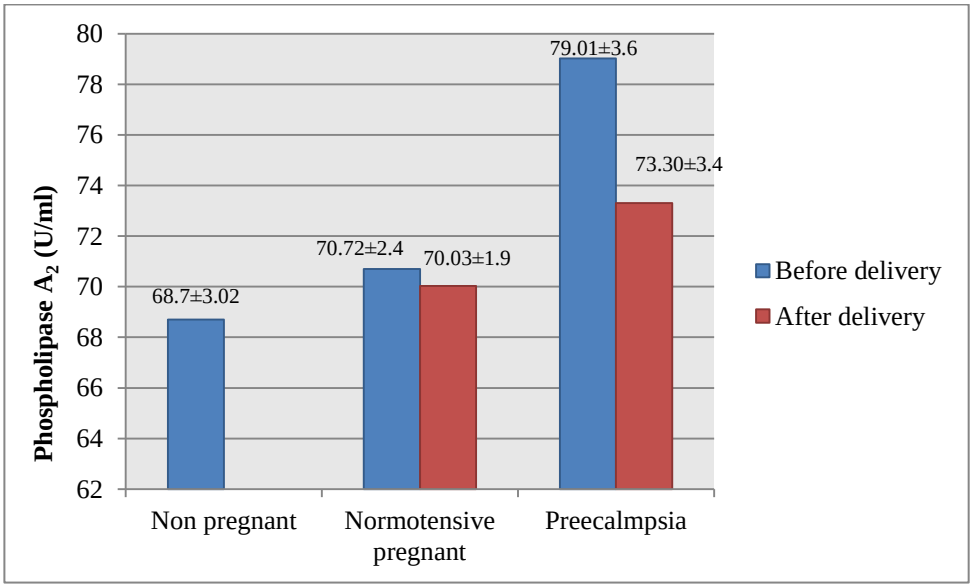


Figure 14: Depicting the phospholipase A_2 activity in non pregnant, before and after delivery of normal pregnant and preeclampsia

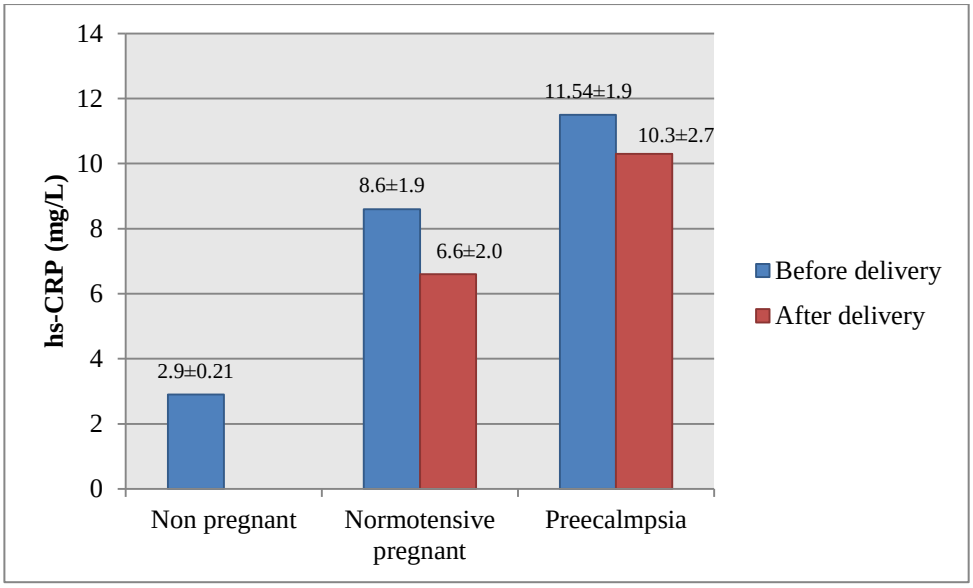


Figure 15: Depicting the high sensitive C-reactive protein levels in non pregnant, before and after delivery of normal pregnant and preeclampsia

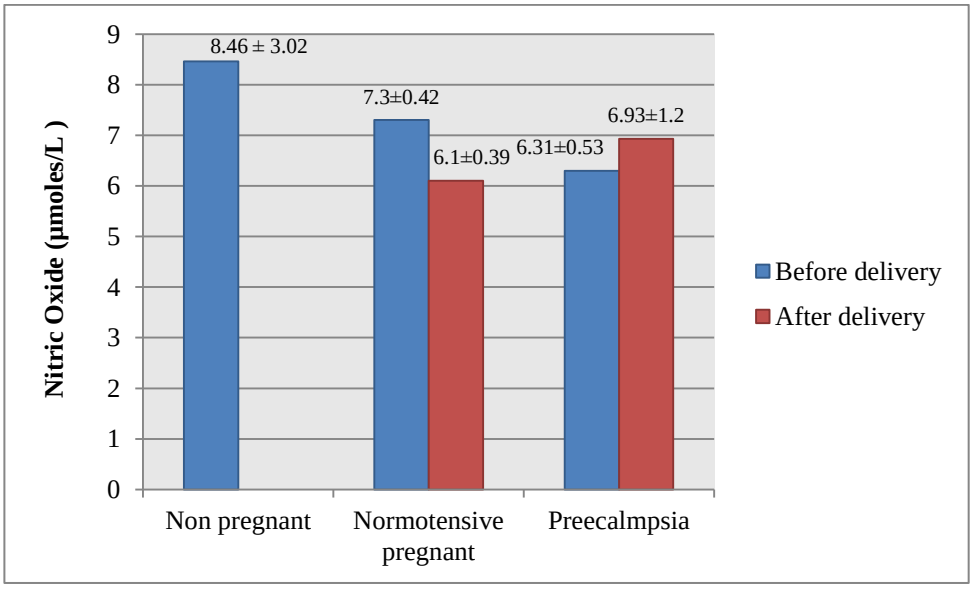


Figure 16: Depicting the nitric oxide levels in non pregnant, before and after delivery of normal pregnant and preeclampsia

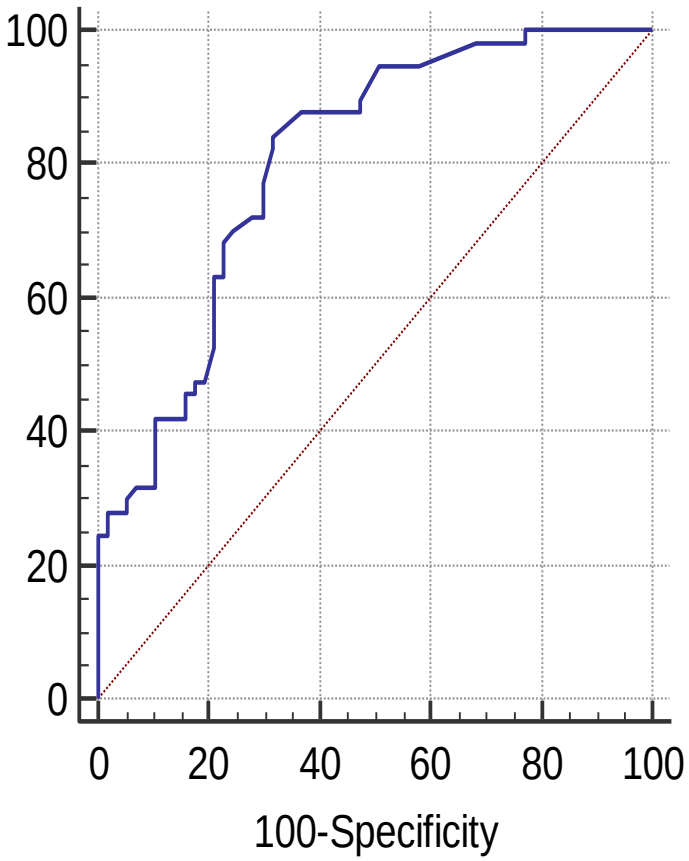


Figure 17: Receiver operating characteristic curve showing the area under curve of malondialdehyde

Graphical representation of specificity on X -axis and sensitivity on Y-axis of the test parameter malondialdehyde indicated sensitivity (84.21), specificity (68.42), and area under curve (0.805) with 95% confidence interval range (0.720-0.873). In receiver operating characteristic curve, the data representing area under curve classified as having good accuracy for the test parameter.

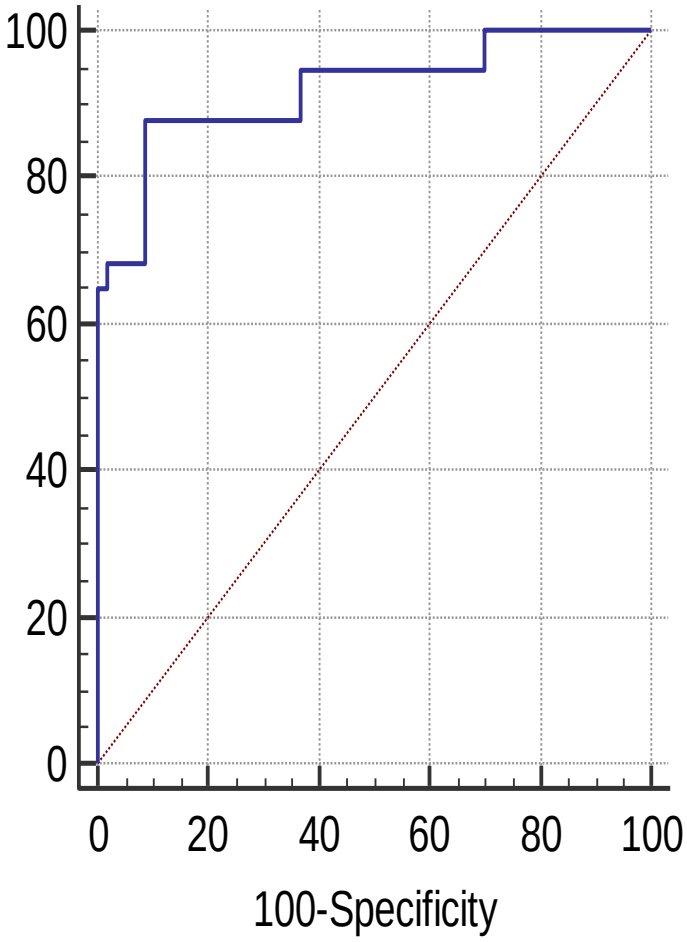


Figure 18: Receiver operating characteristic curve showing the area under curve of ischemia modified albumin

The plot of specificity on X -axis and sensitivity on Y-axis of the test parameter ischemia modified albumin indicated sensitivity (87.72), specificity (91.23) and area under curve (0.920) with 95% confidence interval range (0.854-0.962). In receiver operating characteristic curve, the data representing area under curve classified as having excellent accuracy for the test parameter.

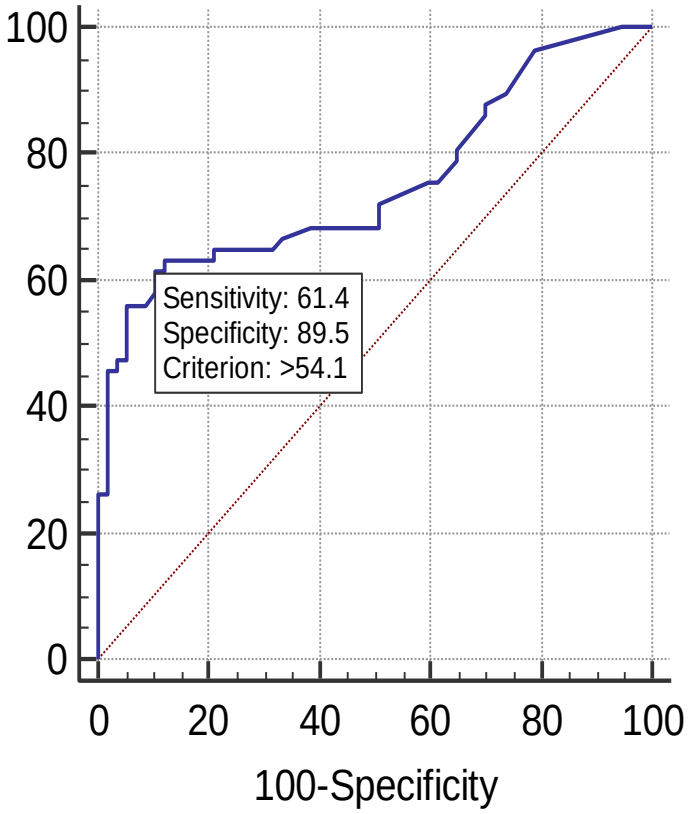


Figure 19: Receiver operating characteristic curve showing the area under curve of xanthine oxidase

Graphical representation of specificity on X -axis and sensitivity on Y-axis of the test parameter xanthine oxidase showed sensitivity (61.40), specificity (89.47) and area under curve (0.753) with 95% confidence interval range (0.663-0.829). In receiver operating characteristic curve, the data representing area under curve classified as having fair accuracy for the test parameter.

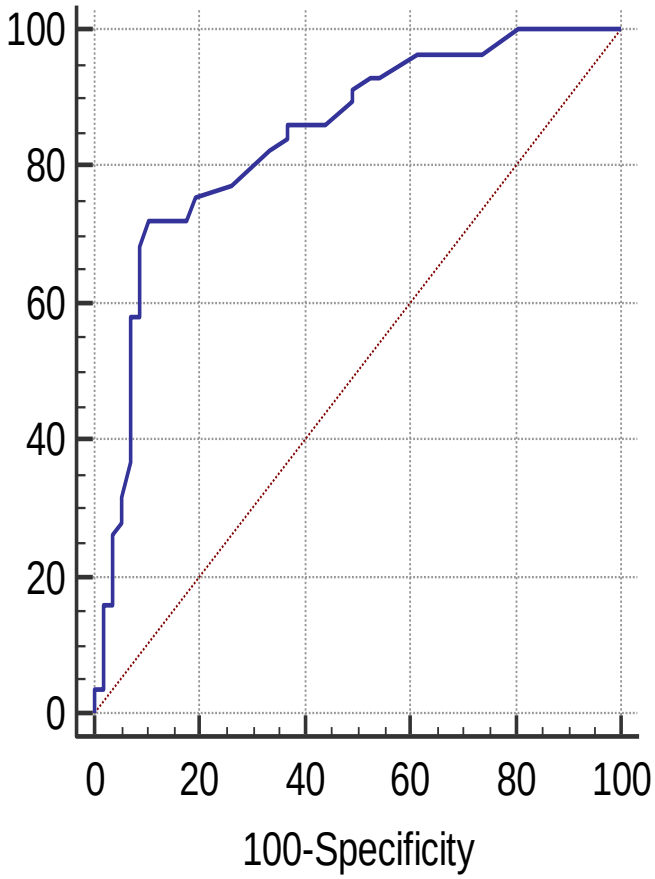


Figure 20: Receiver operating characteristic curve showing the area under curve of uric acid

Graphical illustration of specificity on X -axis and sensitivity on Y-axis of the test parameter uric acid indicated sensitivity (71.93), specificity (89.47) and area under curve (0.841) with 95% confidence interval range (0.761-0.903). In receiver operating characteristic curve, the data representing area under curve classified as having good accuracy for the test parameter.

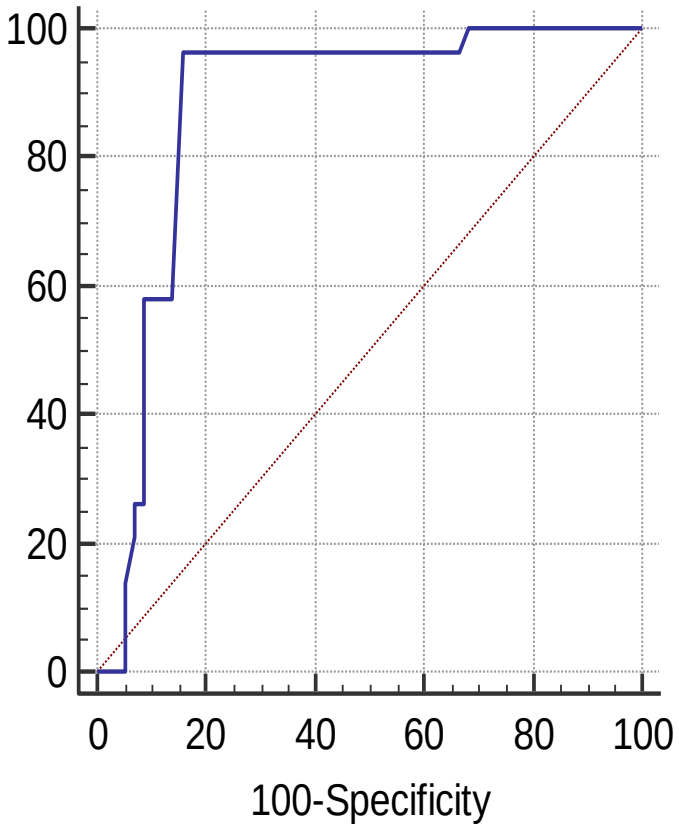


Figure 21: Receiver operating characteristic curve showing the area under curve of catalase

Graphical depiction of specificity on X-axis and sensitivity on Y-axis of the test parameter catalase shown sensitivity (96.49), specificity (84.21) and area under curve (0.876) with 95% confidence interval range (0.801-0.930). In receiver operating characteristic curve, the data representing area under curve classified as having good accuracy for the test parameter.

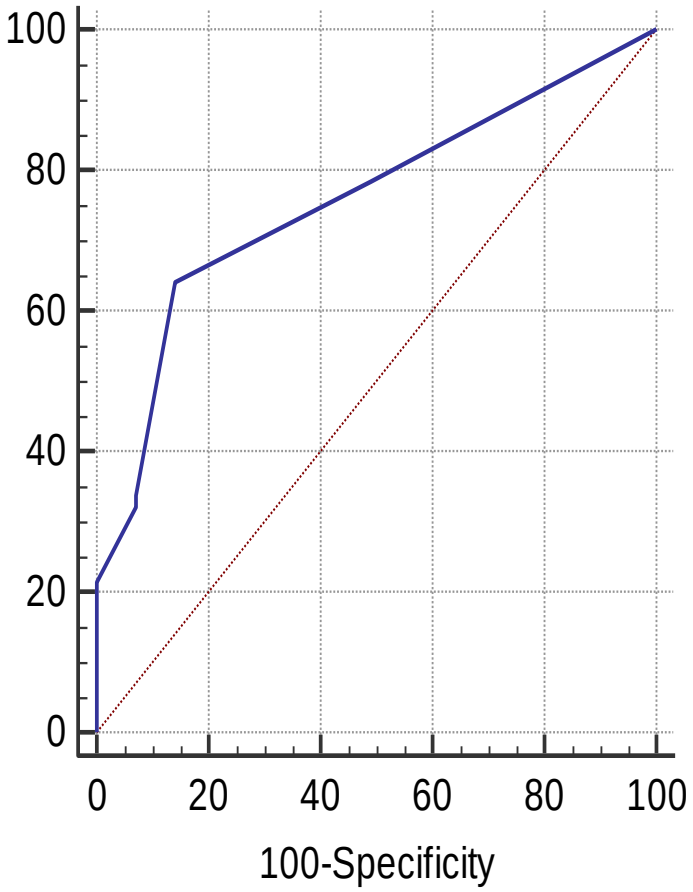


Figure 22: Receiver operating characteristic curve showing the area under curve of elastase

Graphical representation of specificity on X -axis and sensitivity on Y-axis of the test parameter elastase showed sensitivity (64.3), specificity (86) and area under curve (0.758) with 95% confidence interval range (0.669 - 0.834). In receiver operating characteristic curve, the data representing area under curve classified as having fair accuracy for the test parameter.

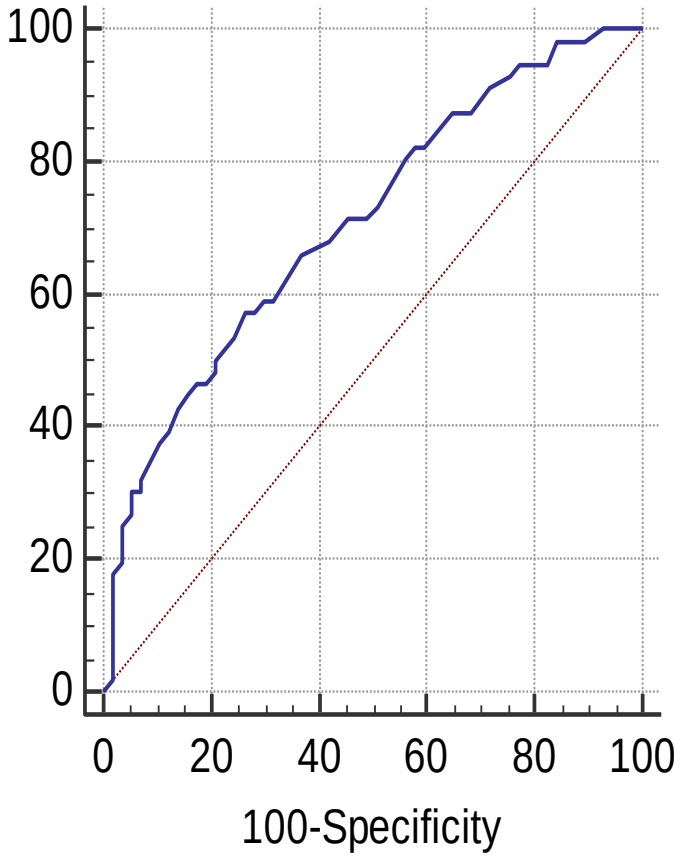


Figure 23: Receiver operating characteristic curve showing the area under curve of hs-CRP

Graphical illustration of specificity on X -axis and sensitivity on Y-axis of the test parameter hs-CRPshowed sensitivity (57.1), specificity (73.7) and area under curve (0.708) with 95% confidence interval range (0.615 - 0.790). In receiver operating characteristic curve, the data representing area under curve classified as having fair accuracy for the test parameter.

Table 22: Comparison of percentage of xanthine oxidase inhibition by allopurinol, quercetin, vitamins and flavonoid extracts at different concentrations

Compounds	Percentage of inhibition at different concentration (µg/ml)							
	6.25	12.5	25	50	100	200	400	800
Allopurinol	17.07± 3	35.37±4.9	48.25±2.7	56.68±3.8	74.24±1.2	81.21±5.1	92.2±4.7	93.3±4.3
Quercetin	3.95±1.1	10.99±1.9	27.5±4.9	45.9±4.5	52.2±4.5	61.32±0.59	65.8±3.3	71.1±4.4
Vitamin C	8.94±1.3	18.3±2.5	25.07±2.7	26.76±4.6	31.8±1.8	46.4±0.68	48.8±3.9	50.7±2.8
Vitamin E	12.2±1.7	18.3±0.84	28.6±3.9	33.9±4.6	42.8±1.3	52.3±6.5	53.5±7.1	54±2.6
Pongamia pinnata	3.3±1.8	6.7±0.5	27.5±4.4	45.9±3.4	52.2±1.0	62.9±2.1	65.9±2.6	69.1±1.1
Morinda citrifolia	8.9±0.9	15.6±72.8	23.9±1.7	33.9±4.1	39.5±1.8	42.9±2.8	45.1±0.9	46.3±1.1
Mangifera indica	8.9±3.9	14.6±5.7	25.1±0.8	30.8±1.3	34.071.8	47.4±14.6	50.4±7.2	53.6±1.4
Zingiber officinale	7.3±0.59	12.2±0.8	21.02±1	27.7±2.6	37.6±3.7	44.5±2.8	49.7±4.2	51.6±0.6

Table 22 illustrating xanthine oxidase inhibition using vitamin C & E and flavonoid extracts from plants such as *Pongamia pinnata L, Morinda citrifolia L, Mangifera indica L, Zingiber officinale Roscoe.* The percentage of inhibition calculated by considering allopurinol as internal standard. In the similar way quercetin was treated as standard flavonoid to compare the inhibitory activity of isolated flavonoids from the selected plants in the study. The concentrations of the inhibitor used were in the range of 6- 800 µg/ml. It was evident that all the compounds exhibited ascending kind of inhibition from the concentration ranging from 50 - 800 µg/ml.

The percentage of inhibition of xanthine oxidase by vitamin C & E clearly evinced that vitamin E is having better inhibition compared to vitamin C. Even though, both the vitamins irrespective of their concentration showed 50% & 54% of inhibition with allopurinol respectively. This showed that vitamins have minimal XO inhibitory activity.

The results prove that flavonoid extract of *Pongamia pinnata L*showed almost similar inhibitory effect compared to standard quercitin. However *Morinda citrifolia L, Mangifera indica L, Zingiber officinale Roscoe* have less inhibitory effect on xanthine oxidase compared to *pongamia pinnata*. Similarly, percentage of inhibition by pure quercetin on xanthine oxidase activity showed approximately less than 40 % inhibition when compared to allopurinol.

The percentage inhibition at 800 µg/ml concentration for allopurinol 93.25%, quercetin 71.1%, vitamin C 50.7%, vitamin E 54%, *Pongamia pinnata L* 69.1%, *Morinda citrifolia L* 46.3%, *Mangifera indica L* 53.6%, *Zingiber officinale Roscoe* 51.6% respectively. This showed other than allopurinol and quercetin *Pongamia pinnata* had maximum inhibitory activity.

The efficacy of quercetin, vitamin E and vitamin C on comparison with optimized flavonoid extract from the above plants on xanthine oxidase inhibitory activity were calculated and expressed as IC_{50} and K_i. Allopurinol a well-known competitive inhibitor of xanthine oxidase considered as positive control for comparison that showed IC_{50} value 0.4mM and K_i 0.13 mM. Inhibitory effect of quercetin on XO showed IC_{50} 2.38mM and K_i 0.37mM. The vitamin C & E inhibition on xanthine oxidase showed IC_{50} 10.6 mM, 1.19 mM and Ki 3.37mM, 0.76mM respectively. *Pongamia pinnata L, Mangifera indica L, Zingiber officinale Roscoe* showed inhibition on xanthine oxidase in the concentration of IC_{50} 8.74 mM, 1.09 mM, 5.4 mM and K_i 0.35 mM, 1.73mM, 2.7mM respectively. But *Morinda citrifolia L* did not show effective xanthine oxidase inhibition.

5.2. DISCUSSION

Our research findings from the demographic data of the study population showed that women with more than 30 years of age group and primigravida were with increased incidence of preeclampsia compared to the normal pregnant women. Other studies have shown that teenage pregnancy and increased maternal age increases risk of preeclampsia[147]. Another observation, we found that preterm delivery as the major consequence of preeclampsia which goes similar with literature [148].

The anemia in pregnancy associated with poor pregnancy outcome[149]. In contrast few reports presented even higher hemoglobin in maternal circulation that linked to intrauterine growth restriction [150]. But the current study indicated no difference in hemoglobin levels, packed cell volume and erythrocyte count in normotensive pregnant women and preeclampsia. Thrombocytopenia might be due to stacking of platelet in the areas with endothelial damage [151] and leukocytosis due to immunological dysfunction[152] in preeclampsia noticed in the study as depicted in the table 1.

The distinguishing biochemical and hematological parameters for further classification of preeclampsia in to mild and severe types tabulated in table 2 & 3. Accordingly no significant difference in gestational age, hematological and liver function investigations between mild and severe preeclampsia were recorded. One of the observations pertaining to bilirubin fractions such as conjugated/direct bilirubin decreased significantly in severe preeclampsia. However an unchanged hemoglobin level seen between mild and severe preeclampsia in spite of low hemoglobin level in both the groups that represents condition of anemia which is on agreement with other research reports[153].

Preeclampsia is associated with various etiological factors which implicated in pathogenesis. It is characterized by increased production of ROS and decreased levels of antioxidant status and hyperuricemia[154]. Preeclampsia is influenced by various factors such as increased oxidative stress, hypoxicated trophoblastic tissue destruction that produces xanthine, hypoxanthine and cytokines leading to inflammatory process[155]. Current study results indicated that increased activity of xanthine oxidase, non-significantly decreased nitric oxide level and marginally elevated uric acid levels.

Improper spiral arteries implantation leads to hypoxia and increased turnover of trophoblastic tissue which might result in increased xanthine and hypoxanthine that serves as substrate for XO which might lead to increased uric acid [77]. The association between preeclampsia and high serum uric acid concentration was reported during the beginning of this century[156]. Reduced uric acid clearance observed in preeclamptic women associated with increased rate of reabsorption and less secretion amounts to more uric acid level in serum[157]. However, there is no data available on measurement of xanthine oxidase that can be considered as enzyme marker in preeclampsia before and after delivery status. Even though in a research report of Karabulut et al. evinced that the increased XO activity in preeclampsia, but data on XO activity is not available in preeclampsia during after delivery. Placental incompatibility in ischemic condition due to free radical formation and increased oxidative stress makes endothelial damage and cell death is the cause for elevation of xanthine oxidase in circulation [49].

Xanthine oxidase (E.C 1.17.3.2) catalyzes the conversion of substrates hypoxanthine/xanthine in to uric acid and hydrogen peroxide. Xanthine dehydrogenase (E.C.1.17.1.4) is NAD+ dependent and xanthine oxidase (E.C.1.17.3.2) uses oxygen [158].

However, limited proteolysis and oxidation of sulfhydryl groups converts irreversibly xanthine dehydrogenase in to xanthine oxidase [48].

Activated leukocytes produce cytokines that in turn increases the xanthine oxidase activity and also reactive oxygen species from endothelium and increases production of uric acid [50, 51]. In our research work, an observation is significantly recorded in terms of elevation of xanthine oxidase in preeclampsia in comparison with normal pregnant women. In the same way uric acid level also increased before delivery. Two fold decrease of xanthine oxidase activity noticed after delivery condition in preeclampsia. Xanthine oxidase activity in preeclampsia increased by 5.26 fold compared to normal pregnant group before delivery. However, after delivery reverts to 2.1 fold in preeclampsia. The level was persistently high compared to healthy pregnant whereas the same enzyme reverts to 2.1 fold after delivery in healthy pregnant women. These results indicated that the rise of XO level in preeclampsia before delivery proves that measurement of xanthine oxidase in preeclampsia is appropriate to consider as a marker since the expression of XO in preeclampsia is evident. However, elevation of uric acid observed in preeclampsia compared to normal pregnant before delivery but the level return to normal in both the groups after delivery.

The present study showed, uric acid level increased before delivery and decreased after delivery in preeclampsia. But in normal Pregnant before and delivery uric acid level was unaltered. This indicates possible involvement of oxidative stress in placenta by contributing xanthine/hypoxanthine and xanthine oxidase activity[40].

Increased conversion of xanthine dehydrogenase in to xanthine oxidase by oxidative stress further adds to increased production hydrogen peroxide which in turn

affects the trophoblast cell function [159, 74]. Thereby oxidative stress has become one of the causative for preeclampsia complications. The present study reported increased xanthine oxidase activity as unique observation with concomitant rise of uric acid in preeclampsia.

Decreased nitric oxide was observed in preeclampsia when compared to normotensive pregnant. It has been proposed that reduced production of vasodilator agent nitric oxide [160] might cause preeclampsia but there are studies showing increased nitric oxide production in preeclampsia suggested to overcome the adverse placental effect[111]. In our study nitric oxide level was increased in normal pregnancy before delivery as an indication of adaptive hemodynamic changes[106] and decreased after delivery in normal pregnant women may be due to down-regulation of maternal NO synthesis [161]. Decreased NO level in preeclampsia and elevated level after delivery in preeclampsia within 48 hours may be an indication of reversible changes of preeclampsia. Elevated ROS production may suppress the expression of endothelial nitric oxide synthase (eNOS). Nitric oxide (NO) combines rapidly with superoxide (O^{2-}) to form peroxynitrite ion ($ONOO^{-}$). Peroxynitrite oxidizes the DNA, lipids, Proteins and also interferes with the vascular signaling pathways [162].

However, in an attempt to evaluate the correlation between xanthine oxidase activity and nitric oxide level a non-significant negative correlation is observed before ($r = -0.260$) and after delivery($r = -0.224$).

The current study further evaluated oxidative stress parameters with special emphasis to xanthine oxidase activity and ischemia modified albumin which may be considered as an oxidative stress marker under prevailing antioxidant status.

Malondialdehyde is a lipid peroxidation marker produced from the peroxidation of polyunsaturated fatty acid was significantly elevated in preeclampsia compared to normotensive pregnant and non-pregnant which is consistent with the previous reports [163,164]. Increased lipid peroxidation products cause peroxidation damage to endothelial membrane which may result in endothelial dysfunction which is associated with reduced nitric oxide[165]. Our study results justify this observation. The significant decrease of MDA levels after delivery in normotensive pregnant and preeclampsia indicated down trend of MDA values after placental removal within 48 hours.

Protein carbonyl is a stable indicator of protein damage in biological system. Reactive oxygen species oxidizes amino acid residues like glutamate, histidine and tryptophan in proteins to form product with carbonyl group. Protein carbonyl was increased by 1.4 fold in normotensive pregnant and 1.5 fold in preeclampsia when compared to non-pregnant as control. In after delivery the decrease in protein carbonyl content were not significant in normotensive pregnant. The striking observation in preeclampsia was protein carbonyl content decreased by 64% in after delivery. Results evinced that decreased protein damage maker indicates revocable changes in after delivery with preeclampsia. The present study showed same pattern as reported by Zusterzeel and his co-workers [166].

Superoxide dismutase (SOD) converts superoxide to water and it acts as first line of defense against free radical scavenging. SOD activity was significantly increased in normotensive pregnant when compared to non-pregnant. But decreased activity was noticed in preeclampsia compared to normotensive pregnant. In after delivery, SOD activity was significantly decreased in preeclampsia and normotensive pregnant groups. Studies conducted by Bakacak M[167] showed decreased SOD activity may be due to

increased Cu/Zn ratio. This altered ratio inactivates Cu/Zn containing antioxidant enzyme superoxide dismutase which may lead to decreased superoxide dismutase. Studies conducted by Ilhan et al.[168] showed that reduced SOD activity may be due to consumption during over production of lipid peroxide in preeclampsia.

Catalase is a heme protein catalyzes cleaving of hydrogen peroxide in to water and oxygen, thus it protects the cell from oxidative damage. In preeclampsia there was decreased enzyme activity when compared to non-pregnant. Its activity did not show significant difference between before and after delivery in normotensive pregnant and preeclampsia. Elevated xanthine oxidase and declined catalase activity indicated the severity of the oxidative stress in terms of hydrogen peroxide and hydroxyl radical in preeclampsia is evident in our study. Decrease in catalase activity may be due inhibition of enzyme by hypochlorite and peroxy nitrite free radicals which were more perhaps in preeclampsia [164, 169].

Glutathione peroxidase (GPx) is a selenium dependent enzyme eliminates hydrogen peroxide and organic hydro peroxides. There was non-significantly decreased GPx activity seen in preeclampsia before delivery when compared healthy control and normotensive pregnant. In after delivery Gpx activity was non-significantly decreased in normotensive pregnant and significantly increased in preeclampsia when compared to before delivery. Decreased glutathione peroxidase may lead to increased generation of reactive oxygen species[170]. Decreased selenium level was associated with decreased glutathione peroxidase activity which may be involved in pathophysiology of preeclampsia [85].

Glutathione reductase replenishes cellular reduced glutathione. Decreased activity in normal Pregnant and increased activity in preeclampsia when compared to healthy control ($p<0.001$) observed in our study. In preeclampsia, activity reduced by two fold in post-delivery indicates the role of placenta. Mohd Suhail and his co-workers found that non-significant decrease of GR activity in preeclampsia compared to non-pregnant[81]. Unlike these reports, significant two fold increase of glutathione reductase is seen in our study during preeclampsia under the study condition compared to normal pregnancy ($p<0.05$) group. Increased GR activity in preeclampsia may be an in vivo defensive response to restore the reduced glutathione level during altered oxidative and antioxidant system may be a compensatory mechanism in response to increased oxidative stress.

Ferric reducing ability of plasma (FRAP) was significantly decreased in normal pregnant and further decrease in preeclampsia before delivery ($p<0.001$) when compared to healthy control. FRAP levels were significantly decreased in preeclampsia when compared to normotensive pregnant ($p<0.05$).FRAP levels did not show significant difference between before and after delivery in preeclampsia as well as normal pregnant. Karacy and his co-workers also observed decreased FRAP level in preeclampsia [39].

Vitamin C levels were significantly decreased in normal pregnant and preeclampsia when compared to healthy control ($p<0.001$). But the decrease in vitamin was not significant during after delivery in normal pregnant and preeclampsia. Kiondo and his co-workers showed decreased vitamin C in normotensive pregnant as risk of developing preeclampsia [171].

Vitamin E levels were significantly increased in normal pregnant when compared to healthy control (<0.05) but there was non-significant decrease in preeclampsia when compared to healthy control. Significantly decreased vitamin E level was seen in preeclampsia when compared to normotensive pregnant (<0.001).In normotensive pregnant and preeclampsia there was no significant difference between before and after delivery. Reduction in vitamin C and E were consistent as shown by other studies [172].

Ischemia modified albumin (IMA) known as hypoxic risk indicator in various diseases and also in preeclampsia during first trimester[104, 173, 98]. In support of this finding, our study results indicated high IMA in preeclampsia. An observation of decreased maternal serum levels of IMA in post-delivery evidenced clinical improvement.

Proportionate rise of MDA and xanthine oxidase in preeclampsia displayed a positive correlation that denoting increased oxidative stress. Positive correlation is also observed between GPx and NO which indicates endothelial dysfunction and proliferates increased formation of super oxide radicals which can inhibit glutathione peroxidase enzyme[174]. Reduced SOD and glutathione peroxidase activity will cause increase in superoxide anion which reacts with nitric oxide to form peroxy nitrite. This in turn decreases the availability of NO. Similar observation also reported by Kenet G[175] and Bilodeau J F [38].

The negative correlation between IMA and GPx noticed in the study .The study rise the probable illustration that normal pregnancy demands high oxygen requirement and evidence increased oxidative stress. Inadequate supply of oxygen to trophoblastic cells results in preeclampsia. The hypoxia and established oxidative stress

alters serum albumin with N-terminal modification in to ischemia modified albumin. Therefore in the study context IMA evolved as good predictive marker of preeclampsia related risk. The impact of superoxide radicals has inhibitory effect on enzymes. Hence GPx activity decreased.

Tatjana N and co-workers reported decreased activity of superoxide dismutase in preeclampsia and also suggested to measure SOD activity to understand low antioxidant status[176]. Thus, SOD can be a predictive enzyme of low antioxidant status. This might result alteration of oxidative stress in preeclampsia.Vanderlelie et al. found that decreased glutathione reductase gene expression in preeclampsia without significant change in glutathione peroxidase[177]. This indicated possible importance of plasma glutathione and preeclampsia toxemic condition on glutathione reductase. In contrast to other studies we found increase of glutathione reductase in preeclampsia compared to normotensive pregnant.

In Reciever operating characteristics curve (ROC)analysisshowed area under curve for ischemia modified albumin (0.92), catalase (0.88), xanthine oxidase (0.8), malondialdehyde (0.804),uric acid (0.84) with sensitivity of 87.7, 96.5, 61.4, 84.21, 71.93 and specificity of 91.2, 84.2, 89.5, 68.4, 89.5respectively.Results indicated ischemia modified albumin as a good marker of intrauterine hypoxic reperfusion risk and abnormal placental development by dysfunction of trophoblastic cells under oxidative stress. ROC analysis showed that there is high production of hydrogen peroxide due to elevated xanthine oxidase activity and catalase is combating it.

Preeclampsia is a multi-factorial pregnancy disorder involves various types of system involvements in attributing to pathophysiology of placenta. Feto-maternal immune reactions in the first week of pregnancy, impaired arterial invasion by

trophoblast and transformation of spiral arteries followed by altered placental perfusion results in chronic hypoxia that triggers intensity of oxidative stress that has impact on placental synctio-trophoblast cell apoptosis and necrosis[178]. The rate of formation of placental debris has pro-inflammatory substances, angiogenic and anti- angiogenic factors etc which leads to endothelial dysfunction and systemic inflammatory response thus placenta play a central role in inflammatory process[179].

An inflammatory response in preeclampsia is usually accompanied by increased concentration of pro-inflammatory signaling molecules like cytokines, activated neutrophils and positive acute phase plasma proteins.Neutrophil activation may occur in the presence of cytokines such as tumor necrosis factor-α (TNF-α) during an inflammatory process. Degranulation of neutrophils releases a serine protease elastase enzyme that prolongs the inflammation by modification of pro-inflammatory cytokines and degrading proteins involved in inflammation. In addition to this, myeloperoxidase also present in neutrophil granules increases oxidative stress by additional production of hydroxyl radical and hypochlorous acid[180]. Hence, neutrophil activation results in vascular damage and dysfunction. Therefore, plasma elastase can be used to assess in vivo neutrophil activation. C-reactive protein is increased rapidly in response to inflammatory stimuli along with elastase in preeclampsia [69]. So that, increased elastase in plasma serves as a predictive marker of pregnancy induced inflammation[181].

A systemic inflammatory response involves leukocytes activation in terms of elastase, acute phase response in terms of hs-CRP and metabolic features of systemic inflammation by means of phospholipase A_2 were presented in the current study. These parameters were compared with hs-CRP as an established reliable inflammatory marker.

In our study, these markers were elevated in normal pregnancy and further accentuated in preeclampsia.

We observed systemic inflammatory response in preeclampsia through hs-CRP similar to the other research reports[182, 120]. The increase in plasma elastase in early onset of preeclampsia has been reported by Gupta AK [118]. Elastase released from polymorph nuclear lymphocytes during inflammatory condition and thus the plasma level increased considerably[183]. Therefore, in the current study plasma elastasewas compared with the hs- CRP a well-known inflammatory marker.

Phospholipase A_2 by virtue of hydrolysis of phospholipid releases arachidonic acid that serves as precursor for the synthesis of eicosanoids which participate in inflammatory process. Phospholipase A_2 enzyme activity was measured to know whether this enzyme can be treated under inflammatory marker. Even though, the importance of phospholipase A_2 in preeclampsia found to be contradictory[184-186].

But our report highlighted increased phospholipase A_2 activity in preeclampsia when compared to normotensive pregnant and non pregnant but did not show diagnostic importance as per ROC analysis.

Study limitations confine to determination of plasma elastase, phospholipase A_2 and hs-CRP from the time of pregnancy to all trimesters to denote the number of chances of pregnancy translated into pre-eclampsia. Our research findings generated a new knowledge about increased plasma elastase and phospholipase A_2 in pregnancy and preeclampsia. The rise of these enzymes and their substantiation in inflammation compared with hs-CRP. Apparently, phospholipaseA_2 activity increased butelastase rise found to be noteworthy since good sensitivity and specificity observed in ROC analysis. Hence plasma elastase can be used as diagnostically important for clinical utility.

Measure of elastase activity represents enhancedmaternal inflammatory process by neutrophil activation and degranulation during before and after delivery within 48 hours.

Flavonoids are polyphenolic compounds with flavones ring structure, ubiquitously distributed in various parts of the plants in the wide range. Flavonoids are classified in to flavones, flavonols, flavonoids, chalcones, anthocyanins, tannins and aurones. They are reported to have antioxidant, enzymes inhibition related to inflammation, cardio protective and bactericidal and vasodialatory function etc[187]. Flavonoids known to possess potential inhibitory action on xanthine oxidase.

Pongamia pinnata L, Morinda citrifolia L, Mangifera indica L, Zingiber officinale Roscoe subjected in the study for isolation of flavonoids compounds to test the inhibitory property on xanthine oxidase enzyme. Seed extract of *Pongamia pinnata Linn* was reported to have inhibition on α-amylase and α-glucosidase activity[188]. Furthermore inhibition of xanthine oxidase measured by using commercially obtained pure flavonoids compounds and suggested the planar flavones (Chrysin, luteolin and flavones), flavonol (quercetin, myricetin, kaempferol, rhamnetin, tangeretin and rutin) were having strong inhibitory effect on xanthine oxidase [56].

Information is limited on xanthine oxidase enzyme inhibition by *Pongamia pinnata* seeds. Our study reported 74% of enzyme inhibition with reference to allopurinol and identified *Pongamia pinnata* seeds flavonoids as the member of xanthine oxidase inhibitors from plant origin.

Afa Palu and his co-researchers reported 64% of xanthine oxidase inhibition using a fruit extract of *Morinda citrifolia L* after processing[58]. Our research findings are similar with the study and were able to obtain 50 % of xanthine oxidase enzyme

inhibition when compared to allopurinol. Mangiferin is a component of leaf known as xanthone C-glycoside of *Mangifera indica L* stands as a first reportto state about inhibitory activity on xanthine oxidase [189]. Our study investigated to explore similar property in the bark and reports nearly 58% of inhibition with allopurinol used as standard inhibitor.

Gouticin a coded herbal formulation contains one of the ingredients *Zingiber officinale Rosoe* reported to have potential inhibition on xanthine oxidase activity. The percentage of inhibition found to be effective and similar with allopurinol an allopathic drug for gouty arthritis. Since gouticin also contains *Apium graveolens, Colchicum autumnale, Tribulus terrestris, Withania Somnifera* along with *Zingiber officinale Roscoe*. In order to authenticate the role of *Zingiber officinale Roscoe* in the gouticin, flavonoids isolated and tested on xanthine oxidase activity using allopurinol as reference inhibitor[190]. Our study showed that nearly 55% of inhibition from the flavonoid extract obtained from the roots of *Zingiber officinale*. In the general screening study of plants on xanthine oxidase inhibition showed the order of priority of flavonoids inhibition on xanthine oxidase with quercetin and allopurinol a potent inhibitor.

In systemic meta-analysis of randomized control trials of various research findings reported that vitamin C supplementation has resulted in the decreased serum uric acid level[191]. In an *in-vitro* study conducted by Feigelson and coworkers on xanthine oxidase inhibition by L-ascorbic acid showed decreased xanthine oxidase activity. In an *in-vitro* study L-ascorbic acid reported to have 51% of xanthine oxidase inhibition at the concentration of 1mg/ml when compared with allopurinol[192]. The

findings of our study apparently similar by means of exhibiting 54% of inhibition at the concentration of 0.8 mg/ml.

In a randomized controlled trial reported the vitamin E inhibitory effect on xanthine oxidase related to gastric lesion prevention[193]. Supplementation of vitamin C & E in cholestasis induced hepato cellular injury, exerted protective benefit through the inhibition of xanthine oxidase as reported by Schimpl G[194]. Our research findings supported this view by showing 58% of inhibition by vitamin E on pure form of xanthine oxidase.

Nevertheless several reports emphasized vitamin C & E inhibits xanthine oxidase activity. None of the report presented remarkable percentage of inhibition other than 52- 55% range. In support of this 2011 WHO report described supplementation of vitamin C & E has no significance in reducing the risk of preeclampsia. The study concludes that plant species under investigation exhibited xanthine oxidase inhibition by optimized flavonoid extract. *Pongamia pinnata L* indicated promising xanthine oxidase inhibition compared to other plant extracts. Vitamin E is also expressed better xanthine oxidase inhibition than vitamin C compared to quercetin and allopurinol. Therefore flavonoids can be used as an alternative to allopurinol a potent inhibitor of xanthine oxidase.

CHAPTER-6

SUMMARY & CONCLUSION

6.0. SUMMARY AND CONCLUSION

6.1. SUMMARY

In this study, we observed leukocytosis and thrombocytopenia in preeclamptic women when compared to normotensive pregnant women. Primigravida and maternal age more than 30 years are at increased risk of developing preeclampsia. However hemoglobin levels had no much difference between the two groups and found to be lower normal. Gamma glutamyl transferase and lactate dehydrogenase activity increased in preeclampsia but it was not significant between mild and severe preeclampsia. Hence it cannot be used as an indicator to classify mild and severe preeclampsia. In preeclampsia xanthine oxidase, malondialdehyde and uric acid levels were significantly increased ($p<0.001$), while total antioxidant status decreased ($p<0.05$) when compared to normotensive pregnant and non-pregnant.

Catalase, glutathione reductase levels were increased ($p<0.005$) and vitamin E, super oxide dismutase levels were decreased ($p<0.001$) in preeclampsia when compared to normal pregnant women. Receiver operating characteristics curve analysis showed area under curve for xanthine oxidase (0.8), malondialdehyde (0.804), uric acid (0.84), ischemia modified albumin (0.92) and catalase (0.88) which indicated as good markers in preeclampsia. Amongst, Ischemia modified albumin is a better marker of intrauterine hypoxic reperfusion risk with sensitivity 87.7% and specificity 91.2%.

Our research findings generated knowledge about elevated plasma elastase, phospholipase A_2 with hs-CRP in preeclampsia which serves as an indicator of inflammation. The raised level of plasma elastase by neutrophil degranulation is a

marker enzyme to represent inflammation in preeclampsia with good sensitivity and specificity but not phospholipase A_2.

To the best of our knowledge this is one of the seldom study that measured oxidative stress parameters, antioxidants, endothelial dysfunction marker, plasma elastase, phospholipase A_2 and hs-CRP during pre and post-partum of normal pregnancy and preeclampsia cases in comparison with non-pregnant as base line.

In the xanthine oxidase inhibition study, pure form of vitamin C and vitamin E has no effective inhibition on pure form of xanthine oxidase *in vitro*. Extrapolation of this observation to *in vivo* condition demands further experimental results. This is in line with WHO 2012 report that states supplementation of vitamin C and vitamin E in preeclampsia to reduce oxidative stress is not recommended. Flavonoid extract from the seeds of *Pongamia pinnata L* showed 74% of xanthine oxidase inhibition.

6.2. CONCLUSION

Preeclampsia is an obstetric emergency for both mother and the fetus with unknown etiology. Delivery is the only effective way in the prompt management. In the present study factors such as oxidative stress, antioxidants and inflammation parameters in preeclampsia and their relationship in pre and post partum were studied.

Our research findings generated knowledge about:

- Xanthine oxidase as an enzyme oxidant marker in preeclampsia.
- Increased uric acid in preeclampsia due to increased xanthine oxidase activity not only due to decreased renal excretion.
- Increased hydrogen peroxide from xanthine oxidase reaction further adds to oxidative stress.
- Ischemia modified albumin can be considered as a better marker of hypoxic reperfusion risk.
- Distinctive observation of elevated catalase activity in pre and post-delivery of preeclampsia within 48 hours noticed despite of gradual reduction of oxidative stress.
- Increased catalase activity in preeclampsia represents combating action against oxidative stress.
- Evidence of endothelial dysfunction observed by reduction of nitric oxide level in preeclampsia.
- The inverse relation between xanthine oxidase and nitric oxide represents an indication of trophoblastic cell destruction and endothelial dysfunction.

- Increased oxidative stress with decreased total antioxidant status and its apparent reversible changes evinced within 48 hours after delivery in preeclampsia illustrated that placental abnormality is the contributing factor in the pathogenesis.
- Elevation of plasma elastase and phospholipase A_2 activities were observed in preeclampsia.
- Plasma elastase measurement has clinical utility when measured along with hs-CRP in inflammation. Increased elastase activity represents enhanced maternal inflammatory process by neutrophil activation and degranulation in preeclampsia.
- Mild systemic inflammation, oxidative stress was seen in normal pregnancy and these further exacerbated in preeclampsia.
- Recorded base line values of the study parameters from non pregnant and before and after delivery of normal pregnant.
- *Pongamia pinnata L* indicated promising xanthine oxidase inhibition compared to flavonoid extracts of the plants studied *in vitro*. Therefore, natural flavonoid components can be used as an alternative inhibitor to allopurinol a known potent inhibitor of xanthine oxidase.
- *In vitro* xanthine oxidase inhibition by vitamin C and E is not supportive for their supplementation to combat oxidative stress due to non effective inhibition (<60%).

CHAPTER-7

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7.0. REFERENCES

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Appendix-1

List of Reprints of the Publication of Ph.D topic

SL No	Title	Authors	Journal name	Indexation
01	Evaluation of Lipid Peroxidation, Protein Carbonyl Content and Total Antioxidant Status in Pre and Post-Delivery of Women with Pre-eclampsia.	**Vanishree B**, CD Dayanand, SR Sheela	American journal of pharmacy and health research. 2014;2: 99-105	Index copernicus
02	Is ischemia Modified Albumin is a Marker in Preeclampsia? A Case Control Study in Kolar Population	C D Dayanand, **Vanishree B** Pushpa Kotur	American journal of pharmacy and health research. 2015: 3(6) 84-90.	Index copernicus
03	Is Xanthine oxidase, a marker in preeclampsia?	**Vanishree B,** CD Dayanand, Pushpa Kotur	Journal of Clinical and Diagnostic Research. 2015: 9(10):1-3. PMCID:4625227	Pubmed EMBASE Scopus Medline Index copernicus
04	Relationship between Xanthine Oxidase, Ischemia Modified Albumin, Nitric Oxide with Antioxidants in Non-Pregnant, Pre and Post-delivery of Normal Pregnant and Preeclampsia.	**Vanishree B,** CD Dayanand, Pushpa Kotur	Indian Journal of Clinical Biochemistry 2016 DOI 10.1007/s12291-016-0599-0.	Pubmed EMBASE Scopus Medline Index copernicus
05	Relationship between plasma elastase, phospholipaseA2 and high sensitivity C - reactive protein in non Pregnant, pre and post-partum of normal Pregnant and preeclampsia	**Vanishree B,** CD Dayanand, SR Sheela	Asian Journal of Pharmeceutical and clinical research. 2017; 10(1):317-20.	Scopus Elsevier, EBSCO, EMBASE Index Copernicus
06	Evaluation of xanthine oxidase inhibitory activity by flavonoids from *pongamia pinnata linn*	**Vanishree B,** CD Dayanand, SR Sheela	Asian Journal of Pharmeceutical and clinical research. 2017; 10(3):1-3.	Scopus EBSCO, Index medicus

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Evaluation of Lipid Peroxidation, Protein Carbonyl Content and Total Antioxidant Status in Pre and Post-Delivery of Women with Preeclampsia

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ABSTRACT

Preeclampsia is a metabolic syndrome and a major cause of maternal, fetal and neonatal morbidity and mortality that amounts to 10% of the pregnancy complications. To determine Malondialdehyde, protein carbonyl and total antioxidant status in preeclampsia and normal pregnant women during pre and post labour within 48 hours. A prospective case control study (n= 60), preeclampsia (n=30) and normal pregnant women (n=30) during pre and post labour within 48 hours. 3 ml of blood samples were collected during pre and post labour of normal pregnant and preeclampsia. MDA, Protein Carbonyl Content and Total Antioxidant Status were measured using spectrophotometric method. Mean ± SD were determined by using student "t" test. The Mean ± SD values of MDA µmol/L (4.7 ±1.8), Protein Carbonyls (168.9±70.5) nmol/, Total Antioxidant Status mmol/L (537 ± 451) in normal pregnant during pre-delivery and MDA µmol/L(2.4 ±0.1), Protein Carbonyls (169±67.2)nmol/L , Total Antioxidant Status mmol/L (634.3 ± 241.2) post-delivery presented. Similarly Mean ± SD values of MDA µmol/L (10.1 ±6.4), Protein Carbonyls (159±123.2)nmol/L , Total Antioxidant Status mmol/L (506.7 ± 287.6) in preeclampsia pre delivery and (6.1±6.1), (98.8±36.8), (680±362.3) post-delivery respectively. A well-known aspect in preeclampsia with increased concentrations of oxidative stress and decreased TAS. However the trend of increased TAS and declined oxidative stress during post labour of normal pregnant and preeclampsia which plays a significant role in pathophysiology of preeclampsia.

Keywords: Preeclampsia, Before delivery, After delivery, Protein carbonyl content

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INTRODUCTION

Preeclampsia is a clinical condition characterized by hypertension and proteinuria. This obstetric complications leading to fetal uterine growth restriction, preterm delivery, maternal and fetal morbidity and mortality[1-2].It occurs in 5-7 % of the pregnancies worldwide.[3]The incidence is still higher in India of around 8-10%.[4]As per the World Health Report the maternal mortality during pregnancy and puerperium is around 12 %.In developing countries, 17% of direct obstetric deaths are as a result of hypertension[5]. The mortality rate of preeclampsia in the developing and developed countries varies approximately eight hundred women die from pregnancy and child birth related complications around the world every day[6]. Although aetiology of preeclampsia is not clear, maternal symptoms are of secondary to endothelial dysfunction causes imbalance to the oxidant and antioxidants ratio in preeclampsia. Propagation of free radicals are known for generation of oxidative stress and thus play a prominent role in preeclampsia. Therefore measurement of indicators of oxidative stress helps to understand an imbalance of oxidants and antioxidants. In the current research, an attempt is made to report the oxidative stress markers for lipid peroxidation as MDA and Protein oxidation as carbonylated proteins in terms of protein carbonyls and also plasma total antioxidant status in pre and post labour of preeclampsia in comparison to normal pregnant group.

MATERIALS AND METHOD

Pregnant women in the third trimester attended to Department of Obstetrics and Gynecology, R.L. Jalappa Hospital and Research Center, Kolar, Karnataka were enrolled for the prospective case control study. Preeclampsia was defined as systolic and diastolic blood pressure greater than 140 mm of Hg and 90 mm of Hg, with significant proteinuria (>300 mg per 24 hr). From this total subjects 30women were clinically diagnosed as Preeclampsia and another 30 was normal pregnant woman were included in the study after obtaining the University Ethical Committee approval and patient information consent. After performing physical examination and obtaining the complete patient history, three ml of blood samples were drawn into Heparinized vaccutainer at pre and post-delivery of women with preeclampsia and normal pregnant women respectively. The content of the tubes were mixed and centrifuged for 15 min at 4000rpm to separate the clear plasma that was used for quantification of Malondialdehyde, Protein carbonyl content and Total antioxidant status. Malondialdehyde (MDA) a marker of lipid peroxidation was measured in plasma by spectrophotometrically using standard as tetra methoxy propane (TMP) as thiobarbituric acid reactive substances on precipitation of proteins with trichloroacetic acid as per

the method described by Sinnbhuber RO et al[7]. Protein carbonyls a marker of protein oxidation was measured in plasma by spectrophotometrically using internal standard oxidized BSA and reduced BSA according to method mentioned by H. Busset al[8] .Oxidized BSA containing additional carbonyls was prepared by reacting (50mg/ml) with hypochlorous acid to final concentration of 5mM. Reduced BSA is prepare by adding 0.1 g sodium borohydride to a solution of 0.5g BSA in 100ml phosphate buffer saline. After 30 minutes, this solution was brought to neutral pH with 2M HCl and then subjected for dialysis. Using reduced BSA from the dialysate and oxidized BSA obtained from the above step used as as standard maintaining constant protein concentration (4mg/ml).Thus prepared standards used during determination of protein carbonyl content by Levin et al[9] method. Plasma Total antioxidant status determined by means of ferric reducing ability of plasma that was measured spectrophotometrically according to the method described by Benzie I F et al[10].The unpaired t test was used to assess the statistical significance of difference between the study groups. A probability level of <0.05 considered as statistically significant. Results are reported as mean standard ± deviation.

RESULTS AND DISCUSSION

Malondialdehyde as lipid peroxidation marker, Protein carbonyl content as a marker of Protein oxidation in oxidative stress and Total Antioxidant status were measured in Preeclampsia and normal pregnant during pre and post labour within 48 hours. Table 1 shows the concentration of MDA, Protein carbonyl content and total antioxidant status in healthy pregnant women and preeclampsia during pre-labour. The results between two groups evinced an observation of increased MDA level in preeclampsia group (10.1±6.4) in comparison to normal pregnancy during pre-labour (4.7±1.8) with p value <0.05 as significance. Study clearly indicates that there are no significant results with respect to protein carbonyls and total antioxidant status between normal pregnancy and preeclampsia during post-delivery. The levels of MDA, Protein carbonyl content and total antioxidant status in normal pregnant women and preeclampsia during post-delivery were shown in Table 2. The decreased levels of protein carbonyl content in preeclampsia (98.8±36.8) compared to the normal pregnancy (169±67.2).MDA levels increased in preeclampsia (6.1±6.1) after delivery when compared to the normal pregnancy (2.4±1.0) which is highly significant with the p value of <0.005.But no difference is observed with respect to Total Antioxidant Status between the two groups during post-delivery. The striking observation is that increasing trend of total antioxidant status in the two groups after delivery within 48 hours with concomitant decrease of lipid peroxidation. Oxidative stress propagates

generation of reactive oxygen species against buffering capacity of antioxidants. The altered homeostasis implicated in the onset of atherosclerosis, cancers, pre-eclampsia, and many other diseases. Oxidative stress markers are also increased in the decidua, placenta, and other maternal tissues[11]. Oxidative stress as a potential indicator in preeclampsia has been studied, MDA is one of the first biomarkers of lipid peroxidation found to elevate in the plasma of women with preeclampsia[12]. Similarly we observed significantly increased MDA levels in preeclampsia .This observation holds good and supported by Petra LM et al[13]. He has explained the mechanism of increase of MDA during cell turn over and decline in antioxidants free radical trapping mechanism. The current study emphasis two fold increase of MDA as a marker of oxidative stress in preeclampsia compared to the control group and nearly fifty percent reduction of MDA was seen between two groups during before and after delivery indicates consistent elevation of MDA. This proves a radical increase in MDA level in control and further increase in Preeclampsia groups as supported by other studies[14].Therefore serum MDA measurement can be used as s prognostic indicator of oxidative stress that also raise an alarm of need of antioxidant supplementation during preeclampsia. Increase in total antioxidant capacity is seen in preeclampsia and healthy pregnant women after delivery when compared to before delivery which might be due to compensatory regulation in response to the oxidative stress[15].There was no difference in Protein carbonyl content between preeclampsia and healthy pregnant women before delivery. As observed in the present study, increased protein carbonyl content in normal pregnancy as well as in preeclampsia was also reported by zusterzeelet al[16]. Increase in MDA is seen in normal pregnancy that generates an imbalance in homeostasis of oxidant and antioxidants. This elevation further aggravates preeclampsia which might result in endothelial damage leading to increased diastolic blood pressure[17] and after delivery MDA level in preeclampsia has not attain to the level of MDA levels in normal pregnancy within forty eight hours. That shows higher level of oxidative stress persists after deivery[18]. In the present study we found that baseline elevation of MDA levels were seen in both the groups with a proportionate variation. The study results also emphasis the reduction in MDA levels in both the groups after delivery with increased TAS level compared to pre labour where it is associated with elevated oxidative stress in preeclampsia. This inverse relationship clearly demonstrates the homeostasis of oxidative stress and antioxidant status. Increased lipid peroxidation and decreased total antioxidant status observed in preeclampsia in comparison to control group in pre delivery. Significant reduction of MDA, protein carbonyl were seen in control and preeclampsia in post-

delivery compared to pre delivery. However protein carbonyl is unaltered in control group but decreased in preeclampsia before and after delivery.

Table 1: Circulating levels of plasma MDA, Protein carbonyl content and total antioxidant status in healthy pregnant women and pregnant women with preeclampsia during pre-labour.

Parameters	pregnant women Pre Labour (Mean±SD)	Pre-eclampsia Pre Labour (Mean±SD)	p value
Protein carbonyl (nmol/L)	168.9±70.5	159±123.2	>0.05
MDA (µmol/L)	4.7±1.8	10.1±6.4	<0.05*
TAS(mmol/L)	537±451	506.7±287.6	>0.05

* P value <0.05 is considered as statistically significant

** Indicates Highly Significant

Table 2: Circulating levels of plasma MDA, Protein carbonyl content and Total antioxidant status in healthy pregnant women and pregnant women with preeclampsia during post labour

Parameters	pregnant women post labour (Mean±SD)	Pre-eclampsia Post labour (Mean±SD)	p value
Protein carbonyl (nmol/L)	169±67.2	98.8±36.8	<0.05*
MDA(µmol/L)	2.4±1.0	6.1±6.1	<0.005**
TAS(mmol/L)	634.3±241.2	680±362.3	>0.05

*P value <0.05 is considered as statistically significant

** Indicates Highly Significant

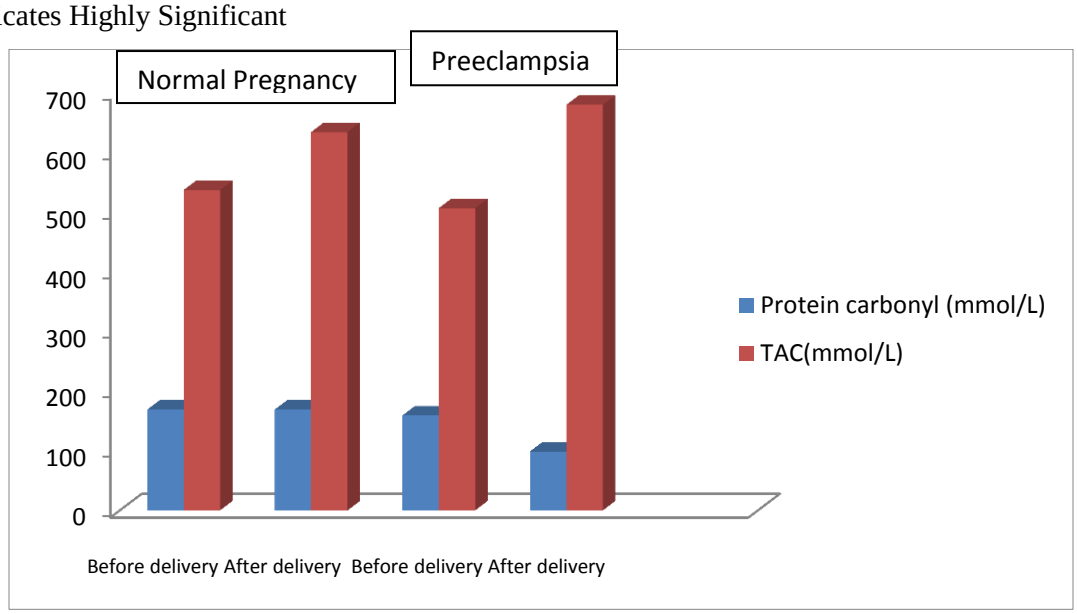

Figure 1: Showing the levels of Protein carbonyl content, TAS between two groups during pre and Post Labour.

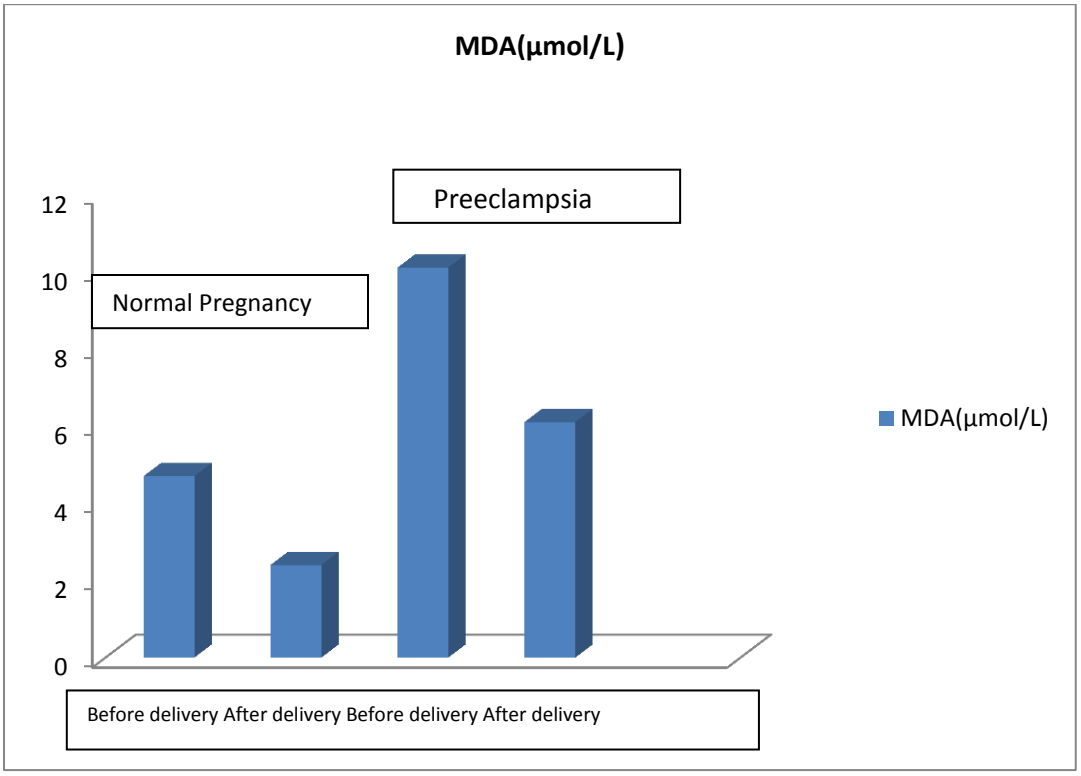

Figure 2: Showing the levels of MDA between the two groups during pre and post labour

CONCLUSION

A well-known aspect in preeclampsia with increased concentrations of oxidative stress and decreased TAS. However the trend of increased TAS and declined oxidative stress during post labour of normal pregnant and preeclampsia which plays a significant role in Pathophysiology of preeclampsia.

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Is ischemia Modified Albumin is a Marker in Preeclampsia? A Case Control Study in Kolar Population-a South West of India

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ABSTRACT

Preeclampsia is a syndrome occurring in pregnancy that affects multiple systemic functions and leads to increased maternal and foetal morbidity as well as mortality. Ischemia Modified Albumin is a well-known marker of cardiac Ischemia. We aimed to test the hypothesis that variation in maternal serum Ischemia Modified Albumin (IMA) in preeclampsia during pre and post labour within 48 hours. A Longitudinal observational study was conducted between June 20 to April 2013in subjects with normal pregnancy (n=30), women with preeclampsia (n=30) and non-pregnant women (n=30) to evaluate the levels of IMA. The women studied were in 20-35thweeks of gestation visited obstetrics clinic in R.L. Jalappa hospital& Research center, Kolar, in a southern state of India. We estimated the maternal serum IMA levels before and after the delivery within 48 hours by using Albumin Cobalt Binding procedure. The results obtained were analysed by using Microsoft Excel 2013 and Quick R. p value < 0.05 is considered as statistically significant. The Median IMA level in the preeclampsia group before delivery 1.071ABSU [inter quartile range (IQR) 0.453-1.708ABSU] and the same within 48hrs after delivery 1.013ABSU [inter quartile range (IQR) 0.526-1.774 ABSU)] was significantly lower (p<0.02) than in normal pregnant group before delivery 0.328 ABSU [inter quartile range (IQR) 0.154-0.592 ABSU] and the same within 48hrs after delivery 0.570ABSU [inter quartile range (IQR) 0.060-0.751 ABSU. However, a significant increase in IMA concentrations (p<0.001) when compared to non-pregnant women 0.101 ABSU [inter quartile range (IQR) 0.049-0.358ABSU]. *In vivo* modification of albumin at the amino terminal residues loose in vitro Cobalt (Co (II)) binding ability. The trend of Modified albumin as IMA in preeclampsia shows the gradual increase after the delivery within 48 hrs. However the levels of IMA in normal pregnancy show a tenfold increase than the non-pregnant women.

Keywords: Ischemia Modified Albumin, Intrauterine Hypoxia, Preeclampsia.

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INTRODUCTION

Human developmental biology has been stirred by the emergence of evidence that the early placental environment is hypoxia[1]. In normal pregnancy intrauterine hypoxic environment, subsequent reperfusion and oxidative stress plays a crucial role in the pathophysiological trophoblast development illustrated by experimental evidences [2-4]. As a measurement of Cardiac ischemia in acute coronary syndromes, Ischemia Modified Albumin (IMA) is measured as a protein marker in several studies[5]. Studies reported with an evidence that during ischemia/ reperfusion, generation of highly reactive hydroxyl radical by reactive oxygen species (ROS) that results in site-specific modifications at N-terminus of the albumin moiety, especially at the N-Asp-Ala-His-Lys sequence, thereby producing IMA [6,7]. The aim of the present study was to determine the Cobalt, Co (II) binding ability to amino-acid residues at the N-terminus of human serum albumin in Maternal Serum of preeclampsia.

MATERIALS AND METHOD

Venous samples were collected from 30 preeclampsia women after confirming hypertension and proteinuria condition and 30 normal pregnant women with single tone pregnancies attending for routine checkup during at 20-35 weeks gestation and from 30 non-pregnant healthy women of reproductive age. In all cases, 5 ml of maternal blood sample was drawn in a vacutainer, allowed to clot and centrifuged at 3000Xg for 15 min to obtain the clear sera. The institutional ethical clearance and written consent forms from all the participants in the study were obtained. Women with known case history of diabetes Mellitus, connective tissue disease, cardiac disease, renal disease and history of recurrent miscarriage were excluded from the study. IMA was measured by the albumin cobalt binding test (ACB test). Serum samples collected from patients with preeclampsia before delivery and follow up of the same patient serum sample after the delivery within 48 hours were frozen at -80^0C within 30 minutes. Frozen samples were gently vortexes after thawing. As per the ACB test, 100µl of patient sample and 50 µl of cobalt chloride (Co (II)) are incubated for 5 minutes. During this incubation, the Co (II), which is a transitional metal that binds to the N-terminal residues of unaltered albumin in the sample; albumin for which the N-terminal residues is altered as a result of ischemic process binds to the Co (II) to a far lesser extent. 25µl of Dithiothreitol (DTT) forms a colored complex with Co (II) that is not bound to the modified N-terminal residues of albumin and this complex is measured at 470nm. The median interquartile range was used to express data and the Mann-Whitney, as appropriate to compare the groups. Two sided *p*-values are reported.

RESULTS AND DISCUSSION

Ischemia Modified Albumin (IMA) was measured in thirty normal pregnant and thirty preeclampsia women before and after the delivery within 48 hours. at a gestation age of 35 weeks that were compared to 30 non–pregnant women as controls. The Median IMA level in the preeclampsia group before delivery 1.0171 ABSU [inter quartile range (IQR) 0.453-1.708ABSU] and the same within 48hrs after delivery 1.013ABSU [inter quartile range (IQR) 0.526-1.774 ABSU)] was significantly higher (p<0.02) than in normal pregnant group before delivery 0.328 ABSU [inter quartile range (IQR) 0.154-0.592 ABSU] and the same within 48hrs after delivery 0.570ABSU [inter quartile range (IQR) 0.064-0.751ABSU]. But a significant lower (p<0.001) IMA concentrations when compared to non-pregnant women 0.101 ABSU [inter quartile range (IQR) 0.049-0.358ABSU] represents the 25th and 75th percentiles, together with the median, with whiskers showing the minimum and maximum serum IMA concentrations in Preeclampsia before and after the delivery, normal pregnant before and after the delivery and in non pregnant women expected under the hypothesis of neutrality with difference in the groups(Table 1 and Figure 1). Human serum Albumin sequence obtained from the database from NCBI in the FASTA format models, the structure is obtained using modular, marked the four amino acid residues N-Asp-Ala-His-Lys using Pymol visualization software tool. Since, characterization of these residues at N-terminal region binds to transition metal cations cobalt and nickel has been elucidated by HPLC, LC-MS and HNMR analysis [9] any modification to these residues produce defective albumin proteins that has the lower binding ability to cobalt metal ions, thus an insight of decreased Cobalt binding proposes a new assay for preeclampsia. Human serum albumin is a major, multi-functional glycoprotein with a single polypeptide chain consists of 585 amino acid residues (Figure 2). It consists of three structurally homologous, largely helical domains (I, II, and III), and each domain consists of two sub domains, A and B. The first four amino acids in the N-terminus is Asp-Ala-His-Lys acts as a specific binding site for transition metals such as cobalt (II), copper (II), and nickel (II). This portion serves as the most susceptible region for degradation compared to other regions of albumin[8]. Studies on the N-terminal binding of human Serum albumin with Co (2+) and Ni (2+) metals have been established by using various advanced techniques like, HPLC, LCMS, and NMR[9]. In ischemic condition, circulating albumin undergoes modification specifically at the amino terminus. This modified protein is termed as “ischemia modified albumin (IMA)” with the decreased binding ability to transition metals (Co (2+) and Ni (2+)). On the basis of this biochemical property, Bar-

Or et al. (1990) developed a rapid colorimetric assay method measuring using an inorganic compound cobalt chloride. Ischemia-induced alterations of the binding capacity of human serum albumin to exogenous cobalt are reported in disease state. The possible in vivo modification to human serum albumin (HSA) includes glycation and oxidation. However, a significant increase in IMA was observed under various pathological conditions such as diabetes, myocardial infraction, etc., is due to the enormous generation of free Radical species under Oxidative stress condition[10]. Thus, generated IMA can be utilized as a protein oxidant marker in the preeclampsia condition. The present study evinced the variation in IMA level in non-pregnant (group 1), and normal pregnant before and after delivery (group 2) and preeclampsia before and after delivery (group 3). According to Ustun Y and his co-workers, IMA levels were significantly higher in the mild and severe preeclampsia groups than in the control group[11]. IMA appear to be significantly increased during pathological pregnancies and thus IMA could be used as a biological marker of preeclampsia[12]. The similar observation also obtained in our study with raised IMA levels in Preeclampsia before delivery and after the delivery within 48 hours. A study conducted on IMA in preeclampsia[13] stated that serum IMA level as a marker of myocardial ischemia; preeclampsia found that there is no significant relationship between IMA levels and preeclampsia, in women with or without small-for-gestation-age (SGA). Contradictory to this observation, in the present study, we found that there is a marked increase in the IMA levels in normal pregnancy and before and after delivery within 48 hours, Compared to non-pregnant group. Even though, further studies are necessary to evaluate the IMA levels at different intervals during pregnancy and after delivery with increased sample size.

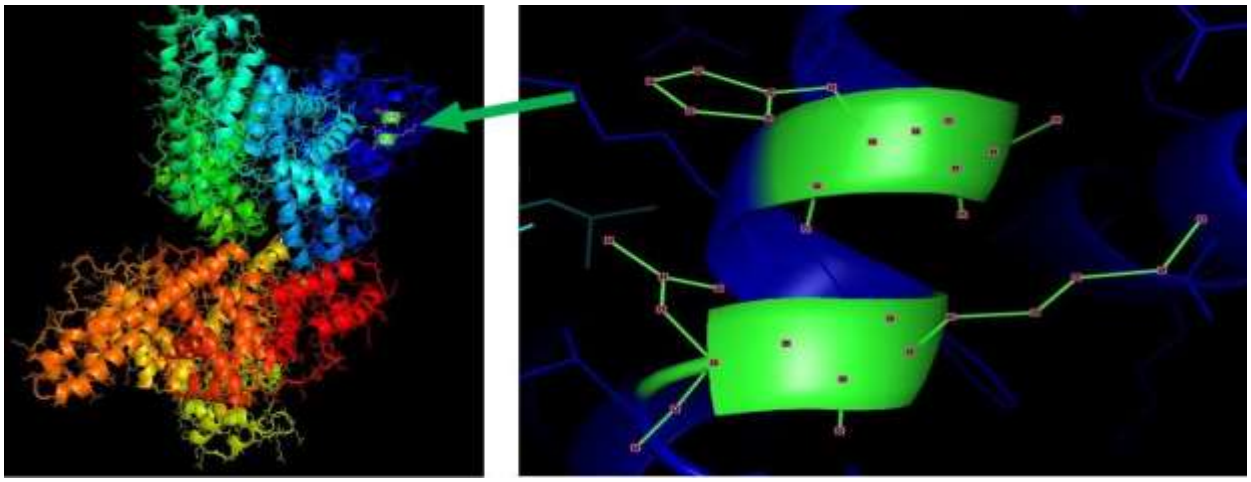

Figure 2: Structure obtained using modular, marked with the four amino acid residues N-Asp-Ala-His-Lys using Pymol visualization software tool

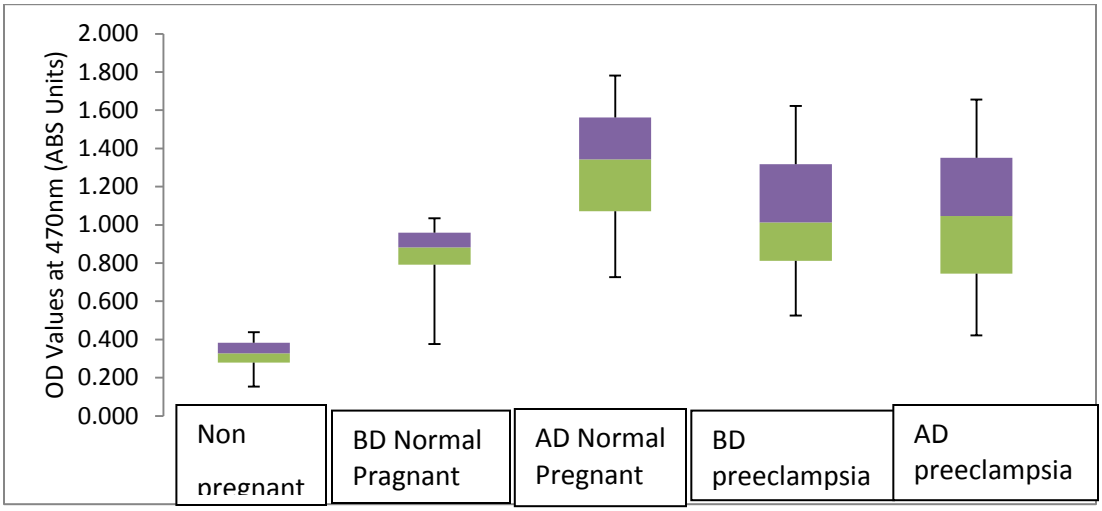

Figure 1: Box plot showing the 25th and 75th percentiles, together with the median, with whiskers showing the minimum and maximum IMA maternal serum concentrations in Preeclampsia before and after the delivery within 48hrs, normal pregnant before and after the delivery within 48hrs., and in non –pregnant women expected under the hypothesis of neutrality with difference in the groups.

Table 1: IMA values in normal pregnancy and preeclampsia before and after delivery

Sl.no	Non pregnant woman	IMA value in normal pregnant woman		IMA value in preeclampsia pregnant woman	
		Before delivery	After delivery	Before delivery	After delivery
ABSU	0.101	0.328	0.570	1.071	1.013
I.Q.R	0.049-0.358	0.154-0.592	0.064-0.751	0.453-1.708	0.526-1.774

CONCLUSION

An *in vivo* modification of albumin at the amino terminal residues loose *in vitro* ligand Cobalt Co(II) binding ability. The trend of Modified albumin as IMA in preeclampsia shows the gradual increase before the delivery. However, the levels of IMA in non-pregnancy shows 3.2- 10 fold decrease than the normal pregnant women and preeclampsia group. Increased IMA concentrations in preeclampsia before and after delivery and that was evaluated in terms of cobalt binding to specific amino acid in albumin.

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Original Article

Biochemistry Section

Is Xanthine Oxidase, a Marker in Pre-eclampsia? A Case-Control Study

VANISHREE BAMBRANA[1], C.D.DAYANAND[2], PUSHPA P KOTUR[3]

ABSTRACT

Introduction: Pre-eclampsia is an obstetrics problem that affects multiple systemic functions and leads to the increased maternal and fetal morbidity and mortality. The objective of the study was to evaluate the plasma levels of Xanthine oxidase (XO) activity, uric acid and Nitric oxide (NO) levels in women with pre-eclampsia and normal pregnancy during antenatal and postpartum period.

Materials and Methods: A case control study was conducted in women with normal pregnancy (n=50) and pre-eclampsia (n=50) before and after delivery. XO activity, uric acid and NO levels were determined from samples at 30-39 weeks of gestation. The current study was conducted in association with Obstetrics and Gynecology Department of R.L. Jalappa Hospital and Research Center. The blood samples were analysed for assay of XO, uric acid and NO. The results were analysed by using SPSS software version 2013. P-value < 0.05 was considered as statistically significant.

Results: The plasma XO activity was elevated (p<0.001) in the pre-eclampsia compared to normotensive pregnant women before delivery and decreased after delivery (p<0.001) significantly. Uric acid level showed a significant increase in pre-eclampsia when compared to the control before delivery (p<0.001) however values were non-significant after delivery.

Conclusion: Placenta plays a key role in the pathophysiology of pre-eclampsia. Placenta removal leads to decrease trend of xanthine oxidase activity, uric acid and elevation of Nitric oxide as reversible changes in pre-eclampsia patients within 48 hours after delivery.

Keywords: Antenatal, Nitric oxide, Postnatal, Uric acid

INTRODUCTION

Pre-eclampsia is a maternal syndrome characterized by hypertension, proteinuria, oedema after 20 weeks of pregnancy [1]. The symptoms of Pre-eclampsia can range from mild to severe due to slow or rapid progress of disease condition. They include persistent headache, blurred vision, Vomiting and abdominal pain [2]. The complications of pre-eclampsia is leading to fetal uterine growth restriction, preterm delivery, maternal and fetal morbidity and mortality [3,4]. It is a multisystem disorder, clearly shows the involvement of utero-placental blood flow, vascular resistance, endothelial integrity, endothelial damage, coagulation system in pre-eclampsia. Potential causes and mechanisms behind pre-eclampsia remain unknown, but the involvement of maternal, immune, genetic factors and placenta have been implicated.

Pre-eclampsia is a leading cause of hypertension results in complication up to 10% pregnancies. Pre-eclampsia and eclampsia accounts for 24% of all maternal deaths in India [5]. In the developing and developed countries, approximately 800 women die from pregnancy and child birth related complications around the world every day [6]. Pre-eclampsia is more common in first pregnancies [7] than the second pregnancies [8]. Early detection and management helps in reducing the complications of pre-eclampsia. Despite its prevalence, pathophysiology is poorly understood and aetiology has to be elucidated.

Xanthine oxidase (XO) is an iron, molybdenum containing flavoprotein which catalyzes the oxidation of xanthine/hypoxanthine into uric acid (2,6,8, trioxy Purine). XO levels are very less in healthy individuals but have shown to be increased in the pathological conditions [9]. Pregnancy induced hypertension is the leading cause of maternal and fetal morbidity and mortality [10]. Pre-eclampsia occurs only in the presence of placenta and resolves after placental delivery. The main hypothesis depends on the decreased placental perfusion due to impaired remodeling of spiral arteries. Inadequacy of placental perfusion might result in hypoxia. This hypoxic interface between maternal-fetus results in destruction of fetal tissue that can release OX, substrate like xanthine/hypoxanthine, cytokines etc [11]. Hypoxia stimulus generates reactive oxygen species like superoxide anion in living cells [12,13].

Reactive oxygen species (ROS) and nitric oxide (NO) interference changes the vascular function. Therefore in the present study, we hypothesized that increased ROS production may reduce availability of nitric oxide and might cause endothelial dysfunction which may play a role in the pathogenesis of pre-eclampsia.

Uric acid is the end product of purine catabolism, its production increased in condition of ischemia reperfusion [14]. Evidence suggests that uric acid has action of antioxidant but under certain conditions the formation of uric acid connected to the production of free radicals [15]. Therefore, present study aims to evaluate the activity of Xanthine oxidase as an enzyme marker in pre-eclampsia. The objectives were to estimate the plasma levels of Xanthine oxidase (XO) activity, uric acid and Nitric oxide (NO) levels in women with the pre-eclampsia and normal pregnancy during before and after delivery.

MATERIALS AND METHODS

Group 1 (n=50) normal pregnant women and Group 2 (n=50) pre-eclampsia in 30-39 weeks of gestation before delivery and same subjects after delivery were recruited in the study from Obstetrics and Gynecology Department of R.L. Jalappa Hospital and Research Center, Kolar, India with the approval of Institutional Ethics Committee. Pre-eclampsia was defined as blood pressure constantly greater than 140/90mm Hg and proteinuria above 0.3 g/24 hours after 20 weeks of gestation. Eligibility for the study was defined as per the Classification of National High Blood Pressure Education Programme working group (NHBPEP). Pregnant women beyond 28 weeks of gestation with pre-eclampsia diagnosed included in the study group as cases and age, gestation matched normotensive pregnant women were included in the controls after obtaining Informed Consent from the patient.

Inclusion criteria were Singleton pregnancy, no fetal anatomical anomaly; nonsmokers were included in the study. Exclusion criteria were chronic hypertension, molar pregnancy, gestational diabetes and multiple gestations.

Three ml of venous blood sample was collected into heparinized tubes and plasma separated by centrifugation at 3000rpm for 15 minutes. OX estimation is done within 2 hours of sample collection. Aliquots were stored at -80ºC until further analysis.

Spectrophotometric continuous rate determination assay of XO through uric acid formation from substrate xanthine recorded at 290nm using a method described by Bergmeyer [16]. Plasma nitric oxide measured in terms of reduction of nitrate into nitrite by reducing agent copper coated cadmium granules using sodium nitrite as standard ($NaNO_2$). The nitrite produced is determined by diazotization of Sulfanilamide in acidic medium and then coupling with Napthyl ethylene Diamine to produce pink colored compound which was measured spectrophotometrically at 540nm [17]. Plasma uric acid oxidized by uricase to produce hydrogen peroxide and allontoin. Peroxidase acts on hydrogen peroxide and oxidizes 3, 5-dichloro-2 –hydro benzenesulfonic acid and 4-aminophenazone to form a red–violet quinoneimine compound which was measured at 505 nm [18].

STATISTICAL ANALYSIS

The unpaired t-test was used to assess the statistical significance of difference between the study groups. A probability level of $p<0.05$ considered as statistically significant. Results were presented as mean±SD. Pearson correlation coefficient was used to measure the correlation between NO and XO.

Parameters	Group (1) Healthy pregnant women Before Delivery (Mean±SD)	Group(2) Pre-eclampsia Before Delivery (Mean±SD)	p-value
Nitric oxide (µmoles/L)	7.29±3.38	6.24±3.8	>0.05
Xanthine Oxidase (Units/L enzyme)	39.10±54.04	205±197.02	<0.001**
Uric Acid (mg/dl)	4.20±1.54	6.44±2.21	<0.001**

[Table/Fig-1]: Plasma Xanthine oxidase, Nitric oxide and Uric acid levels of normal pregnant and pre-eclamptic cases before delivery

**p-value <0.001 is considered as highly significant

Parameters	Group (1) Healthy pregnant women After Delivery (Mean±SD)	Group (2) Pre-eclampsia After Delivery (Mean±SD)	p-value
Nitric oxide (µmoles/L)	6.12±3.031	7.12±9.9	>0.05
Xanthine Oxidase (Units/L enzyme)	17.9±14.2	96.6±141.3	<0.001**
Uric Acid (mg/dl)	4.27±2.41	4.5±1.9	>0.05

[Table/Fig-2]: Plasma Xanthine oxidase, Nitric oxide and Uric acid levels of normal pregnant and pre-eclamptic cases after delivery

**p-value <0.001 is considered as highly significant

RESULTS

The mean and standard deviation values of XO and uric acid (39.10 U/L ±54.04, 205U/L ±197.02 p<0.001), (4.20mg/dl±1.54, 6.44 mg/dl±2.21 p<0.001) were higher in pre-eclamptic group compared to the control. There was decreased Nitric oxide 7.29µ moles/L ±3.38, 6.24µmoles/L ±3.8 (p>0.05) in pre-eclampsia when compared to healthy pregnant before delivery but it was not significant. Similarly Xanthine oxidase 17.9U/L±14.2, 96.6 U/L±141.3 (p<0.001), Uric acid 4.27mg/dl±2.4 4.5mg/dl±1.9 (p>0.05), and Nitric oxide 6.12µmoles/L±3.031, 7.12µmoles/L±9.9 (p>0.05), in healthy pregnant and pre-eclampsia after delivery within 48 hours were presented in [Table/Fig-1,2] respectively. Maternal plasma xanthine oxidase activity, nitric oxide and uric acid are measured before delivery and after delivery from Group (1) and Group (2) are presented in [Table/Fig-3-5] respectively.

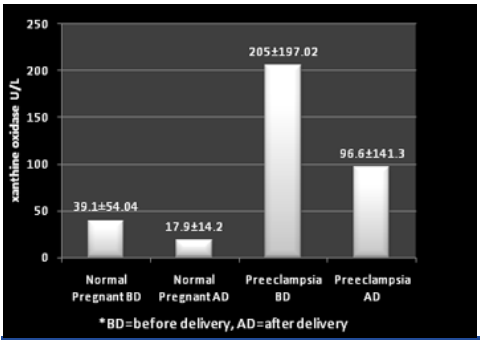
DISCUSSION

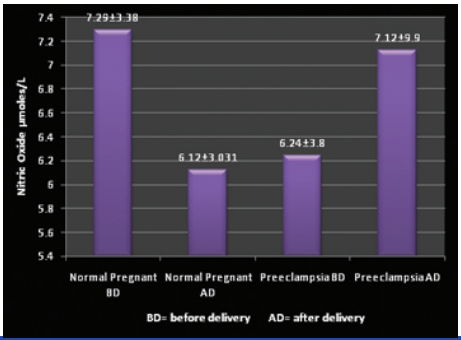
Pre-eclampsia is characterized by increased production of ROS and decreased levels of antioxidant status and hyperuricemia [19]. Pre-eclampsia is influenced by various factors such as increased oxidative stress, hypoxicated trophoblastic tissue destruction that produces xanthine, hypoxanthine and cytokines leading to inflammatory process [20]. Current study results indicated that increased activity of XO, non-significantly decreased nitric oxide level and marginally elevated uric acid levels observed.

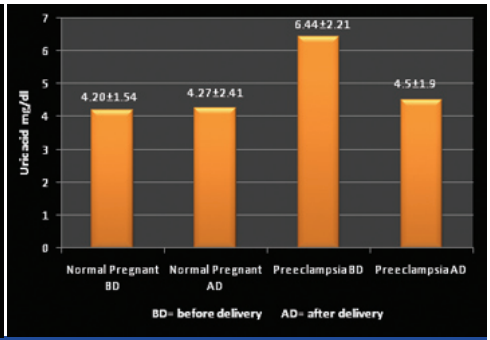
Improper spiral arteries implantation leads to hypoxia and increased turnover of trophoblastic tissue which might result in increased xanthine and hypoxanthine that served as substrate for XO that might lead to increased uric acid [21]. However, there is no data available on measurement of XO that can be considered as enzyme marker in pre-eclampsia before and after delivery status. Even though in a research report of Karabulut et al., evinced the increased XO activity in pre-eclampsia, but data on XO activity is not available after delivery condition of pre-eclampsia [11].

XO (E.C 1.17.3.2) catalyzes the conversion of substrates hypoxanthine/xanthine in to Uric acid and hydrogen peroxide. Xanthine dehydrogenase (E.C.1.17.1.4) is NAD+ dependent and xanthine oxidase (E.C.1.17.3.2) uses oxygen exists during purification protocol [22]. However, limited proteolysis and oxidation of sulfhydryl groups converts irreversibly Xanthine dehydrogenase in to XO [23]. Placental incompatibility in ischemic condition due to free radical formation and increased oxidative stress makes endothelial damage and cell death the cause for elevation of XO in circulation [11].

Activated leukocytes produce cytokines that in turn increases the XO activity and also ROS from endothelium and increases production of uric acid [24,25]. In our research work, an observation is significantly recorded in terms of elevation of XO in pre-eclampsia in comparison with normal pregnant women. In the same way uric acid level also increased before delivery. Two fold decrease of XO activity noticed after delivery condition in pre-eclampsia. XO activity in pre-eclampsia increased by 5.26 fold compared to normal pregnant group before delivery. However, after delivery reverts to







[Table/Fig-3]: Showing the levels of Xanthine oxidase normal pregnant and pre-eclamptic cases before delivery. **[Table/Fig-4]:** Showing the levels of Nitric oxide between normal pregnant and pre-eclamptic cases before delivery. **[Table/Fig-5]:** Showing the levels of Uric acid between normal pregnant and pre-eclamptic cases before delivery

2.1 fold in pre-eclampsia. The level was persistently high compared to healthy pregnant whereas the same enzyme reverts to 2.1 fold after delivery in healthy pregnant women. These results indicated that the rise of XO level in pre-eclampsia before delivery proves that measurement of XO in pre-eclampsia is appropriate to consider as a marker since the expression of XO in pre-eclampsia is evident. However, elevation of uric acid observed in pre-eclampsia compared to normal pregnant before delivery but the level return to normal in both the groups after delivery.

Nitric oxide has not shown significant changes in both the groups before and after delivery. It has been proposed that reduced production of vasodilatory agent nitric oxide [26] might cause pre-eclampsia but there are studies showing increased nitric oxide production in pre-eclampsia suggested to overcome the adverse placental effect [27]. In our study nitric oxide level was increased in normal pregnancy before delivery as an indication of adaptive haemodynamic changes [28] and decreased after delivery in normal pregnant women may be due to down-regulation of maternal NO synthesis [29]. NO level was elevated after delivery in pre-eclampsia within 48 hours may be an indication of reversible changes of pre-eclampsia. Elevated ROS production may suppress the expression of endothelial nitric oxide synthase (Enos). Nitric oxide (NO) combines rapidly with superoxide (O2-) to form peroxynitrite ion (ONOO-). Peroxynitrite oxidizes the DNA, lipids, Proteins and also interferes with the vascular signaling pathways [10].

However in an attempt to evaluate the correlation between xanthine oxidase activity and nitric oxide level a non-significant negative correlation is observed before (r= -0.260) and after delivery (r= -0.224).

LIMITATION

However, limitations of the study were measurement of XO level from time of pregnancy to at all level of trimesters to understand whether or not gradual increase of XO activity as a marker to denote the number of chances translated into pre-eclampsia. Further study can be designed by culturing trophoblastic cells and expose them to free radical stress environment to measure XO activity. Understanding of pre-eclampsia at early stage is a good indication to decide suitable treatment strategies to prevent its onset and pathological changes.

CONCLUSION

Our research findings generated knowledge about increased XO activity and uric acid in pre-eclampsia in comparison with normal pregnant women. The inverse relation between XO and NO found in our study may be an indication of trophoblastic cell destruction and endothelial dysfunction.

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ORIGINAL ARTICLE

Relationship Between Xanthine Oxidase, Ischemia Modified Albumin, Nitric Oxide with Antioxidants in Non Pregnants, Pre and Post-delivery of Normal Pregnants and Preeclampsia

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Abstract Preeclampsia is a multisystem disorder involves altered homeostasis of oxidants–antioxidants, inflammatory process and endothelial dysfunction. The present study aim was to determine the levels of oxidative stress parameters (malondialdehyde, protein carbonyl, ischemia modified albumin and xanthine oxidase), nutrient antioxidants (vitamin C and vitamin E), enzyme antioxidants (catalase, superoxide dismutase, glutathione peroxidase glutathione reductase), total antioxidant status (TAS) and its association with nitric oxide. The study population consists of three groups, non pregnants (Group 1, n = 57), normotensive pregnants (Group 2, n = 57) and Preeclampsia (Group 3, n = 57). Group 2 and 3 were followed after delivery within 48 h. In preeclampsia xanthine oxidase, malondialdehyde and uric acid levels were significantly increased ($p < 0.001$), while TAS decreased ($p < 0.05$) when compared to normotensive pregnant and non pregnant. Catalase, glutathione reductase levels were increased ($p < 0.005$) and vitamin E, super oxide dismutase levels were decreased ($p < 0.001$) in preeclampsia when compared to normal pregnants. Receiver operating characteristics curve analysis showed area under curve for xanthine oxidase (0.8), malondialdehyde (0.804), Uric acid (0.84), ischemia modified albumin (0.92) and catalase (0.88) which indicated as good markers in preeclampsia. Amongst, ischemia modified albumin is a better marker of intrauterine hypoxic reperfusion risk with sensitivity 87.7 % and specificity 91.2 %. The increased hydrogen peroxide from xanthine oxidase adds to oxidative stress and increased catalase activity in preeclampsia represents combating action. Increased oxidative stress, decreased TAS and its apparent reversible changes evinced within 48 h after delivery in preeclampsia illustrated that placental abnormality is the contributing factor in the pathogenesis.

Keywords Ischemia modified albumin · Xanthine oxidase · Preeclampsia · Normotensive pregnant · Nitric oxide

Introduction

Preeclampsia is an obstetric problem associated with hypertension, proteinuria and edema after 20 weeks of pregnancy. The symptoms include persistent headache, blurred vision, Vomiting and abdominal pain. The complications of preeclampsia may result in fetal uterine growth restriction, preterm delivery, maternal and fetal morbidity and mortality [1]. In the developing and developed countries, approximately eight hundred women die from pregnancy and child birth related complications every day. However, from this data nearly 10–25 % of preeclampsia cases results in maternal death [2].

The complications of Preeclampsia decreases after delivery. Although, its exact mechanism is unknown, it has been suggested that free radicals generated during oxidative stress implicated in promotion of maternal vascular malfunction by affecting endothelial cells. Oxidative stress is prominent when the balance between the reactive oxygen species (ROS) overrides the antioxidant capacity of the target cell. Thus, altered equilibrium leads to tissue injury

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and damages cellular biomolecules. Therefore, a homeostasis between oxidants and antioxidants is crucial in health and disease [3].

Membrane lipid peroxidation markers, protein markers and markers denoting nucleic acid damage are increased in plasma of preeclamptic women. Defensive role of nutrient antioxidants such as carotenoids, vitamin E, L-ascorbic acid and enzyme antioxidants status minimize oxidative damage by virtue of their capacity to scavenge free radicals generated during cellular metabolic process [4]. Karacy et al. [5] reported the oxidative stress in terms of malondialdehyde (MDA) and antioxidants in preeclampsia. In our previous study we reported that xanthine oxidase (XO) can be considered as a good marker for preeclampsia but it was not statistically assessed and compared with other oxidative stress parameters. And also activity was not evaluated in the non pregnant basal group. As an extension of this previous research work we measured xanthine oxidase activity compared with other oxidative stress parameters and antioxidants in large sample size. The possibility of considering xanthine oxidase as an enzyme marker of oxidative stress along with other oxidative stress markers in preeclampsia in pre and post-delivery has become the requisite [6]. Therefore, the present study was conducted to evaluate xanthine oxidase (XO) as a proxidant enzyme and also, ischemia modified albumin (IMA) as a hypoxic risk factor in preeclampsia with its clinical importance by comparing with other known oxidative stress markers. Similarly, serum catalase as an antioxidant enzyme with other antioxidants in preeclampsia during pre and post-delivery conditions within 48 h needs to be elucidated. This research gap necessitates a reason for this study.

Materials and Methods

The study was conducted by the Department of Biochemistry in collaboration with the Department of Obstetrics and Gynecology R.L. Jalappa Hospital and Research Center, Kolar, India after obtaining Institutional Ethical Committee approval. The enrollment of the study population was commenced after obtaining individual Informed Consent. The study was conducted between August 2013 to December 2015.

The study population was divided into 3 groups. Group 1 (n = 57) comprising of non-pregnants as control population; group 2 (n = 57) as normotensive pregnants and group 3 (n = 57) as preeclampsia cases. G2 and G3 were in 30–39 weeks of gestation before delivery and same subjects followed after delivery within 48 h. G2 and G3 subjects were clinically diagnosed from Obstetrics and Gynecology. The age matched control populations (G1) were randomly recruited from the healthy volunteers of Sri Devaraj Urs Medical College and were in 20–30 years.

Pre-eclampsia was diagnosed based on the criteria of National High Blood Pressure Education Programme working group involving blood pressure $\geq$140/90 mm Hg and proteinuria ($\geq$300 mg/24 h) after 20 weeks of gestation.

Inclusion criteria were singleton pregnancy, no fetal anatomical anomaly; nonsmokers were included in the study. Exclusion criteria were patient with any history of renal disease, thyroid disorder, chronic hypertension, gestational diabetes, epilepsy, and hypertensive encephalopathy and cardio vascular disease.

Four mL of fasting blood sample were collected from subjects under the study groups using appropriate vacutainer under aseptic condition. Samples for vitamin E assay were centrifuged, clear plasma obtained was transferred to screw capped vials wrapped with aluminum foil in order to minimize the exposure to light and prevent vitamin E loss. The samples were stored frozen at −80 °C until analysis.

Fine chemicals like xanthine, xanthine oxidase, glutathione peroxidase, glutathione reductase, Nitro blue tetrazolium, Phenazenemethosulphate, Sulphanilamide, NADH, Glutathione (reduced and oxidized), NADPH, Vitamin E, Ascorbic acid, metaphosphoric acid were obtained from Sigma Aldrich. All other reagents used were of analytical grade.

Methods

Malondialdehyde reacts with thiobarbituric acid to form a pink colored complex; absorbance was measured at 535 nm spectrophotometrically [7].

Protein carbonyl reacts with 2, 4, di-nitro phenyl hydrazine (DNPH) forming a Schiff base to produce yellow hydrazine. The absorbance was measured spectrophotometrically at 370 nm [8].

Xanthine oxidase catalyzes the conversion of xanthine to uric acid and hydrogen peroxide. The increase in absorbance (ΔA) was measured at 290 nm against control [9].

The concentration of ischemia modified albumin was determined by addition of a known amount of cobalt (II) to a serum specimen and measured unbound cobalt (II) by colorimetric assay using dithiothreitol. The absorbance of the intensity of the color produced measured against control at 470 nm [10].

Uric acid is converted by uricase to allantoin and hydrogen peroxide, which under the catalytic influence of peroxidases, oxidizes 3, 5-dichloro-2 hydroxy benzene sulphonic acid and 4 amino phenazone to form red violet

quinine imine absorbance measured at 505 nm spectrophotometric ally [11].

Nitric oxide (NO) in plasma measured by the reduction of nitrate into nitrite by copper coated cadmium granules as a reducing agent using sodium nitrite as standard (NaNO2). The nitrite produced is estimated by diazotization of Sulfanilamide in acidic medium and then coupling with Napthyl ethylene Diamine to produce pink colored compound. The absorbance was measured spectrophotometrically at 540 nm [12]. Ferric Reducing Ability of Plasma (FRAP) reduces ferric ions to ferrous ion at low pH. The absorbance of violet colored ferrous tripyridyltriazine complex measured at 593 nm [13].

Superoxide dismutase (SOD) is measured based on the inhibition of the formation of Phenazenemethosulphate-Nitro blue tetrazoliumformazon complex. The color formed at the end of the reaction can be extracted into butanol and measured at 560 nm [14].

Glutathione peroxidase (GPx) catalyzes the oxidation of reduced glutathione by hydrogen peroxide. In the presence of glutathione reductase and NADPH, the oxidized glutathione is converted to the reduced form with simultaneous oxidation of NADPH measured as decrease in absorbance at 340 nm [15].

Catalase rapidly breaks down hydrogen peroxide leading to decrease in absorbance. A difference in the absorbance at 240 nm per minute is measure of catalase activity [16].

Plasma ascorbic acid oxidized by Cu^{2+} to form dehydroascorbic acid which reacts with 2, 4, DNPH to form a red color bis-hydrazone. The absorbance is measured at 520 nm [17].

Vitamin E measurement based on Emmerie-Engel reaction, which is based on reduction of ferric iron to ferrous iron by tocopherols which then forms red colored complex with α, α1-dipyridyl. Tocopherols and carotenes were first extracted into petroleum ether and absorbance was read at 460 nm to measure the carotenes. A correction was made for this after adding ferric chloride and read at 520 nm [18].

Statistical Analysis

The results were expressed as mean ± standard deviation and analysed using one way ANOVA test with post hoc Bonferroni analysis to compare the values between the three groups. Pearson correlation analysis was used to find the correlation between the various parameters. Receiver operating characteristics (ROC) curve analysis was done to assess diagnostic utility of a parameter in the study. A probability p value of <0.05 was considered as statistically significant. Statistical analysis was performed with the licensed version of SPSS 20.

Results

The demographic data and results of the study were tabulated in Table 1. The subgroups of the study were age matched. The biochemical parameters under evaluation belong to oxidative stress group (MDA, IMA, protein carbonyl and XO) were significantly higher in preeclamptic cases in pre delivery, whereas the same parameters significantly decreased in post-delivery within 48 h. Similarly, antioxidant status representing parameters were Vitamin C, vitamin E, FRAP, and enzyme antioxidants were SOD, GR, GPx, Catalase. Results indicated inverse relation between oxidative stress and antioxidant status in preeclampsia patients. Declined antioxidant power (FRAP) with preeclampsia in pre delivery gradually increased after

Table 1 Comparison of the biochemical parameters between groups in the study population

Parameters	G_1 (mean ± SD)	G_2 (mean ± SD)	G_3 (mean ± SD)
Malondialdehyde (μmoles/L)	4.53 ± 2.2	6.8 ± 5.4	15.4 ± 9.7
Protein carbonyl (nmol/L)	98.95 ± 39.9	137.21 ± 55.3	153.9 ± 111.6
Ischemia modified albumin (ABU)	0.55 ± 0.33	0.77 ± 0.24	1.24 ± 0.30
Xanthine oxidase (U/L)	3.04 ± 1.0	37.07 ± 51.2	193.6 ± 191
Uric acid (mg/dL)	3.8 ± 1.2	4.04 ± 1.5	6.5 ± 2.2
Nitric oxide (μmoles/L)	8.46 ± 3.9	7.3 ± 3.2	6.3 ± 4
Ferric reducing ability of plasma (μmoles/mL)	1953 ± 634.8	1203 ± 1248	685.6 ± 646.1
Glutathione peroxidase (U/L)	546.7 ± 385.4	606.32 ± 423	493.8 ± 428
Glutathione reductase (U/L)	30.5 ± 24.3	12.1 ± 7.6	22.5 ± 23.3
Superoxide dismutase (U/mL)	7.7 ± 4.7	11.6 ± 2.8	8.5 ± 4.6
Catalase (U/mL)	114.2 ± 64.5	46.5 ± 57.1	84.7 ± 56.7
Vitamin C (mg/dL)	1.31 ± 0.4	0.6 ± 0.3	0.5 ± 0.2
Vitamin E (mg/L)	10.1 ± 2.1	13.5 ± 9	9.3 ± 4.7

G_1 non pregnant, G_2 normotensive pregnant, G_3 preeclampsia

Table 2 Comparisons of biochemical parameters showing significance between groups in study population

Parameters	G_1 versus G_2	G_1 versus G_3	G_2 versus G_3
Malondialdehyde (μmoles/L)	0.180	0.000	0.000
Protein carbonyl (nmol/L)	0.023	0.000	0.723
Ischemia modified albumin (ABU)	0.000	0.000	0.000
Xanthine oxidase (units/L enzyme)	0.266	0.000	0.000
Uric acid (mg/dL)	1.000	0.000	0.000
Nitric oxide (μmoles/L)	0.337	0.007	0.426
Ferric reducing ability of plasma (μmoles/mL)	0.000	0.000	0.007
Glutathione peroxidase (U/L)	1.000	1.000	0.602
Glutathione reductase (U/L)	0.000	0.098	0.018
Superoxide dismutase (Unit/mL enzyme)	0.000	0.894	0.000
Catalase (unit/mL enzyme)	0.000	0.023	0.003
Vitamin C (mg/dL)	0.000	0.000	0.156
Vitamin E (mg/L)	0.008	1.000	0.001

G_1 non pregnant, *G_2* normotensive pregnant, *G3* preeclampsia

$p < 0.05$ considered as statistically significant

Table 3 Circulating oxidative stress and antioxidant parameters during before and after delivery in normal pregnant and preeclamptic women

Parameters	Normal pregnant (G_2)		Significance	Preeclampsia (G_3)		Significance
	Pre-delivery	Post-delivery		Pre-delivery	Post-delivery	
Malondialdehyde (μmoles/L)	6.85 ± 5.42	5.4 ± 4.99**	0.000	15.42 ± 9.7	10.5 ± 9.4**	0.000
Protein carbonyl (nmol/L)	137.21 ± 55.3	127.1 ± 48.7	0.125	153.9 ± 111.6	98.6 ± 43.8**	0.000
IMA (ABU)	0.77 ± 0.24	0.75 ± 0.27	0.914	1.24 ± 0.30	1.03 ± 0.143	0.000
Xanthine oxidase (U/L)	37.07 ± 51.2	17.1 ± 13.7*	0.002	193.6 ± 190.8	99.99 ± 139.1**	0.000
Uric acid (mg/dL)	4.0 ± 1.5	4.16 ± 2.3	0.692	6.4 ± 2.2	4.7 ± 1.9**	0.000
Nitric oxide (μmoles/L)	7.3 ± 3.2	6.1 ± 2.9*	0.002	6.31 ± 4.01	6.93 ± 9.4	0.617
FRAP (μmoles/mL)	1203 ± 1247.6	1123.5 ± 1026.7	0.474	685.5 ± 646.1	748.9 ± 418.6	0.391
Glutathione peroxidase (U/L)	606.3 ± 423	493 ± 428	0.000	493.8 ± 428.1	803.7 ± 486.4**	0.093
Glutathione reductase (U/L)	12.1 ± 7.6	7.8 ± 3.7**	0.000	22.51 ± 23.32	10.1 ± 3.1	0.169
Superoxide dismutase (U/L)	11.6 ± 2.8	9.9 ± 3.62*	0.003	8.5 ± 4.6	7.5 ± 4.4*	0.003
Catalase (U/L)	46.46 ± 57.1	42.11 ± 37.7	0.623	84.7 ± 56.7	88.8 ± 56.7	0.642
Vitamin C (mg/dL)	0.58 ± 0.32	0.67 ± 0.62	0.319	0.47 ± 0.24	0.59 ± 0.60	0.109
Vitamin E (mg/L)	13.5 ± 8.95	13.96 ± 9.3	0.655	9.3 ± 4.7	9.99 ± 4.6	0.276

* Probability value of $p < 0.05$

** Probability value of $p < 0.005$

delivery that represents the possible reversible changes. In the post-delivery condition antioxidant data represents improvement in pregnancy induced hypertension on removal of placenta towards normal.

Statistical significance and correlation analysis between the parameters of the groups studied were depicted in Tables 2, 3, and 4. Data analysis evinced significantly positive correlation between NO versus GPx, FRAP versus MDA, FRAP versus XO and significantly negative correlation observed between IMA versus GPx, GR versus SOD.

ROC curve analysis for significant parameters was presented in Table 5. The data shows the sensitivity specificity and also the superiority of the IMA as a marker when compared to XO, uric acid, MDA and catalse in preeclampsia.

Discussion

Preeclampsia is associated with various etiological factors which implicated in the pathogenesis. Hypoxic risk is a major cause for the development of oxidative stress that affects integrity of trophoblastic tissues which lead to elevated xanthine and hypoxanthine and also uric acid. The

Table 4 Correlation between the significant parameters in preeclampsia

Parameters	Correlation coefficient	*p* value
MDA versus FRAP	0.277	0.039
IMA versus GPx	−0.433	0.001
GR versus SOD	−0.455	0.000
NO versus GPx	0.426	0.001
FRAP versus XO	0.275	0.040

MDA malondialdehyde, *FRAP* ferric reducing ability of Plasma, *IMA* ischemia modified albumin, *GPx* glutathione peroxidase, *GR* glutathione reductase, *SOD* superoxide dismutase, *NO* nitric oxide, *XO* xanthine oxidase

* Probability value of $p < 0.05$

association between preeclampsia and high serum uric acid concentration was reported during the beginning of this century. Reduced uric acid clearance observed in preeclamptic women associated with increased rate of reabsorption amounts to hyperuricemia [19].

The present study showed elevated uric acid level before delivery and decreased after delivery in preeclampsia. But in normal pregnants before and after delivery Uric acid level was unaltered. This indicates possible involvement of oxidative stress on placenta by means of contributing xanthine/hypoxanthine and xanthine oxidase activity.

Increased conversion of XDH into xanthine oxidase by oxidative stress further adds to increased production of hydrogen peroxide which in turn affects the trophoblast cell function [6, 20]. Thereby oxidative stress has become one of the causative factor for preeclampsia complications. The present study reported increased xanthine oxidase activity as unique observation with concomitant rise of uric acid in preeclampsia.

Malondialdehyde is a lipid peroxidation marker produced from the peroxidation of polyunsaturated fatty acid was significantly elevated in preeclampsia compared to normotensive pregnant and non-pregnant which is consistent with the previous work [5]. Increased lipid peroxidation products cause peroxidation damage to endothelial membrane which may result in endothelial dysfunction which is associated with reduced nitric oxide [21]. Our study results justifies this observation. The Significant decrease of MDA levels after delivery in normotensive pregnant and preeclampsia indicated down trend of MDA values after placental removal within 48 h.

Protein carbonyl is a stable indicator of protein damage in biological system. Reactive oxygen species oxidizes amino acid residues like glutamate, histidine and tryptophan in proteins to form product with carbonyl group. Protein carbonyl content was significantly increased in preeclampsia ($p < 0.001$) when compared to non pregnants. However the increase of protein carbonyls was found non-significant between normotensive pregnant and preeclampsia ($p > 0.05$). The data from normotensive pregnants and preeclampsia in comparison with control group suggests increase of oxidative stress. Even though, protein carbonyl content in after delivery of normotensive pregnants was non-significant, 64 % of protein carbonyls decreased in preeclampsia groups. Results evinced that decreased protein damage maker indicates revocable changes in after delivery with preeclampsia. The present study showed same pattern as reported by Zusterzeel et al. [22].

Superoxide Dismutase converts superoxide to water and it acts as first line of defense against free radical scavenging. SOD activity was significantly decreased in normotensive pregnant when compared to non pregnant. Decreased activity was also noticed in preeclampsia compared to normotensive pregnant. In after delivery, SOD activity was significantly decreased in preeclampsia and normotensive pregnant groups. Studies conducted by Bakacak et al. [23] showed decreased SOD activity may be due to increased Cu/Zn ratio. This altered ratio inactivates Cu/Zn containing antioxidant enzyme superoxide dismutase which may lead to decreased superoxide dismutase.

Catalase is a heme protein catalyzes cleaving of hydrogen peroxide into water and oxygen, thus it protects the cell from oxidative damage. In preeclampsia there was decreased enzyme activity when compared to non-pregnant. Its activity did not show significant difference between before and after delivery in normotensive pregnant and preeclampsia. Elevated xanthine oxidase and declined catalase activity indicated the severity of the oxidative stress in terms of hydrogen peroxide and hydroxyl radical in preeclampsia is evident in our study. Decrease in catalase activity may be due to inhibition of enzyme by hypochlorite and peroxy nitrite free radicals which were more perhaps in preeclampsia [21].

Table 5 ROC curve analysis of the parameters under preeclampsia

Parameters	Sensitivity	Specificity	Area under curve	95 % CI
IMA	87.7	91.2	0.920	0.854–0.962
Catalase	96.5	84.2	0.88	0.801–0.930
MDA	84.2	68.4	0.81	0.720–0.873
XO	61.4	89.5	0.753	0.663–0.829
UA	71.9	89.5	0.84	0.761–0.903

GPx is a selenium dependent enzyme eliminates hydrogen peroxide and organic hydro peroxides. There was non-significantly decreased GPx activity seen in preeclampsia before delivery when compared to healthy control and normotensive pregnant. In after delivery Gpx activity was non significantly decreased in normotensive pregnant and significantly increased in preeclampsia when compared to before delivery. Decreased glutathione peroxidase may lead to increased generation of reactive oxygen species. Decreased selenium level was associated with decreased glutathione peroxidase activity which may be involved in pathophysiology of preeclampsia [24].

Glutathione reductase replenishes cellular reduced glutathione. Decreased activity in normal Pregnants and increased activity in preeclampsia equivalent to healthy control ($p < 0.001$) observed in our study. In preeclampsia, activity reduced by two fold in post-delivery indicates the role of placenta. Suhail et al. [25] found that non-significant decrease of GR activity in preeclampsia compared to non pregnants. Unlike these reports, significant two fold increase of glutathione reductase is seen in our study during preeclampsia under the study condition compared to normal pregnancy ($p < 0.05$) group. Attaining GR activity equivalent level to non-pregnant could be an in vivo defensive response to restore the reduced glutathione level during altered oxidative and antioxidant system may be a compensatory mechanism in response to increased oxidative stress.

FRAP was significantly decreased in normal pregnant and preeclampsia before delivery ($p < 0.001$) when compared to healthy control. FRAP levels were significantly decreased in preeclampsia when compared to normotensive pregnant ($p < 0.05$) similar with the observations of Zusterzeel et al. [22]. FRAP levels did not show significant difference between before and after delivery in preeclampsia as well as normal pregnant Karacy et al. [5] also observed decreased FRAP level in preeclampsia. Vitamin C levels were significantly decreased in normal pregnant and preeclampsia when compared to healthy control ($p < 0.001$). But there was no significant difference between before and after delivery in normal pregnant and preeclampsia.

Vitamin E Levels were significantly increased in normal pregnant when compared to healthy control (<0.05) but there was non-significant decrease in preeclampsia when compared to healthy control. Significantly decreased vitamin E level was seen in preeclampsia when compared to normotensive pregnant (<0.001). In normotensive pregnant and preeclampsia there was no significant difference between before and after delivery. Reduction in vitamin C and E were consistent as shown by other studies [26].

Ischemia modified albumin known as hypoxic risk indicator in various diseases and also in preeclampsia during first trimester [27]. In support of this finding, our study results indicated high IMA in preeclampsia. An observation of decreased maternal serum levels of IMA in post-delivery evidenced clinical improvement. Elevation of MDA and XO activity represents oxidative stress and showed weak relationship with ferric reducing ability of plasma. FRAP is contributed by super oxide dismutase, glutathione peroxidase, catalase, bilirubin, uric acid, reduced glutathione, vitamin E, vitamin C, free cu^{2+}, zn^{2+}, Mn^{2+}, Fe^{2+}, and selenium [28]. Uric acid concentration is known as contributing factor to reducing ability in vitro, but biological significance of uric acid contribution to the antioxidant potential is unclear in vivo [29] and generates research gap. The possible reason for FRAP decline may be due to altered antioxidants in single or in combination.

Positive correlation is also observed between GPx and NO which indicates endothelial dysfunction and proliferates increased formation of super oxide radicals which can inhibit Glutathione peroxidase enzyme [30]. Reduced SOD and glutathione peroxidase activity will cause increase in superoxide anion which reacts with nitric oxide to form peroxy nitrite. This in turn decreases the availability of NO [13].

The negative correlation between IMA and GPx noticed in the study. The study rise the probable illustration that normal pregnancy demands high oxygen requirement and evidence increased oxidative stress. Inadequate supply of oxygen to trophoblastic cells results in preeclampsia. The hypoxia and established oxidative stress alters serum albumin with N-terminal modification into ischemia modified albumin. Therefore in the study context IMA evolved as good predictive marker of preeclampsia related risk. The impact of superoxide radicals has inhibitory effect on enzymes. Hence GPx activity decreased. Vanderlelie et al. [31] found that decreased glutathione reductase gene expression in preeclampsia without significant change in glutathione peroxidase. This indicated possible importance of plasma glutathione and preeclampsia toxemic condition on glutathione reductase. In contrast to other studies we found increase of glutathione reductase in preeclampsia compared to normotensive pregnants. A weak linear correlation was seen between protein carbonyl and MDA in preeclampsia which was not significant.

In ROC curve analysis, area under curve for ischemia modified albumin (0.92), Catalase (0.88), xanthine oxidase (0.8), malondialdehyde (0.804), Uric acid (0.84) with sensitivity of 87.7, 96.5, 61.4, 84.21, 71.93 and specificity of 91.2, 84.2, 89.5, 68.4, 89.5 respectively. Results indicated ischemia modified albumin as a good marker of intrauterine hypoxic reperfusion risk and abnormal placental development by dysfunction of trophoblastic cells under oxidative stress. ROC analysis showed that there is increased production of hydrogen peroxide due to elevated

xanthine oxidase activity in preeclampsia and catalase is combating it. Limitations of the study were measurement of IMA and XO level from time of pregnancy to all level of trimesters to understand whether or not gradual increase of IMA levels and XO activity as a marker to denote the number of chances of pregnancy translated into pre-eclampsia. Culturing trophoblastic cells and exposing to free radical stress environment to measure XO activity.

Conclusion

Our research findings generated knowledge about IMA as an intrauterine risk factor and XO as an enzyme oxidant marker. Increased xanthine oxidase activity and uric acid seen in preeclampsia with decreased total antioxidant status. Distinctive observation of elevated catalase activity in pre and post-delivery of preeclampsia within 48 h noticed despite of gradual reduction of oxidative stress. Endothelial dysfunction evidenced by reduction of nitric oxide level in preeclampsia during pre and post-delivery. The inverse relation between XO and NO in our study represents an indication of trophoblastic cell destruction and endothelial dysfunction. Therefore, the over-all study concludes an inverse relation between oxidative stress and antioxidant status in preeclampsia in comparison with normal pregnant and non pregnant suggests restoration of plasma antioxidants level.

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Compliance with Ethical Standards

Conflict of interest Mrs. Vanishree Bambrana, Dr. C. D. Dayanand and Dr. Pushpa Kotur declare that they have no conflict of interest. Financial support for this work borne by authors.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

Informed Consent Informed consent was obtained from all individual participants included in the study.

Research Involving Human Participants and/or Animals This is non interventional study. Sample collection from human participants under taken after obtaining Institutional Ethical Committee approval. An appropriate individual patient informed consent used for sample collection. This article does not contain any studies with animals performed by any of the authors.

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Research Article

PHOSPHOLIPASE A_2, PLASMA ELASTASE ACTIVITY IN PRE- AND POST-PARTUM OF PRE-ECLAMPTIC WOMEN

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ABSTRACT

Objective: The objectives of the present study were to evaluate the activity of phospholipase A_2, plasma elastase enzymes and to assess relation with an inflammatory marker high sensitive C-reactive protein (hs-CRP) in nonpregnant before and after delivery of normotensive pregnant and pre-eclamptic women.

Methods: The study population consists of three groups: Nonpregnant (Group 1, n=57), normotensive pregnant (Group 2, n=57), and pre-eclamptic women (Group 3, n=57). Groups 2 and 3 were followed after delivery within 48 hrs. Phospholipase A_2, plasma elastase, and hs-CRP levels were determined spectrophotometrically.

Results: The plasma elastase, phospholipase A_2 activity, and hs-CRP were elevated in pre-eclampsia significantly ($p<0.05$), nonsignificant rise in normotensive pregnant before delivery condition compared to nonpregnant women. However, plasma elastase in normal pregnancy and pre-eclampsia were decreased by 1.2- and 2.07-fold, respectively, after delivery. Whereas phospholipase A_2 and hs-CRP found to be nonsignificantly decreased in the postdelivery status of the both the groups. Receiver operating characteristics curve analysis showed that elastase enzyme has diagnostic importance to assess inflammation on the basis of area under curve (0.758).

Conclusion: Our research findings generated knowledge about raised level of plasma elastase enzyme by neutrophil degranulation represents inflammation in pre-eclampsia. Elevated elastase, phospholipase A_2 with hs-CRP in pre-eclampsia serves as indicators of inflammation.

Keywords: Elastase, High sensitive C - reactive protein, Phospholipase A_2, Pre-eclampsia.

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INTRODUCTION

Pre-eclampsia is a multisystem obstetric problem associated with hypertension, proteinuria, and edema after 20 weeks of pregnancy [1]. The symptoms are persistent headache, blurred vision, vomiting, and abdominal pain [2]. The pre-eclampsia complications may result in fetal uterine growth restriction, preterm delivery, maternal and fetal morbidity and mortality [3,4]. Research reports are available on inflammatory response [5], leukocyte activation [6], dyslipidemia [7], oxidative stress, and intrauterine hypoxia [8] as a potential cause for generation of free radicals and inflammatory mediators.

Neutrophil elastase gene located on chromosome 19 at p13.3. Protein expressed is a serine protease (EC No. 3.4.21.37) consisting of 218 amino acid residues with a molecular weight 29.5 kDa. It exerts biological role in the degradation of collagen IV and elastin in causing inflammation [9]. Phospholipase A_2 gene located on chromosome number 1 at q31.1. The expressed protein is heat stable, calcium-dependent enzyme (EC No. 3.1.1.4) with molecular weight 85 kDa. Phospholipase A_2 enzyme cleaves arachidonic acid at Sn2 position in phospholipid; the released product serves as precursor for leukotriene's synthesis which acts as inflammatory mediators [10].

High sensitive C-reactive protein (hs-CRP) is a plasma globulin fraction synthesized in the liver during acute inflammation phase in response to pneumococci infection where it binds to carbohydrate moiety of the capsule of bacteria. Circulating level of CRP in the range of 0.5-10 mg/L considered as hs-CRP, whereas the concentration in the range of 10-1000 mg/L is a nonspecific indicator of inflammation [11].

In our earlier research reports, we presented increased concentration of oxidative stress and decreased total antioxidant status [12] and also increased xanthine oxidase activity with the inverse relation of nitric oxide in pre-eclampsia [13,14]. There is seldom information available with respect to phospholipase A_2 and plasma elastase in pre-eclampsia. Therefore, in the present study, an attempt was made to estimate the phospholipase A_2 and plasma elastase activities in comparison with hs-CRP to assess inflammation in pre-eclampsia and normotensive pregnant women. These parameters were also measured in both the groups after delivery within 48 hrs made the study distinct.

METHODS

The study was conducted between December 2014 and May 2016 by the Department of Biochemistry in collaboration with the Department of Obstetrics and Gynecology, R. L. Jalappa Hospital and Research Center, Kolar, Karnataka, India. It was conducted after obtaining the Institutional Ethics Committee approval from Sri Devaraj Urs Medical College. The participants of the study population were enrolled after obtaining individual patient written informed consent.

The study population was allocated into three groups. Group 1, nonpregnant (n=57); Group 2, normotensive pregnant (n=57); Group 3 pre-eclamptic women (n=57). G_2 and G_3 were in 30-39 weeks of gestation before delivery and were followed after delivery within 48 hrs. G_2 and G_3 subjects were clinically diagnosed from obstetrics and gynecology. The age-matched control populations (G_1) were randomly recruited from the healthy volunteers of the Sri Devaraj Urs Medical College and were in age group of 20-30 years.

Pre-eclampsia was diagnosed with blood pressure ≥140/90 mm Hg, proteinuria ≥300 mg/24 hrs after 20 weeks of gestation, were included as per national high blood pressure education program working group. The subjects with any history of renal disease, thyroid disorder, chronic hypertension, gestational diabetes, epilepsy, hypertensive encephalopathy, and cardiovascular disease were excluded from the study.

About 4 ml of blood sample was collected from each subject under the study groups using appropriate vacutainer under aseptic conditions to obtain serum and plasma. These samples were stored at −80°C until further analysis.

Fine chemicals such as N-Succinyl-ala-ala-ala-p-Nitroanilide (SAAANA), leukocyte elastase, phospholipid, and phospholipase A_2 were obtained from Sigma-Aldrich, USA. hs-CRP immunoturbidimetric kit was commercially obtained from Euro-Diagnostic System, Chennai, India. All other chemicals and reagents used were of analytical grade.

Elastase hydrolyses synthetic substrate SAAANA to give rise to products N-Succinyl-ala-ala-ala and p-nitroanilide. The absorbance of p-nitroanilide was measured in a spectrophotometer at 410 nm wavelength [15].

The CRP-ultrasensitive is a quantitative turbidimetric test for the measurement of low levels of CRP in human serum or plasma. Latex particles coated with specific antihuman CRP are agglutinated when mixed with samples containing CRP. The agglutination causes an absorbance change, dependent on the CRP contents of the patient sample that can be quantified by comparison from a calibrator of known CRP concentration.

Phospholipase A_2 hydrolyzes the Sn2-fatty-acyl ester bond of phosphorglyceride liberating free fatty acid and lysophospholipid. Based on the absorbance change of bromothymol blue, indicators with the concentration of hydrogen ion released from enzyme catalyzed reaction were measured at 620 nm [16].

The results were tabulated as mean±standard deviation (SD) and analyzed using one-way ANOVA test with *post-hoc*-Dunnett analysis to compare the values between the three groups. Paired *t*-test was used to compare the changes before and after delivery in normal pregnancy and pre-eclampsia. A probability value of $p<0.05$ was considered as statistically significant. Receiver operating characteristics (ROC) curve was plotted to find out the diagnostic importance of the parameters measured under study. Statistical analysis was performed with licensed version of SPSS 20.

RESULTS

Table 1 depicted the mean±SD of the elastase, phospholipase A_2, and hs-CRP as an inflammatory indicator. Considerable increase in elastase (5.8 U/ml±3.01), phospholipase A_2 (70.7 U/ml±18.4), and hs-CRP (8.6 mg/L±14.1) in normal pregnancy when compared to nonpregnant and further increase of these parameters (26.85±79.31, 79.02±27.6, 11.5±14.2), respectively, in pre-eclampsia when compared to normal pregnancy. Analysis of the results showed that the marked rise in the elastase, phospholipase A_2 activity in pre-eclampsia noticed when compared with the well-known inflammatory marker hs-CRP. In pre-eclampsia, there was 6.7-fold increase of elastase enzyme and 4.0-fold increase of hs-CRP observed when compared to nonpregnant women.

Table 2 shows the comparison of study parameters among nonpregnant, normotensive pregnant, and pre-eclampsia. Phospholipase A_2 and elastase were significantly increased ($p<0.05$) in pre-eclampsia, and the same parameters were statistically nonsignificant in normotensive pregnant when compared to nonpregnant women. Whereas hs-CRP level significantly increased in normal pregnancy ($p<0.05$) and pre-eclampsia ($p<0.001$) when compared with that of nonpregnant.

Table 3 depicts the elastase, phospholipase A_2, and hs-CRP levels during pre- and post-delivery of normal pregnant and pre-eclampsia cases. These parameters were increased in pre-eclampsia prominently compared to normal pregnant during predelivery. However, elastase activity decreased by 1.2 and 2.07-fold, respectively, in normal pregnancy and pre-eclampsia during after delivery, whereas phospholipase A_2 and hs-CRP were found to be nonsignificantly decreased ($p>0.05$) during postdelivery.

Table 4 illustrates the ROC curve features such as sensitivity, specificity, and area under curve for hs-CRP, phospholipase A_2, and plasma elastase in pre-eclampsia. ROC analysis showed that plasma elastase had area under the curve of 0.758. Phospholipase A_2 did not show any appreciable diagnostic importance. Figs. 1-3 shows the levels of phospholipase A_2, plasma elastase, and hs-CRP in nonpregnant before and after delivery of normal pregnant and pre-eclamptic cases.

DISCUSSION

Pre-eclampsia is a multifactorial pregnancy disorder involving various types of system attributing to the pathophysiology of placenta. Feto-maternal immune reactions in the 1st week of pregnancy, impaired arterial invasion by trophoblast, and transformation of spiral arteries followed by altered placental perfusion. It results in chronic hypoxia

Table 1: Comparison of the biochemical parameters between groups in the study population

Parameters	Mean±SD		
	G_1 (n=57)	G_2 (n=57)	G_3 (n=57)
Elastase (U/ml)	4.01±1.31	5.8±3.01	26.85±79.31
Phospholipase A_2 (U/ml)	68.7±22.85	70.7±18.4	79.02±27.6
hs-CRP (mg/l)	2.9±1.54	8.6±14.1	11.5±14.2

G_1: Nonpregnant, G_2: Normotensive pregnant, G_3: Pre-eclampsia, SD: Standard deviation, hs-CRP: High sensitive C-reactive protein

Table 2: Comparisons of biochemical parameters showing significance between groups in study population by *post-hoc*-Dunnett multiple comparisons

Parameters	G_1 versus G_2	G_1 versus G_3
Elastase (U/ml)	NS	<0.05*
Phospholipase A_2 (U/ml)	NS	<0.05*
hs-CRP (mg/l)	<0.05*	<0.001**

G_1: Nonpregnant, G_2: Normotensive pregnant, G_3: Pre-eclampsia. *Statistically significant, **Highly significant, NS: Nonsignificant, hs-CRP: High sensitive C-reactive protein

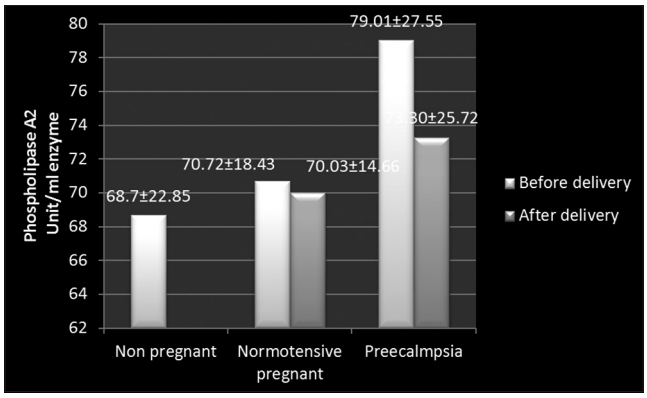

Fig. 1: It shows the phospholipase A_2 activity in nonpregnant before and after delivery of normal pregnant and pre-eclamptic women

Table 3: Elastase, phospholipase A_2, and hs-CRP parameters during before and after delivery in normotensive pregnant and pre-eclamptic women

Parameters	Mean±SD					
	Normotensive pregnant (n=57)			Pre-eclampsia (n=57)		
	Predelivery	Postdelivery	p	Predelivery	Postdelivery	p
Elastase (U/ml)	5.80±3.019	4.6±1.64	<0.05*	26.85±79.30	12.91±7.72	>0.05
Phospholipase A_2 (U/ml)	70.72±18.43	70.03±14.66	>0.05	79.01±27.55	73.30±25.72	>0.05
hs-CRP (mg/L)	8.6±14.15	6.6±15.2	>0.05	11.5±14.24	10.3±20.4	>0.05

*Statistically significant. SD: Standard deviation, hs-CRP: High sensitive C-reactive protein

Table 4: ROC curve analysis of the parameters in pre-eclampsia

Parameters	Sensitivity (%)	Specificity (%)	AUC	95% CI
hs-CRP	57.1	73.7	0.708	0.615-0.790
Phospholipase A_2	35.7	86	0.580	0.484-0.672
Plasma elastase	74.3	86	0.758	0.669-0.834

AUC: Area under curve, hs-CRP: High sensitive C-reactive protein, CI: Confidence interval, ROC: Receiver operating characteristics

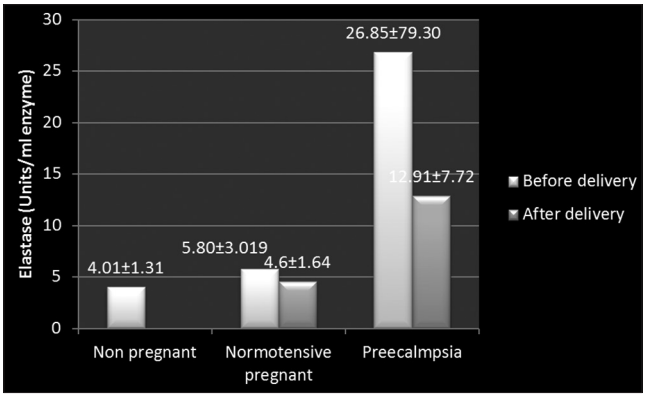

Fig. 2: It Illustrates the plasma elastase activity in nonpregnant before and after delivery of normal pregnant and pre-eclamptic cases

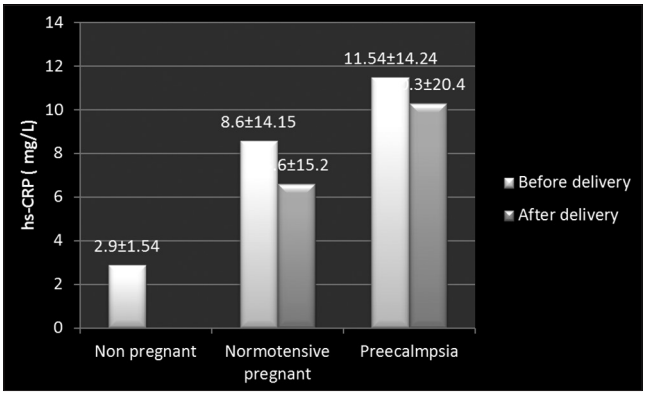

Fig. 3: It presents the high sensitive C-reactive protein levels in nonpregnant before and after delivery of normal pregnant and pre-eclamptic cases

that triggers intensity of oxidative stress that has an impact on placental syncytiotrophoblast cell apoptosis and necrosis [17]. The rate of formation of placental debris has pro-inflammatory substances, angiogenic, and antiangiogenic factors, etc., which lead to endothelial dysfunction and systemic inflammatory response, thus placenta play a central role in inflammatory process [18].

An inflammatory response in pre-eclampsia is usually accompanied by increased concentration of pro-inflammatory signaling molecules such as cytokines, activated neutrophils, and positive acute phase plasma proteins. Neutrophil activation may occur in the presence of cytokines such as tumor necrosis factor-α during an inflammatory process. Degranulation of neutrophils releases elastase enzyme that prolongs the inflammation by modification of pro-inflammatory cytokines and degrading proteins involved in the inflammation. In addition to this, myeloperoxidase also present in neutrophil granules increases oxidative stress by the additional production of hydroxyl radical and hypochlorous acid [19]. Hence, neutrophil activation results in vascular damage and dysfunction. Therefore, plasma elastase can be used to assess *in vivo* neutrophil activation. CRP is increased rapidly in response to inflammatory stimuli along with elastase in pre-eclampsia [20]. So that, increased elastase in plasma serves as a predictive marker of pregnancy induced inflammation [21].

A systemic inflammatory response involves leukocyte activation by elastase, acute phase response by hs-CRP, and metabolic features of systemic inflammation by phospholipase A_2 were presented in the current study. Elastase and phospholipase A_2 activity were compared with reliable inflammatory marker hs-CRP. In our study, these biochemical parameters were elevated in normal pregnancy and further accentuated in pre-eclampsia.

We observed the systemic inflammatory response in pre-eclampsia through hs-CRP which is similar to the research reports of von Versen-Hoeynck 2009 [22], Belo 2009 [23]. The increase in plasma elastase in early onset of pre-eclampsia has been reported by Gupta 2006 [24]. Elastase released from polymorphonuclear lymphocytes during inflammatory condition; hence, the plasma levels were increased considerably [25]. Therefore, in the current study, plasma elastase was compared with the hs-CRP.

Phospholipase A_2 by virtue of hydrolysis of phospholipid releases arachidonic acid that serves as precursor for the synthesis of eicosanoids which participates in the inflammatory process. Phospholipase A_2 enzyme activity was measured to know whether this enzyme can be included under inflammatory marker for consideration. Statistical significance of phospholipase A_2 activity was seen in nonpregnant and pre-eclampsia cases.

Even though, the importance of phospholipase A_2 in pre-eclampsia found to be contradictory [26-28], but our results highlighted increased phospholipase A_2 activity in pre-eclampsia compared to nonpregnant women. However, phospholipase A_2 did not show diagnostic importance as per ROC analysis. Limitation of the study confines to the determination of plasma elastase, phospholipase A_2, and hs-CRP from the time of pregnancy to all trimesters to denote the number of chances of pregnancy translated into pre-eclampsia.

CONCLUSION

Our research findings generated a new knowledge about plasma elastase and phospholipase A_2 increase in normal pregnancy and further rise in pre-eclampsia. Elevation of these enzymes evidences inflammation

in pre-eclampsia when tested along with hs-CRP. Phospholipase A_2 activity increased but did not show diagnostic importance, whereas plasma elastase can be considered for diagnostic utility. Elevated elastase activity represents enhanced maternal inflammatory process by neutrophil activation and degranulation in pre-eclampsia.

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Research Article

EVALUATION OF XANTHINE OXIDASE INHIBITORY ACTIVITY BY FLAVONOIDS FROM *PONGAMIA PINNATA LINN*

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ABSTRACT

Objective: Flavonoids from the crude seeds extract of *Pongamia pinnata* L., dried fruit powder of *Morinda citrifolia* L., bark of *Mangifera indica* L., and rhizome of *Zingiber officinale* Rosc. were screened for xanthine oxidase (XO) inhibition at different concentration. The inhibitory potential of quercetin and allopurinol were used for the determination of 50% inhibitory concentration (IC_{50}) and K_i values.

Methods: Isolation of flavonoids from the plant extracts was processed by column chromatography and tested for XO inhibitory activity in the range of 6-800 µg/ml.

Results: The results demonstrated that optimized flavonoids extract of *P. pinnata* L. exhibited promising XO inhibition. *P. pinnata* L., *M. indica* L., and *Z. officinale* Rosc. had IC_{50} in the concentration of 8.74 mM, 1.09 mM, 5.4 mM and Ki 0.35 mM, 1.73 mM, 2.7 mM, respectively.

Conclusion: The study showed that plant species under investigation exhibited XO inhibition by optimized flavonoid extract. *P. pinnata* L. indicated promising XO inhibition compared to other plant extracts. Flavonoids can be used as a potent inhibitor of XO an alternative to allopurinol.

Keywords: Xanthine oxidase, Quercetin, Allopurinol, *Pongamia pinnata*, Oxidative stress.

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INTRODUCTION

Traditional system of medicine comprises use of variety of bioactive compounds from the plants. The expeditions in search of medicinal values of plants are continuous process for therapeutic benefit. Plants sources are rich in pharmacologically active compounds such as phenolics, tannins, flavonoids, and lignin [1]. Such kind of active constituents distributed in different parts of the plants and posses properties of antimicrobial, antimutagenic, anticarcinogenic, anti-inflammatory, vasodilatation, and enzyme inhibitory activity [2].

Pongamia pinnata L. is an angiosperm also known as Indian beach tree belongs to family Fabaceae. It is known to have anti-inflammatory, antiulcer, antidiarrheal, antibacterial, anticonvulsion, wound healing, hypoglycemic, etc. [3].

Morinda citrifolia L. also known as Indian mulberry or noni belongs to family Rubiaceae. Different parts of this plant used as antioxidant, anti-inflammatory, antihypertensive, antidiarrheal, antithrombotic, anticancerous, xanthine oxidase (XO) inhibitory and hypoglycemic, etc. [4,5].

Mangifera indica L. is well known as mango that belongs to family Anacardiaceae. The different parts of the plants used as remedial measure for infection, hypertension, insomnia, asthma, rheumatism, hemorrhage, and anemia [6,7].

Zingiber officinale Rosc. is called as ginger belongs to family Zingiberaceae. The rhizome contains zingiberene and gingerols. Precisely 6-gingerol is a constituent responsible for pungent taste. It is used as antipyretic, analgesic, hypotensive, antiplatelet aggregation, and thromboxane synthesis [8].

The aim of the study was to screen, *in vitro* efficacy of flavonoid components isolated from the seeds of *P. pinnata* L., dried fruit powder of *M. citrifolia* L., bark of *M. indica* L., and rhizome of *Z. officinale* Rosc. However, there is a need to determine XO enzyme inhibition to evaluate the protective effect on inflammation and oxidative stress.

Increased purine catabolism produces more reactive oxygen species as hydrogen peroxide and elevated uric acid level [9]. Furthermore, increased uric acid could be due to elevated XO activity. Hence, this has become subject of active research to screen for natural ingredients for therapeutics has become the need for the study. *In vitro* screening for XO inhibitory activity using vitamin C, vitamin E, flavonoid extracted from Indian conventional plants, along with allopurinol might be useful in extrapolating reduction of oxidative stress.

METHODS

Collection of plant material

Screening of XO inhibition was carried out using Indian conventional plants known to have medicinal property. They are *P. pinnata* L. (seeds), *M. citrifolia* L. (fruit), *M. indica* L. (bark), *Z. officinale* Rosc. (rhizome) were collected and authenticated from the Horticulture College, Tamaka, Kolar.

Preparation of plant extract

The methonolic extraction of *P. pinnata* L., *M. citrifolia* L., *M. indica* L., and *Z. officinale* Rosc. were carried out by following procedure. The plant materials were cleaned, air dried at room temperature in dark, ground to fine powder using pestle and mortar. Until further processing, the powder was stored in sterile amber colored bottle in dark at room temperature.

5 g of powder obtained was dissolved in 50 ml absolute methanol and subjected for filtration using whatman number 1 filter paper. The filtrate was concentrated in vacuum evaporator under reduced pressure and air dried. Thus obtained powder was stored in sterile bottles at 4°C until further use [10].

Isolation and purification of flavonoids from the plant extracts by column chromatography

A glass column measuring 50×2 cm dimension developed using methanol with silica gel adsorbent on glass wool and allowed to settle by gravity flow. Column was allowed to equilibrate with suitable methanol as elution solvent. The even surface of the silica gel was protected by placing whatman number 1 filter paper disc. In this process, 1 g/ml of processed crude extract was applied for separation. All the eluted fractions were tested for flavonoid content, the active fractions were pooled and air dried under sterile conditions. The concentrated dried powder subjected for qualitative confirmation of flavonoids using dimethyl sulfoxide as a dissolving solvent and quercetin as an internal standard [11].

Qualitative detection of flavonoids

2 ml of above extract was treated with few drops of 20% sodium hydroxide which produced intense yellow color and on further addition of dilute hydrochloric acid became colorless confirming the presence of flavonoids [12].

XO inhibition assay

XO inhibition assay was done as per the method described by Bergmeyer. Test solution contained 1.9 ml of phosphate buffer, 0.5 ml of xanthine substrate, and 0.5 ml of inhibitor of interest. Contents were mixed by inversion and equilibrated to 25°C. To this equilibrated solution, added 0.1 ml of XO enzyme obtained from Sigma-Aldrich, USA. Blank contained 0.1 ml of distilled water in place of enzyme solution. Immediately mixed the contents and recorded the change in absorbance per minute for approximately 5 minutes at 290 nm in PerkinElmer Lambda 35 spectrophotometer. The maximum linear rate of absorbance change obtained per minute of test considered for calculation [13]. Concentration of inhibitors such as quercetin, allopurinol, vitamin C, vitamin E, and flavonoid extract of plants used in the range of 6-800 µg/ml were tested for XO inhibition.

5% methanol was used to solubilize vitamin E, quercetin, and flavonoids extract of plants except vitamin C and allopurinol which was dissolved in water. The distinct percentages of inhibition, 50% inhibitory concentration (IC_{50}) and Ki were calculated.

RESULTS

Table 1 illustrating XO inhibition by pure chemical allopurinol, quercetin flavonoid, vitamin C and E and flavonoid components extracted from plants such as *P. pinnata* L., *M. citrifolia* L., *M. indica* L., and *Z. officinale* Rosc. The percentage of inhibition was calculated. In the similar way quercetin treated as internal standard flavonoid to compare the inhibitory activity of isolated flavonoids from the plants in the study. During the inhibition study, the results obtained using inhibitory components on XO activity was in the range of 6-800 µg/ml. It was evident that all the compounds exhibited ascending kind of inhibition from the concentration ranging from 50 to 800 µg/ml.

The percentage of inhibition of XO under assay condition by vitamin C and E clearly evinced that these vitamins had 50-60% of inhibition compared to allopurinol. The result showed that these vitamins have property of bringing minimal inhibition on enzyme activity irrespective of their concentration.

The results on percentage of inhibition of XO by isolated flavonoids of *P. pinnata* L. along with quercetin had similar inhibition property. However, *M. citrifolia* L., *M. indica* L., and *Z. officinale* Rosc. have less inhibitory effect on XO compared to *P. pinnata*. This observation suggested that flavonoid content of *P. pinnata* L. is almost similar to quercetin in action compared to others. Percentage of inhibition by quercetin on XO activity, when compared to allopurinol, showed approximately 40% less inhibition.

The percentage of inhibition at 800 µg/ml concentration for allopurinol was found 93.25%, quercetin 71.1%, vitamin C 50.7%, vitamin E 54%, *P. pinnata L* 69.1%, *M. citrifolia* L. 46.3%, *M. indica* L. 53.6%, *Z. officinale* Rosc. 51.6%. Results indicated other than allopurinol and quercetin, *P. pinnata* L. had maximum inhibitory activity.

The investigation on the efficacy of quercetin, vitamin E and vitamin C on comparison with optimized flavonoid extract from the above plants on XO inhibitory activity were calculated and expressed concentration that reduces half of enzyme activity (IC_{50}) and inhibitory constant (Ki). Allopurinol a well-known competitive inhibitor of XO considered as positive control for comparison that showed IC_{50} value 0.4 mM and Ki 0.13 mM. Inhibitory effect of quercetin on XO showed IC_{50} value 2.38 mM and Ki 0.37 mM. The vitamin C and E inhibition on XO in the concentration of IC_{50} 10.6 mM, 1.19 mM and Ki 3.37 mM, 0.76 mM, respectively. *P. pinnata* L., *M. indica* L., and *Z. officinale* Rosc. showed XO inhibition in the concentration of IC_{50} 8.74 mM,1.09 mM, 5.4 mM and Ki 0.35 mM, 1.73 mM, 2.7 mM, respectively. However, *M. citrifolia* L. did not show effective XO inhibition.

DISCUSSION

Flavonoids are closely related polyphenolic compounds with flavone ring structure, ubiquitously distributed in various parts of the plants in the wide range. Flavonoids are classified into flavones, flavonols, flavonoids, chalcones, anthocyanins, tannins, and aurones. They are reported to have antioxidant, enzymes inhibition related to inflammation, cardioprotective and bactericidal property, etc. [14]. Flavonoids known to have potential inhibitory action on XO responsible for oxidative injury.

P. pinnata L., *M. citrifolia* L., *M. indica* L., and *Z. officinale* Rosc. subjected in the study for isolation of flavonoids compounds to test the inhibitory property on XO enzyme. *P. pinnata* L. seeds material reported to have inhibition on α-amylase and α-glucosidase activity [15]. Furthermore, inhibition of XO measured using commercially obtained pure flavonoids compounds and suggested the planar flavones (chrysin, luteolin, and flavones), flavonol (quercetin, myricetin, kaempferol, rhamnetin, tangeretin and rutin) were having strong inhibitory effect on XO activity (Nagao *et al*., 1999).

Information is limited on XO enzyme inhibition by *P. pinnata* L. seeds hence this study reported 74% of enzyme inhibition with reference to

Table 1: Comparison of percentage of xanthine oxidase inhibition by allopurinol, pure and isolated flavonoids and vitamins at different concentrations

Compounds	Percentage of inhibition at different concentration of inhibitor							
	6.25 µg/ml	12.5 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml	400 µg/ml	800 µg/ml
Allopurinol	17.1±2.7	35.37±4.9	48.3±2.7	56.7±3.8	74.2±1.2	81.2±5.1	92.2±4.7	93.3±4.3
Quercetin	3.95±1.1	10.99±1.9	27.5±4.9	45.9±4.5	52.2±4.5	61.3±0.59	65.8±3.3	71.1±4.4
Vitamin C	8.94±1.3	18.3±2.5	25.1±2.7	26.8±4.6	31.8±1.8	46.4±0.68	48.8±3.9	50.7±2.8
Vitamin E	12.2±1.7	18.3±0.84	28.6±3.9	33.9±4.6	42.8±1.3	52.3±6.5	53.5±7.1	54±2.6
P. pinnata	3.3±1.8	6.7±0.5	27.5±4.4	45.9±3.4	52.2±1.0	62.9±2.1	65.9±2.6	69.1±1.1
M. citrifolia	8.9±0.9	15.6±72.8	23.9±1.7	33.9±4.1	39.5±1.8	42.9±2.8	45.1±0.9	46.3±1.1
M. indica	8.9±3.9	14.6±5.7	25.1±0.8	30.8±1.3	34.1±1.8	47.4±14.6	50.4±7.2	53.6±1.4
Z. officinale	7.3±0.59	12.2±0.8	21.02±1	27.7±2.6	37.6±3.7	44.5±2.8	49.7±4.2	51.6±0.6

P. pinnata: Pongamia pinnata L., *M. citrifolia: Morinda citrifolia* L., *M. indica: Mangifera indica* L., *Z. officinale: Zingiber officinale* Rosc.

allopurinol and identified *P. pinnata* L. seeds flavonoids as the member of XO inhibitors from plant origin.

Palu *et al.* reported 64% of XO inhibition using a fine powder of fruit of *M. citrifolia* L. after processing [16]. Our research findings are similar with the study and able to obtain 50% of XO enzyme inhibition when compared to allopurinol. Mangiferin is a component of leaf known as xanthone C-glycoside of *M. indica* L. stands as a first report to state about inhibitory activity on XO [17]. Our study investigated to explore similar property in the bark and reports nearly 58% of inhibition with allopurinol used as standard inhibitor.

Gouticin a coded herbal formulation contains one of the ingredient is *Z. officinale* Rosc. which is reported to have potential inhibition on XO activity. The effective percentage of inhibition found to be similar with allopurinol an allopathic drug for gouty arthritis. In addition to this, gouticin also contains *Apium graveolens, Colchicum autumnale, Tribulus terrestris,* and *Withania somnifera* along with *Z. officinale* Rosc. [18]. Our study showed that nearly 55% of inhibition from the flavonoid extract obtained from the rhizome of *Z. officinale* compared with allopurinol.

In systemic meta-analysis of randomized control trials of various research findings reported that vitamin C supplementation as lowering serum uric acid level [19]. In an *in vitro* study on XO inhibition by L-ascorbic acid in trace amounts resulted decreased XO activity [20]. In our study, L-ascorbic acid reported to have 51% of XO inhibition at the concentration of 1.0 mg/ml when compared with allopurinol. The findings of our study are apparently similar by means of exhibiting 54% of inhibition at the concentration of 0.8 mg/ml [21].

In a randomized controlled animal study reported that the vitamin E inhibitory effect on XO related to gastric lesion prevention [22-24]. In cholestasis induced hepatocellular injury, supplementation of vitamin C and E as antioxidants exerted protective benefit through the mechanism of inhibition of XO and xanthine dehydrogenase reported [17]. In the same line of investigation our research findings supported this view by showing 58% of inhibition by vitamin E on pure form of XO.

Nevertheless several reports emphasized vitamin C and E inhibits XO activity. None of the report presented remarkable percentage of inhibition other than 52-55% range. In support of this WHO 2012 report described supplementation of vitamin C and E has no significance in reducing the risk of oxidative stress.

CONCLUSION

Flavonoids can also be used as natural inhibitor of XO. *P. pinnata* L. had maximum inhibitory activity. However, *M. citrifolia* L. did not show effective XO inhibition.

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