CHAPTER ONE

INTRODUCTION

Natural or herbal products used in the management of diseases in folkloric medicine have been reported (Dias *et al.,* 2012). The practice of using these products is common in the developing countries for medical care, because they are not expensive and are culturally acceptable as well as compatible with human body system (Pal and Shukla, 2003). According to the World Health Organization (2002), 80% of the populace living in Africa use natural/traditional medicines for primary health care. Also, in United States of America, natural products are used by approximately 20% of the population (Ekor, 2014). Natural products can be classified as bio-based materials (bioplastics), bodily fluids (milk and plant exudates) and plant materials (fruits, roots and leaves) (Bhat *et al.,* 2005). An example of plant material based natural product is *Citrus limon* fruits.

Citrus limon fruit also known as "lemon" is from citrus family *Rutacea* (Ihesie, 2015). The fruit is native to South Asia, primarily North Western India (Morton and Miami, 1987). However, it is widely cultivated globally (Johnson, 2018). In Nigeria, it is locally known as "lemonu/ osan gaingain" (Yoruba), "lemun tsami" (Hausa) and "oro nkrisi" (Igbo) (Gawab, 2016). *Citrus limon* juice (CLJ) is rich in vitamin C (53 mg/100 g), thus providing 64% of the daily requirement (Bruno *et al.,* 2006). Other essential nutrients the juice provides include vitamin A (22 IU/100 g), folate (11 µg/100 g), potassium, calcium, sucrose, phosphorus and essential oils (Mohanapriya *et al*., 2013). The phytochemicals in CLJ includes flavonoids, polyphenols and terpenes (Rauf *et al.,* 2014). The juice has higher concentration of citric acid when compared with other citrus fruits (Penniston *et al.,* 2008). Previous report has observed CLJ and its major bioactive components oleic, palmitic and stearic acids to lower low-density lipoprotein and total cholesterol due to its hypolipidaemic property (Karacor and Cam, 2015). It also promotes platelet aggregation thereby enhancing wound healing (Oguwike and Onubueze, 2013). Its ability to cause weight loss (Fukuchi *et al.,* 2008; Samundy *et al.,* 2016) and prevent kidney stones (Touhami *et al.,* 2007) has been documented.

The CLJ is widely used by women of reproductive age for various purposes (Busch, 2017). It is used by non-pregnant women for detoxification and weight loss (Samundy *et al.,* 2016). It is also used for the treatment of severe bleeding/miscarriage (Singh *et al.,* 1984) and nausea thereby alleviating morning sickness in the first trimester of pregnancy (Lixandru, 2016). Few reports have shown that it is generally safe for consumption in pregnancy (Lixandru, 2016; Karabagias, 2017) suggesting that it creates a conducive maternal environment for foetal development (Louik *et al.,* 2010).

Maternal internal environment from which the developing foetus derives its nutrients is affected by exposure to substances ingested by the mother, oxidative status as well as hormonal imbalances which are able to modify its growth and development (Fowden *et al*., 2008). These modifications give rise to lifelong alterations in the physiology, metabolism as well as epigenome of an offspring and the phenomenon is known as developmental programming (Waterland and Garza, 1999). Exposures that affect maternal internal environment such as type and quantity of dietary intake as well as maternal lifestyle are all referred to as insults (Fowden *et al*., 2006a). Nutritional imbalance and metabolic disruption of maternal origin during early stage of development has been reported to cause lifelong health challenges in offspring (Sutton *et al*., 2016). These subsequently affect offspring health and enhance the risk of diseases in later life (Waterland and Michels, 2007). Changes in the maternal internal environment may be passed on to the foetus through the placenta.

The placenta is the primary interface amid the foetus and the mother (Roos *et al*., 2009). It maintains foetal growth by facilitating nutrients substrate transfer and wastes involving the maternal and foetal circulations (Roos *et al*., 2009). It also modulates maternal immune responses in order to avoid rejection of the conceptus (Zhang *et al*., 2015). The placenta also produces hormone like peptides and steroids as well as cytokines and glycoproteins which impacts on maternal, placental and foetal metabolism (Jansson and Powell, 2013). The foremost nutrient substrates required for placental and foetal growth are oxygen (Carter, 2009), glucose (Hay, 2006), amino acids (Battaglia and Regnault, 2001) and fatty acids (Duttaroy, 2009). The transport processes of these nutrients depend on placental morphology and vascularity (Zhang *et al*., 2015) as well as physiological and biochemical characteristics such as receptors, enzymes, hormones and nutrients transporters (Lager and Powell, 2012).

Maternal insults are transferred to the developing foetus via alterations in placental functions (Fowden *et al*., 2009), thereby affecting the growth and development of foetal tissues (Myatt, 2006). For instance, reduction in maternal lipids such triglycerides lead to reduction in placental transport and deposition of fats in the foetus resulting in to reduced birth weight and foetal microsomia (Gallo *et al*., 2017). Apart from the maternal insults, growth of the foetus is regulated by the nutrients availability in maternal circulation, which is transferred into foetal circulation through the placenta (Zhang *et al*., 2015). The placenta must therefore function optimally in order to supply nutrients to the foetus maximally. For example, placental restriction and insufficiency occurs when there are alterations in placental growth (Mayhew *et al*., 2003), substrate transport capacity (Zamudio *et al*., 2010) and oxidative status (Erel *et al*., 2001). This suggests that even when maternal nutrient is optimally available, placental insufficiency may place a restriction on the amount and quality of nutrients that are transferred to the foetus resulting into foetal growth restriction (Zhang *et al*., 2015). Previous studies reveal that poor maternal nutritional status result into development of Intrauterine Growth Restriction (IUGR) in the developing world, of which in the developed countries, it is an upshot of placental insufficiency (Morrison *et al*., 2009). Thus, the maternal internal environment affects placental *milieu*, consequently causing alterations in the amount of specific nutrients and growth-regulating hormones provided to the foetus during development (Sferruzzi-Perri and Camm, 2016). Placenta is therefore termed the link between maternal insult and adverse placental and pregnancy/foetal outcomes.

Pregnancy/foetal outcome refer to end results of fertilisation processes, which are related to appropriate intrauterine growth and placental efficiency (Fowden *et al*., 2006). Foetal outcomes can be deduced from anthropometric markers such as birth weight, crown-rump length, abdominal and head circumferences, while placental outcomes can be inferred from placental weight, chorionic surface area, placental coefficient as well as foeto-placental ratio (Shastry and Bhat, 2015). These markers are used as a representation of intrauterine environment as well as placental efficiency (Hayward *et al*., 2016). For instance, reduced birth weight may be an indication of failure of placenta to adapt to changes in maternal internal environment (Vaughan *et al*., 2008), which has been a major cause of cardiovascular diseases (Leon *et al*., 1998), obesity (Boney *et al*., 2005) and diabetes (Harder *et al*., 2007) in adult life.

Hence, foetal and placental outcomes are dependent on maternal environment and placental efficiency.

1.2 Statement of problem

Indiscriminate consumption of natural products with acclaimed beneficial effects in pregnancy is common (Arias and Ramon-Laca, 2005). Many of these ingested natural products by mothers have detrimental effect on foetal growth and development thus resulting in altered birth morphometry. Altered birth morphometry is linked with higher risk of ill health and adult diseases in offspring, leading to increased global burden of diseases (Fowden *et al.*, 2009).

1.3 Justification for the study

The CLJ has numerous beneficial effects on the body (Olukanni *et al.*, 2013). It is commonly consumed during pregnancy to alleviate morning sickness and to prevent severe bleeding especially during the first trimester (Lixandru, 2016). Nevertheless, it is a known hypolipidemic agent with potent effect on body fat loss (Samundy *et al.*, 2016). The ability of hypolipidemic agents to alter internal *milieu* as well as transport of nutrients raises a concern about the growth and development of the foetus which depends on optimal transplacental transport of nutrients. There is dearth of scientific knowledge on the association between *Citrus limon* juice, its major bioactive constituents and the placental as well as foetal outcomes.

1.4 Aim of the study

The study was designed to investigate the effects of maternal administration of *Citrus limon* juice, oleic, palmitic and stearic acids on placental efficiency and foetal morphometry in Wistar rats.

1.5 Specific objectives of the study

The specific objectives of the study were to determine the effects of *Citrus limon* juice and its major bioactive constituents on;

1. Serum levels of pregnancy hormones,
2. Placental and birth morphometric indices,
3. Maternal serum and placental lipid profile as well as oxidative status,
4. Placental expression of nutrients transporters such as Glucose Transporter -1 (GLUT-1), Small Neutral Amino Acid Transporter-1 (SNAAT-1) and Fatty Acid Transporter -1 (FATP-1).

CHAPTER TWO

LITERATURE REVIEW

2.1. *Citrus limon* (lemon)

Citrus limon is an example of plant based natural products, from citrus family *Rutaceae*. The tree is about 3m in height with leaves that are dark green in colour (Oguwike and Onubueze, 2013). The fruits range from greenish yellow to bright yellow, while the juice is yellow in colour, the fruits are oval in shape with a base that could be pointed or round depending on the variety (Mohanapriya *et al.*., 2013). In Nigeria, it is called oroma-nkrisi (Igbo), lemun tsami (Hausa) and lemonu / osan gaingain (Yoruba) (Gawab, 2016). There are similarities between lemons and limes in terms of composition and colour (Mohanapriya *et al.*., 2013).

Citrus limon has anticancer and antidiabetic properties, this is due to the phytochemicals present in the fruit (Burt, 2004 and Ortuno *et al.*., 2006). Its antibacterial property has also been identified (Kawaii *et al.*., 2000).

Figure 2.1: A fruiting *Citrus limon* tree with a blossom (black arrow) (Darwin, 2008)

Figure 2.2: Outer surface and cross section of *Citrus limon* fruit (Adapted from BBKSS, AGRO LTD, 2018)

2.1.1 Scientific Classification

• Kingdom – Plantae, Angiosperms, Eudicots, Rosids

• Order – Sapindales

• Family – Rutaceae

• Genus – Citrus

• Species – C. × limon

• Binomial name - Citrus × limon.

(Mohanapriya *et al.*, 2013)

2.1.2 Nutritional value of *Citrus limon*

Table 2.1: Nutritional value of raw lemon without peel

Nutrients	Value / 100 g	Daily requirements (%)
Energy	121 kJ	
Carbohydrates	9.32 g	
Sugars	2.50 g	
Dietary fibre	2.8 g	
Fat	0.30 g	
Protein	1.10 g	
Thiamine (Vit. B_1)	0.040 mg	3 %
Riboflavin (Vit. B_2)	0.020 mg	1 %
Niacin (Vit. B_3)	0.100 mg	1 %
Pantothenic acid (B_5)	0.190 mg	4 %
Vitamin B_6	0.080 mg	6 %
Folate (B_9)	11 µg	3 %
Vitamin C	53.0 mg	88%
Calcium	26 mg	3 %
Iron	0.60 mg	5 %
Magnesium	8 mg	2 %
Phosphorus	16 mg	2 %
Potassium	138 mg	3 %
Zinc	0.06 mg	1 %

(USDA, 2008)

2.1.1 Health Benefits of *Citrus limon* Juice

Citrus limon juice is harnessed for treating throat infections, dental anomalies and burns due to the vitamin c components in the juice (Ihesie, 2015). It is also used in the management of indigestion and constipation because it aids digestion (Busch, 2017). The CLJ is well known for treatment and prevention of kidney stones (Touhami *et al.*, 2007), it prevents cardiovascular diseases (Riaz and Khan, 2013) and lowers body temperature (Karabagias, 2017). The health benefits of CLJ have been reported to be due to the nourishing elements in the juice (Ihesie, 2015). Flavonoids in *Citrus limon* are compounds that have antioxidant and cancer fighting properties (Oguwike and Onubueze, 2013).

2.2. Developmental and Intrauterine Programming

Intrauterine programming is the process by which insults during critical period of development results into permanent alterations in structure and function of tissues (Fowden *et al.,* 2008). It was originally known as "Barker Hypothesis (Barker, 2007), although this phenomenon was proposed in the 1990s (Barker, 1995), it was first identified in 1873 in birds (Gray, 1963) and humans (Wood and Foster, 1998). The term programming was first introduced by Lucas in (1991), however after the Dutch famine figures emerged (Ravelli, 1976), the term developmental origins of health and disease was adopted (Barker, 2004). The result observed in conjunction with other studies (Nijland *et al.,* 2008) prove the period of organogenesis and tissue differentiation (perinatal period) to be vulnerable to adverse environmental conditions (Padmanabhan *et al.*, 2016).

Maternal nutritional imbalance, endocrine-disrupting substance exposure, disease conditions, lifestyle adoptions and medical treatments during pregnancy has been shown to alter foetal developmental trajectory (Padmanabhan *et al.,* 2016). The consequences of some of these insults are observed immediately after birth such as spina bifida (which is as a result of thalidomide medication for the treatment of morning sickness) (Finnell *et al.,* 2002), while others are manifested at adulthood such as coronary and metabolic diseases (Padmanabhan *et al.,* 2016). In addition, the consequences of the insults could lead to placental alterations such as IUGR (Puttabyatappa *et al.,* 2015), thereby resulting into adult reproductive and metabolic abnormalities.

2.3. Placenta

The placenta derived its name from Greek word *plakuos* meaning flat cake; it is named after its gross anatomical appearance (Liddell and Scott, 2012). It is a materno-foetal organ that begins development during blastocyst implantation. It provides nutrition, gas exchange, removal of waste, endocrine and immune support to the developing foetus (Roos *et al*., 2009). It is also a source of hematopoietic stem cells (Hill, 2019). In humans, the length of placenta is about 22 cm and it is 2-3 cm thick, the weight is approximately 500 grams and dark reddish-blue/ crimson colour in appearance. It is linked to the developing foetus by an umbilical cord of about 30–90 cm long and 1-2 cm in diameter. The placenta is made up of two surfaces; maternal and embryonic which are delivered at parturition (Hill, 2019).

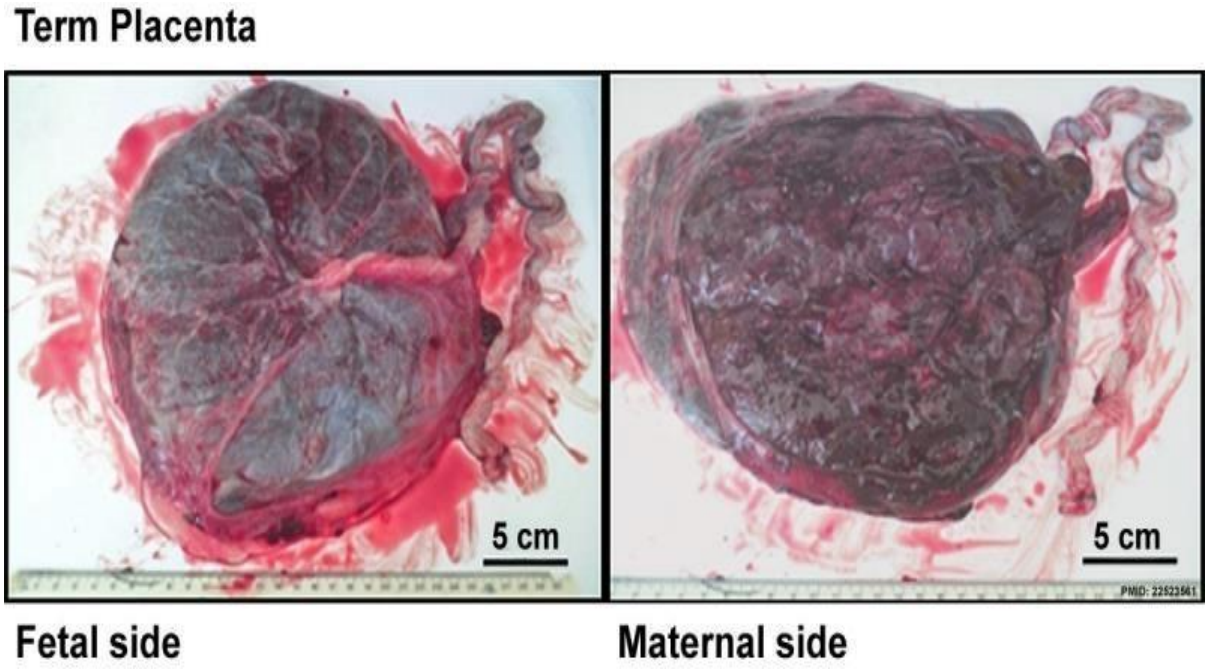


Fig 2.3: Structure of term placenta showing foetal and maternal side (Hill, 2019)

2.3.1 Embryogenesis of placenta

The placenta begins development at a period (7^{th}-10^{th} week) during which the blastocyst is implanted into the endometrial lining of the uterus. Blastocyst outer layer forms the trophoblast, which is further divided into two layers (cytotrophoblast and syncytiotrophoblast) by differentiation and fusion, this process occurs throughout placental development. The syncytiotrophoblast (syncytium) provides the barrier necessary for transport processes in the placenta (Ahokas and McKinney, 2008). The placentation is classified according to the kind of layers amid maternal and foetal circulations (Loke and King, 1995). In human, rats and mice, the placenta is categorised as haemochorial, due to the direct interaction between the maternal blood and foetal trophoblast, thereby enabling nutrients transfer and supply from mother to the developing foetus (Hill, 2019). In dogs and cats, it is classified as endotheliochorial because maternal endometrial blood vessels interact with the chorion, where as in pigs and cows it is categorised as epitheliochorial because maternal epithelium is in direct contact with the chorion (Hill, 2019).

The umbilical cord encloses a paired arteries and a vein. The arteries are involved in the transportation of waste materials and deoxygenated blood to the placenta, while the vein transport oxygenated blood to the foetus (Hill, 2019). Foetal and placental vascularisation (in stages) begins at day 21 after conception. Branching angiogenesis (second stage of blood vessels formation) begins after the onset of vasculogenensis (the first stage of blood vessels formation), this helps to increase capillary density (Hendricks *et al*., 1989). The third and final stage of blood vessel growth occurs around 24-26 weeks of gestation, at this stage terminal villi forms and the capillaries grow longitudinally (Kingdom *et al*., 2000). The process is correlated with increase surface area to volume ratio which promotes nutrient exchange (Benirschke and Kaufmann, 2000). Proper vascularisation determines the optimal growth and function of the placenta (Risau, 1997). Common pregnancy disorders involving poor foetal growth, such as foetal growth restriction and preeclampsia is linked to alterations in vascularisation (te Velde *et al*., 1997).

2.3.2 Functions of placenta

a. Transport of respiratory gases

Swift diffusion of respiratory gases (oxygen and carbon dioxide) ensues due to the permeability of placental membrane to these gases and blood flow to the placenta (Gude *et al*., 2004). Their exchange is due to the affinity of foetal and maternal haemoglobin to the gases.

b. Carbohydrates transport and metabolism

Glucose (main carbohydrate) is the main source of energy to the foetus. It is transported to the foetus across the placenta. All the glucose used by the foetus is a derivative of the maternal circulation because the foetus is capable of very little gluconeogenesis. Its transport is usually via the glucose transporters (GLUTs).

The glucose from the mother passes through the microvillous membrane of the syncytiotrophoblast (rate-limiting step) to the foetal capillary endothelial cells via the basement membrane (Bauman *et al*., 2002). The GLUT-1 in maternal and foetal membranes of the placenta is responsible for glucose transport at term (Illsley, 2000). GLUT-3 also transports glucose at term but it is found only in the endothelial cells and it regulates glucose concentration in endothelia cells and foetal blood (Illsley, 2000). GLUT-4 is localised in the placental stromal cells and is associated with glucose transport and conversion of glucose to glycogen (Xing *et al*., 1998). At term, GLUT-8 is found in human placenta but has less importance in first trimester (Limesand, *et al*., 2004). GLUTs distribution varies between trimesters which suggest differences in function at these stages (Illsley, 2000).

c. Metabolism and transport of Amino acids

Amino acids are substantial substrates required for synthesis of protein and nucleic acids in the foetus although they can be metabolised by the foetus. More than 20 amino acids present in the circulation are of two types; essential that is not produced by human tissues and nonessential that are synthesised from intermediates of glycolytic and citric acid cycle. Amino acids are transported to the foetus through the microvillous and basal membranes of the syncytium. Amino acids in foetal circulation are greater than maternal circulation (Yudilevich and Sweiry, 1985). Transporters of amino acid are involved in active exchange of these acids to the developing foetus (Cariappa *et al*., 2003).

In humans, the mostly expressed amino acids transporters are system y^+, X_{AG}, ASC and system A (Cariappa *et al*., 2003).

d. Lipids transport and metabolism

Lipids consist of compounds such as free fatty acids, triglycerides, cholesterol and so on and are mostly bound to plasma proteins (Gude *et al*., 2004). Free fatty acids, for instance are bound to serum albumin, while cholesterol and triglycerides form lipoprotein complexes. Lipoprotein lipase localised in the micro villous membrane of syncithiotrophoblast releases free fatty acids from the lipoprotein complexes in the maternal circulation, thereby allowing fatty acids and glycerol to be easily transported via syncytiotrophoblast by diffusion or through fatty acid binding proteins (rate limiting step) (Haggarty, 2002). Cholesterol can be synthesised by the placenta, but the foetus utilises the one in the maternal circulation (Gude *et al*., 2004). Liver and biliary system eliminates bile acids and biliary pigments in adults, but due to immature of foetal liver, the placenta plays its role (Marin *et al*., 2003).

e. Water, inorganic ions, minerals and vitamins transport

Hydrostatic and osmotic pressure determines the transport of water across the placenta. Water can be transported via passive transport or trophoblast expressed water channels (Stulc, 1997). There are similarities in sodium and chloride concentration in foetal and maternal circulations, although potassium, calcium and phosphate concentrations are more in foetal than maternal circulation (Shennan and Boyd, 1987). Transport of these ions is via active transporters (Sibley *et al*., 2002). Irons are also transported towards foetal circulations via the placenta (Gude *et al*., 2004).

f. Placental endocrine functions

As a result of absence of nerves in the placenta, the interaction between it and the mother and/or foetus is therefore through blood-borne substances (hormones). The placenta produces hormones that functions locally in the uterus or within the placenta itself (Gude *et al*., 2004). It also secretes growth factors and some other pregnancy associated proteins (Gude *et al*., 2004).

The corpus luteum secretes progesterone at the earlier stage of the pregnancy, but becomes atrophied by the 9th week of pregnancy, during this period placenta assumes its role and function to inhibit oestrus, uterine contraction and release of luteinizing hormone from pituitary gland (Gude *et al*., 2004).

It also secretes circulating oestrogens (oestrone, oestradiol and oestriol), which function to improve the growth of reproductive organs in the mother (Page, 1993).
Human Chorionic Gonadotrophin (hCG) is synthensize mainly in the first 12 weeks of pregnancy but the concentration is dependent on the stage of the pregnancy (Gude *et al*., 2004). The hCG helps to prolong corpus luteum during the first 12 weeks of pregnancy and cytotrophoblast cell fusion, it also aids functional differentiation of villous trophoblast which can also be stimulated by oestradiol and glucocorticoids (Malassine and Cronier, 2002).

Placental lactogen is structurally related to growth hormone and prolactin, it is associated with modulation of embryonic development and can also stimulate the production of other regulatory substances (Handwerger and Freemark, 2000). Its angiogenic function has also been highlighted (Corbacho *et al*., 2002). Placental growth hormone regulates maternal modification in pregnancy and placental development (Lacroix *et al*., 2002). Compounds like cytokines produced by the placenta and extraplacental membranes are involved in parturition (Keelan *et al*., 2003), while others such as eicosanoids and nitric oxide are associated with control of blood flow to the placenta (Gude *et al*., 1998).

g. Placental protective functions

Small xenobiotics pass through the placenta through simple diffusion or placental transport systems. However, the reduction in the transfer of potentially toxic substances protects the foetus from certain xenobiotics in maternal circulation by activating export pumps (Marin *et al*., 2003). Apart from the pumps, cytochrome P450 enzymes also help to protect the foetus from toxic substances (Pasanen, 1999). However, some substances such as alcohol, thalidomide and anticonvulsants could pass through the placenta to cause teratogenic effects (Gude *et al*., 2004). Generally, placenta does not readily transfer bacteria/virus from mother to foetus, though HIV transmission could occur in positive mothers (Soilleux and Coleman, 2003). Poor pregnancy outcomes have been associated with trophoblast viral infection (Arechavaleta-Velasco *et al*., 2002).

2.4 Adaptation of the placenta in developmental programming

Due to the crucial intrauterine development of an individual, placenta has been an organ of interest and curiosity (Anjankar *et al*., 2014). Intrauterine status of a foetus has been observed to be a mirrored by the placenta and also gives an accurate record of prenatal experiences of an infant (Sirpurkar *et al*., 2015). The placenta regulates transfer of nutrient by either morphological or functional adaptations so as to ensure optimal growth of the foetus (Sandovici *et al.,* 2012). These adaptations arise in response to maternal or foetal signals that eventually leads to changes in placental efficiency (Fowden *et al.,* 2009). Placental efficiency refers to birthweight to placental weight ratio (BW:PW ratio) (Wilson and Ford, 2001), it is a representation of placental adaptation (Fowden *et al.,* 2009). Failure of placenta to adapt to intrauterine environment may result into alteration in foetal size (Sibley *et al.,* 2010). More so, this suggests that placental efficiency is crucial to the programming of the foetus *in utero*, eventually increasing the incidence of cardiovascular and metabolic disease in later life (Hayward *et al.,* 2016).

2.4.1. Role of placental nutrient transporters

Optimal foetal growth is associated with nutrient availability in maternal circulation and the ability of these nutrients to be transfered into foetal circulation via the placenta. Transporters, electrochemical gradients and diffusion channels are of essence in nutrients substrate transfer across the placenta. Size, morphology, total surface area of the syncytium, abundance and activity of nutrient transporters as well as uterine and feto-placental blood flow are crucial for suitable nutrient transfer to the foetus through the placenta (Desforges and Sibley, 2010). The rate of foetal growth is coordinated by the nutrients available in maternal circulation via alterations in placental nutrient transporter which serve as a control mechanism. For instance, growth restriction occurs when nutrients are limited and it accelerates when nutrients are in surplus (Jansson and Powell, 2006).

The primary energy source that is essential for foetal and placental growth is glucose; its transport is by facilitated diffusion via glucose transporters family (GLUT) (Baumann, *et al.,* 2002). GLUT-1, the primary placental glucose transporter is

abundantly expressed in first trimester and at term (Illsley, 2000). It is greatly expressed in the microvillous membrane (Jansson *et al.,* 1993). GLUT-3 is expressed in the microvillous membrane of the syncytiotrophoblast, cytotrophoblast and endothelia cells (Brown *et al.,* 2011), while insulin- dependent GLUT-4 is expressed in the cytosol of the syncytiotrophoblast (Ericsson *et al.,* 2005).

Amino acids are crucial in the development of foetal tissue. The placenta expresses different amino acid transporters of which system A and L are widely investigated (Jansson, 2001). System A, a sodium-dependent transporter is associated with the transfer of small neutral amino acids for example alanine into the cell (Jansson, 2001), while System L is a sodium- independent transporter that facilitates transport of large neutral amino acid such as leucin (Verrey and System, 2003). System A is highly expressed in microvillous membranes of the syncytiotrophoblast (Hoeltzli and Smith, 1989), whereas systems L are mostly expressed in microvillous membrane and basal membrane (Cleal *et al.,* 2011). Three subtypes of System A (SNAT-1, SNAT-2, and SNAT-4) are expressed by placenta at term (Desforges *et al.,* 2009). Fatty acids promote the development of brain and fat deposition, because triglycerides cannot cross the syncytiotrophoblast, they are firstly converted to free fatty acids by placental triglycerides lipase (King, 2006), thereafter fatty acid transporters and binding proteins ensures their uptake by the placenta (Duttaroy, 2009).

Cholesterol is a chief component of cell membranes and precursor for steroid hormones, although the foetus is capable of synthesizing cholesterol endogenously (Herrera, 2002), but the majority of the cholesterol utilised by the foetus is from maternal circulation. Their transport is through lipoprotein complexes (Woollett, 2011) and the syncytiotrophoblast highly expresses lipoprotein specific receptors (Wadsack *et al.,* 2003). Cholesterol is transferred to the foetus through binding cassette transporter which are mostly expressed in the endothelial cells (Stefulj *et al.,* 2009) as well as the microvillous membrane and basal membrane (Aye *et al*., 2010).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Experimental animals

Male (180-200 g) and female (100-150 g) Wistar rats obtained from the Central Animal House, College of Medicine, University of Ibadan were used for the studies. The male rats were proven breeders, while the females were virgin rats with regular oestrous cycles. The rats were housed in the Postgraduate Animal House, Department of Physiology, College of Medicine, University of Ibadan, in aerated plastic cages and had access to rodent's pelletized feed (Ladokun feed, Ibadan) and water *ad libitum.* Animals were acclimatized to the environmental condition of the animal house for two weeks before the start of the study. Ethical approval was obtained from University of Ibadan, Animal Care and Use Research Ethics Committee (UI-ACUREC), with voucher number UI-ACUREC/19/0014 and the study was carried out according to the guidelines of animal protection and welfare.

3.2 Preparation of *Citrus limon* juice (CLJ)

Fresh fruits of *Citrus limon* (lemon) were obtained from a farm in Ibadan, the fruits were authenticated at Forestry Research Institute of Nigeria (FRIN) with voucher number FHI.110938 (figure 4.1). The fruits were washed, cut into halves and the juice was expressed into a glass beaker. The expressed juice was filtered using a clean sieve and the filtrate was collected into clean bottles. An aliquot of the filtrate was concentrated using a rotary evaporator in order to determine the concentration. The filtrate was given to the animals at a dose of 1 mL/kg orally (Khan *et al.,* 2010).

3.3 Reagents

Analytical grade of 9-octadecenoic acid (oleic acid), octadecanoic acid (stearic acid) and n- Hexadecanoic acid (palmitic acid) that was used for the purpose of the study were obtained from Sigma Aldrich chemicals Ltd (USA).

3.4 Experimental protocol

The research was carried out as two studies: preliminary study (study one), in which the effects of *Citrus limon* juice on oestrus cycles, reproductive hormones and organs in female Wistar rats were assessed and study two, in which the effects of maternal administration of *Citrus limon* juice and its major components on placental efficiency and foetal morphometry in Wistar rats, including the mechanism of action were evaluated.

3.5 Preliminary study

Twenty female Wistar rats (120-150 g) with normal oestrus cycle pattern were used for the study. They were grouped into four of five animals each; control, 50% CLJ, 75% CLJ and 100% CLJ. Control animals received distilled water, while group two, three and four received 50%, 75% and 100% concentration of the juice for a period of 3 weeks.

3.5.1 Animal grouping

The experimental animals were grouped as follows:

1. Control - received 1 mL/kg of distilled water
2. 50% CLJ - received 1 mL/kg of 50% *Citrus limon* juice
3. 75% CLJ - received 1 mL/kg of 75% *Citrus limon* juice
4. 100% CLJ - received 1 mL/kg of 100% *Citrus limon* juice

3.6 Determination of Gas chromatography and mass spectroscopy (GC-MS)

Analysis of individual constituents in *Citrus limon* juice was done using an Agilent 7890AGC interfaced to an Agilent 5973N mass selective detector. The temperature of the gas chromatography oven was initially 50^0C maintained for 1 minute, then increased to 100^0C for 5 minutes, thereafter to 300^0C for 5 minutes, the total run time was 48 minutes. The injector temperature was 250^0C with a split ratio of 1:30 and MS detector at 280^0C. Electronic integration was measured to get compositions with flame ionization detection at 280^0C. The peak numbers, relative abundance of constituents and retention time was recorded. The data was retrieved via GC-MS solution software and National Institute Standards and Technology (NIST) library was used to identify the corresponding peaks.

3.7 Determination of oestrus cycle pattern

The oestrous cycle pattern of the animals was studied using the Marcondes' technique (Marcondes *et al.*, 2002). This was done by determining the phase of oestrous cycle daily (7:00- 8:00 am) throughout the duration of the experiment. Normal saline was introduced into the vagina using a Pasteur pipette after gentle handling in order to prevent stress to the animals. The Pasteur pipette delivered normal saline into the vagina and obtained the lavage. The obtained smear was placed on clean glass slides and mounted on the stage of a light microscope (Olympus, Japan). Slides were viewed under a magnification of ×40. The cell types as well as proportion were used to identify the phase of oestrus cycle in the rats. A smear of proestrus comprises of non-cornified nucleated cells; a smear of oestrus comprises of non-nucleated cornified cells; a smear of metestrus comprises of equal proportion of leukocytic, cornified and nucleated cells and a smear of diestrus comprises of leukocytic cells (Long and Evans, 1922).

3.8 Body weight measurements

Body weight of the animals was taken weekly and prior to sacrifice with a digital electronic weighing scale (Lisay, China). Thereafter, mean progressive body weight was calculated and recorded.

3.9 Sacrifice

After completion of study one, the animals were sacrificed in the proestrus phase of their estrous cycle. The animals were weighed and then anaesthetized using intraperitoneal injection of thiopentone sodium (50 mg/kg) (Pereda *et al.,* 2006). Animals were surgically dissected to reveal the thoracic cavity wherein the heart lies and blood was collected into a plain sample through cardiac puncture. The ovary, uterus, kidney, liver and heart of each animal were removed, freed from adherent fat and weighed with sensitive weighing scale. Organs collected were immersed in 10% formalin for histological assessments.

3.10 Experimental protocol for study two

Twenty-five mature female Wistar rats (100-120 g) with normal oestrus cycle pattern and ten proven male breeders (180-200 g) were used for the study.

3.10.1 Animal grouping

The experimental animals were grouped as follows:

1. Control - received 1 mL/kg of distilled water
2. CLJ - received 91.0 mg/kg *Citrus limon* juice
3. OA - received 27.8 mg/kg of oleic acid
4. PA - received 23.4 mg/kg of palmitic acid
5. SA - received 17.6 mg/kg of stearic acid

All administration was done orally from Gestation Day 1 to 20 (GD1-20) and the doses of the major components in the juice (oleic, palmitic and stearic acids) was calculated based on the percentage composition in the juice.

3.11 Co-habitation and confirmation of mating

Female rats in the proestrus phase were introduced to male rats at ratio 2:1 (female to male). On the following morning after pairing, vaginal lavage was obtained with Pasteur pipette filled with 0.1 ml normal saline by gently inserting the pipette tip into the rat vagina. The withdrawn vaginal content was put on a glass slide and the smear was spread out evenly. The glass slide was examined using the ×10 and ×40 objective lenses of light microscope (Olympus, Japan) to determine the presence of spermatozoa. Confirmation of mating was established by the observation of spermatozoa in vaginal smear. The day on which spermatozoa were observed in the vaginal smear was designated as Gestation Day 1 (GD1) for each rat.

3.12 Determination of maternal feed intake and blood glucose level

Twenty four hours maternal feed intake/ consumption in the rats were measured by subtracting the leftover weight from the initial weight of the feed introduced to the animals (Laaksonen *et al.*, 2013). At the end of the study, maternal fasting blood glucose level was measured from the tail vein by using glucometer (Accu-chek Active, USA).

3.13 Caesarean section

Non-survival caesarean section was performed at the end of study two under thiopentone anaesthesia (50 mg/kg, i.p.) (Pereda *et al.*, 2006). The animals were surgically opened around the pelvic region to fully expose the gravid uterus, which was carefully removed from the root. The pups with its respective placentas were carefully dissected from the uterus (figure 3.1a). Pup and placenta morphometry were estimated. Two placentas per sex and dam were immersed in 10% neutral buffer formalin for immunohistochemical and histological assessments, while five other placentas were homogenised in phosphate buffer (pH 7.4) for redox status, lipid profile, metabolic hormones and protein analysis.

3.14 Determination of placental morphometry

The placental weight was determined by weighing the placenta of each pup on a digital electronic weighing scale (Lisay, China). Placental volume was measured using Archimedes principle (figure 3.1b) by dropping each placenta into a measuring cylinder containing a known volume of phosphate buffer solution. The displaced volume of phosphate buffer was recorded as the volume of placenta. Placental thickness was measured with the use of a Vernier calliper by measuring from the widest surface to the concave surface (figure 3.1c). Placental circumference was measured by placing a piece of thread round the perimeter of the widest surface of the placenta (figure 3.1d). This length of the thread that represents the perimeter of the placenta was then measured using a ruler. This was done because the rat's placental surface has at least two different diameters and the circumference cannot be calculated from the diameter since it is not a perfect circle. Placental chorionic surface area was calculated from this value.

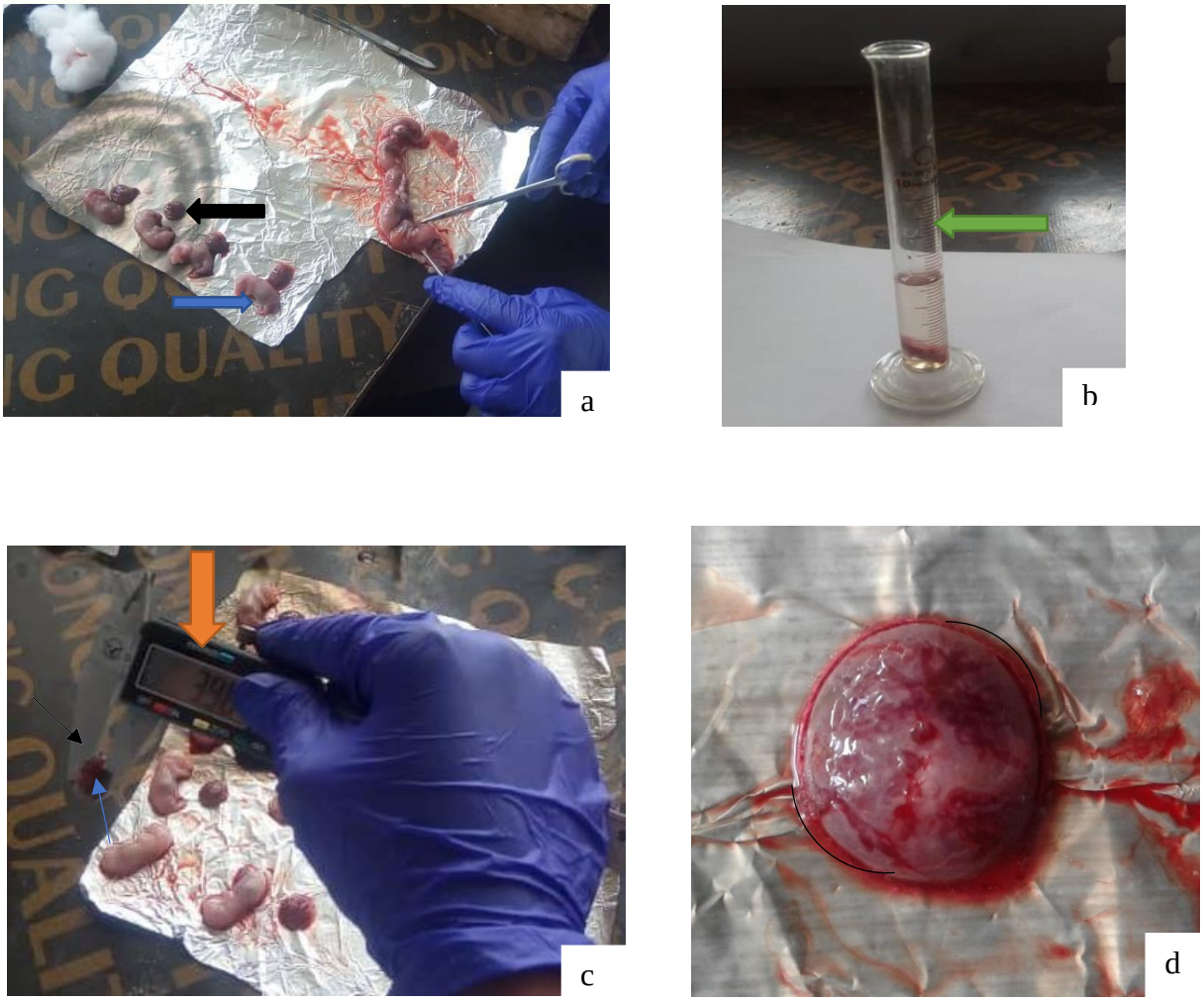


Figure 3.1: a. Dissection of pups (blue arrow) and placentas (black arrow) from the uterus. b. Placental volume determination using Archimedes' principle (green arrow).
c. Measurement of placental thickness from the widest surface **(**blue arrow) to the concave surface (black arrow) with a vernier calliper (orange arrow).
d. Placental circumference determination using a piece of thread round the perimeter of the placenta (black curve).

3.15 Pup morphometry

Pup morphometry was carried out immediately after the pups were obtained by Caesarean section. The pups/foetus were weighed on an electronic scale (Lisay, China). The head diameter was measured from one ear to the other ear, abdominal diameter was measured at the centre of the waist, Ano-Genital Distance (AGD) was measured from the genital ridge to the anus and crown- rump length was measured from nose tip to tail root of the rat. All measurements were recorded using a digital Vernier calliper (Mitutotyo, Japan), while the pups were gently but firmly held. Head circumference ($2\pi r$), abdominal circumference ($2\pi r$) and anogenital distance index (Gallavan *et al.,* 1999) were calculated from the data obtained.

3.16 Blood collection and preparation of serum

The blood collected as earlier stated was left to stand for about 45 minutes, after which was centrifuged at 3000 rpm for 15 minutes. The serum portion was separated and stored at -20^{o}C for ELISA analysis. Serum concentration of oestradiol, follicle stimulating hormone and luteinizing hormone were assayed in female non pregnant rats while progesterone, chorionic gonadotropin, leptin and insulin were assayed in pregnant dams.

3.17 Organ harvest

At the end of study two, placentas were harvested from the dams at Gestation Day 20 (GD 20) and weighed on the digital electronic scale (Lisay, China). The placentas were immersed in 10% neutral buffer formalin for immunohistochemical and histological assessments.

3.18 Histological assessment of the organs

Fixation

The organs obtained at sacrifice were immediately fixed in 10% formalin for at least 5 hours.

Dehydration

The tissues were dehydrated by passing them through graded alcohol (70%, 80%, 90% and 2 changes of 100% for an hour each). This procedure was undertaken so as to remove the inherent water content in a gradual way considering osmotic dynamics.

Clearing

The tissues were cleared of the ethanol that they have been bathed so as to initiate and complete the process that would make cells transparent at microscopic level. This was done by placing them twice in xylene solution for one hour each. Thereafter, it was placed twice in the wax bath for one hour each.

Embedding

The tissues were embedded in molten paraffin wax (mould) which served as support to the tissues for the stage sectioning. The embedded tissues were left until the wax solidified. The tissues were then cut into blocks and they were held in position by paraffin wax. Afterward, the blocks were clamped and positioned for sectioning.

Microtomy

The blocks were pruned, cut and split at 3-5µ. The split tissue was placed in 20% alcohol on water at 50^{o}C, then drained and it was put on a microscope slide for 60 minutes. The satisfactory sections were selected with a clean microscope slide that has been previously lined with glycerine egg albumin. The slides carrying the sections were labelled with diamond pencil, arranged in a slide carrier and put in an oven to dry.

Staining

The slides were stained with Haematoxylin and Eosin (H&E) so as to determine the general morphology. The tissues were dewaxed in xylene twice for 3 minutes each and sections were immersed in water. They were then rinsed in graded alcohol for two minutes each, after which they were bathed in water and stained with Harris-haematoxylin for 5 minutes. They were bathed in water again and differentiated in 1% alcohol in 3 dips. They were placed in running water for 180 seconds and counterstained with aqueous eosin for another 180 seconds and then dehydrated with ascending grades of alcohol for a minute each. They were then immersed in xylene and placed on the microscope for histological assessments. Photomicrograph of the sections was gotten at different magnifications in order to observe morphological changes.

3.19 Determination of serum hormonal and placental nutrient transporter levels

Serum levels of hormones, lipid profile and placental metabolic proteins/ nutrient transporters were assayed by ELISA. The protocols used for the assay were specified by the kit manufacturers. The kits and their manufacturers are listed as follows:

Table 3.1: ELISA kits used and their manufacturers list

S/N	Kit	Manufacturer
1	Estradiol	Calbiotech Inc., ES180S Austin Dr, Spring Valley, CA92020, United States of America.
2	Follicle stimulating hormone	Calbiotech Inc., ES180S Austin Dr, Spring Valley, CA, 91978, United States of America.
3	Luteinizing hormone	Calbiotech Inc., ES180S Austin Dr, Spring Valley, CA, 91978, United States of America.
4	Progesterone	Calbiotech Inc., PG1295 Austin Dr, Spring Valley, CA, 92020, United States of America.
5	Chorionic gonadotropin	Calbiotech Inc., HC251F Austin Dr, Spring Valley, CA, 92020, United States of America.
6	Leptin	Elabscience Biotechnology Inc., E-EL-R0582, United States of America.
7	Insulin	Calbiotech Inc., IS130D Austin Dr, Spring Valley, CA, 92020, United States of America.
8	Placental lactogen	Elabscience Biotechnology Inc., E-EL-H1971, United States of America.

9	Placental lipase	Biorex Diagnostics Limited, BXC0511A, Unit 2C Antrim Technology Park, Muckamore, BT411QS, United Kingdom.
10	GLUT-1	Elabscience Biotechnology Inc., E-EL-R0429 96T, United States of America.
11	Total triglyceride	Fortress Diagnostics Limited, BXC0271, Unit 2C Antrim Technology Park, Antrim, BT411QS, United Kingdom.
12	Total cholesterol	Fortress Diagnostics Limited, BXC0261, Unit 2C Antrim Technology Park, Antrim, BT411QS, United Kingdom.
13	HDL-cholesterol	Fortress Diagnostics Limited, BXC0422A, Unit 2C Antrim Technology Park, Antrim, BT411QS, United Kingdom.
14	LDL-cholesterol	Fortress Diagnostics Limited, BXC0431, Unit 2C Antrim Technology Park, Antrim, BT411QS, United Kingdom.

3.19.1 ELISA Procedure

Direct competitive and sandwich ELISA were used in this study for the quantitative analysis of the analyte. Generally, the essential reagent required for immunoenzyme methods include the microtitre plate, high affinity and specific antibody, the attached enzyme, the enzyme substrate, wash buffer, stop solution and absorbance based microplate reader.

Overall procedure

1. Attachment of capture antibody specific to target protein to a microplate
2. Addition of standards and samples containing unknown amount of the target protein which binds to the capture antibody
3. Washing to remove unbound substances
4. Addition of a detection antibody that binds to the immobilized target protein
5. Washing away excess detection antibody and addition of HRP conjugate
6. Addition of HRP substrate for indirect detection of bound protein

The following materials were provided in each of the kits used;

- Clear 96-well plate
- Coating buffer (10 mM phosphate buffer, pH 7.4)
- Blocking buffer (Assay Buffer)
- Wash buffer (Tris-buffered or phosphate-buffered saline with 0.05% Tween 20)
- Plate sealers
- Reagent reservoirs
- Coating antibody
- Detection antibody
- Streptavidin-HRP
- TMB substrate solution
- Stop solution (1.8 N H_2SO_4)

Absorbance-based microplate reader, distilled water and samples are readily available in the laboratory.

Procedure

1. Coating Solution was prepared by diluting the capture antibody in coating buffer. This was done according to the manufacturer dilution recommendations for each analyte.
2. Plates were coated with 100 µL per well of Coating Solution. Plates were Covered and incubated at 2–8 °C.
3. Wells were aspirated and wash one time with >200 µL of wash buffer per well. Thereafter, the wells were inverted and tapped on absorbent paper to remove excess liquid.

4. Plate were blocked with 200 µL per well with blocking buffer for 1 hour at room temperature.

5. The wells were aspirated, inverted and tapped on absorbent paper to remove excess liquid.

6. Standards and sample dilutions were prepared in blocking buffer.

7. 100 µL of standards (in duplicate) and samples was pipette into designated wells, incubated for 1 hour at room temperature with gentle continual shaking (~500 rpm).

8. Wells were aspirated and washed 5 times with >200 µL of **wash buffer** per well, thereafter, wells were inverted and tapped on absorbent paper to remove excess liquid.

9. Detection antibody solution was prepared by diluting the detection antibody in blocking buffer. Recommended antibody dilution was done according to the manufacturer's instruction per kits.

10. 100 µL of the detection antibody solution was added into each well and incubated for 2 hours at room temperature with gentle continual shaking (~500 rpm).

11. Wells were aspirated and washed 5 times with >200 µL of wash buffer per well. Thereafter, wells were inverted and tapped on absorbent paper to remove excess liquid.

12. Working solution of Streptavidin-HRP with blocking buffer was made by diluting 1:5,000. For instance, to make enough for 1 plate, 2 µL of streptavidin-HRP was added to 9.998 mL of blocking buffer.

13. 100 µL of working streptavidin-HRP solution was added into each well and incubated for 30 minutes at room temperature with gentle continual shaking (~500 rpm).

14. Wells were aspirated and washed 5 times with >200 µL of wash buffer per well, thereafter the wells were inverted and tapped on absorbent paper to remove excess liquid.

15. 100 µL of TMB substrate solution was added to each well and incubated for 30 minutes at room temperature.

16. 100 µL of stop solution was added to each well.

17. Absorbance was read at 450 nm within 30 minutes of adding stop solution.
18. The result was calculated using log-log constructed from the standard curve.

Assessment of Total Triglyceride

The contents of the kits include the following;

- R1 Buffer (1×105 ml) –Pipes buffer pH 7.8 (50 mmol/L)
  Magnesium ion (17.5 mmol/L)
  P-chlorophenol (2mmol/L)
- R2 Enzyme reagent (10×10ml) – Glycerolkinase (800U/L)
  Lipoprotein lipase (15000U/L)
  Glycerol-3-p-oxidase (4000U/L)
  Peroxidase (440U/L)
- R4 Standard (1×5mL) – Triglyceride (200 mg/dL)

Triglyceride test principle is based on enzymatic colorimetric test.

Procedure

Materials provided in the kit include working solution, controls and 0.9% Nacl.

To prepare the working reagent, the enzyme reagent (R2) was reconstituted: one vial of the reagent was mixed with 10ml of buffer R1. The working reagent is stable for 21days when stored at 2-18°C.

1. 10 µL of sample or standard was pipette into test tube
2. 1000 µL of working solution of sample or standard was pipette into the test tube
3. After incubating at 37°C for 5 minutes, within 60 minutes the absorbance was read of sample against standard.
4. The results was calculated using the following formula;

$$\textbf{Triglyceride concentration} = \frac{\Delta \text{ ab Sample}}{\Delta \text{ ab Standard}} \times \textbf{Standard Concentration}$$

Assessment of Total Cholesterol

The contents of the kits include the following;

- R1 Buffer (1×105 ml) –Pipes buffer pH 7.8 (50 mmol/L)
  Phenol (60 mmol/L)
- R2 Enzyme reagent (10×20ml) – 4 aminoantipyrine (0.3 mmol/L)
  Cholesterol oxidase (>100µL)
  Cholesterol esterase (>150µL)
  Peroxidase (>800µL)
- R4 Standard (1×5mL) – Cholesterol (200 mg/dL)

Enzyme reagents R2 with the corresponding volume of buffer R1 was dissolved, thoroughly mixed was allowed to stand for 15 minutes before use. This working reagent will be stable for 21 days at4°C.

Procedure

1. 10 µL of distilled water was pipette into blank test tube
2. 10 µL of sample and standard was pipette into the tubes
3. 1000 µL of enzyme reagent was pipette into the blank, standard and sample thereafter, the absorbance was read of sample against standard and blank.
4. The results was calculated using the following formula;

$$\textbf{Cholesterol concentration} = \frac{\Delta \text{ ab Sample}}{\Delta \text{ ab Standard}} \times \textbf{Standard Concentration}$$

Assessment of High Density Lipoprotein and Low Density Lipoprotein

Method

Low Density Lipoprotein (LDL) and Very Low Density Lipoprotein (VLDL) in the samples were precipitated from solution when the precipitant is added. With the aid of centrifugation, the precipitate was separated from the supernatant (contains only HDL-cholesterol). The supernatant was assayed for cholesterol and the concentration obtained was that of the HDL-cholesterol present in the sample.

Procedure for use of LDL Precipitant

1. 500µL of LDL precipitant was pipette into centrifuge tubes
2. 200µL of sample was pipette into centrifuge tubes
3. The solution was mixed and incubated for 10 minutes at room temperature
4. The solution was centrifuged for 10 minutes at 4000 rpm
5. The supernatant was carefully collected.

3.20 Determination of redox status in ovarian and placental tissue

The harvested ovaries and placentas were homogenised in phosphate buffer saline, the homogenate was centrifuged at 10,000 rpm for 15 minutes at 4^{o}C. The supernatant collected was used for the assays of malondialdehyde, total antioxidant capacity, superoxide dismutase, catalase and glutathione peroxidase using spectrophotometry.

Assessment of ovarian and placental lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) a product of lipid peroxidation was used to assay ovarian and placental lipid peroxidation (Ohkawa *et al.,* 1979). Malondialdehyde is recognised as the product of lipid peroxidation that results with thiobarbituric acid to give a pink complex absorbing maximally at 535 nm. Adam-Vizi and Seregi (1982) method was used to determine the levels of malondialdehyde in the homogenate.

Assessment of Total Antioxidant Capacity

Total antioxidant capacity was determined by the use of Total Antioxidant Status (TAS) assay kit (Fortress Diagnostics, United Kingdom). The kit uses the principle of colorimetry and the test was conducted based on the manufacturer's instructions.

Assessment of Superoxide Dismutase (SOD) level

The activity of SOD was assessed by Misra and Fidovich (1972) method. The ability of SOD to inhibit autoxidation of epinephrine (pH 10.2) is the principle adopted. Oxygen radicals produced by the interaction of Fe^{++} and H_2O_2 causes oxidation of epinephrine to adrenochrome in Fenton's reagent, whereas xanthine oxidase reaction (co-oxidation of epinephrine via xanthine oxidase acting on xanthine) was observed to cause oxidation of epinephrine by superoxide anions. Hence, availability of SOD which removes superoxide radical by catalysing its dismutation to O_2 and H_2O_2 makes it possible to test the involvement of superoxide in enzymatic oxidation of epinephrine.

Assessment of catalase activity

Catalase enzyme was measured by the method of Sinha (1972). Formation of chromic acetate from dichromate (in acetic acid) heated in the presence of hydrogen peroxide is the principle adopted. The chromic acetate generated was then evaluated colorimetrically at 570-610nm. Thereafter the hydrogen peroxide in the catalase preparation was allowed to split for duration, addition of dichromate/acetic acid to the

mixture at a known period put an end to the reaction. Thereafter, the remaining hydrogen peroxide in the mixture (solution) was determined by measuring the chromic acetate present after heating the solution.

Assessment of glutathione peroxidase activity

Glutathione peroxidase catalyses hydrogen peroxide to water and oxygen or alcohol and oxygen. The assay was based on colorimetry (Rotruck *et al.*, 1973).

3.21 Procedure for immunohistochemistry

Tissue sections were prepared as described previously (3.16). Sections were transferred onto charged glass slides. The slides were dried overnight. The following day, microscope slides were labelled with Hb pencil, they were dewaxed in Xylene twice for 5 minutes each.

Slides were transferred into 100 % ethanol for 2 changes, 5 minutes each and then transferred once through 90 % and 80 % ethanol for 300 seconds each. This process is for slides rehydration. The slides were thereafter placed in distilled water for 5 minutes, they were immersed in wash buffer for additional 5 minutes and then rinse with distilled water. Citrate buffer was used to retrieve the antigen. Slides were placed in a staining chamber that contained 300 mL citrate buffer (pH 6.0, 10mM), it was then incubated at 95-100^{0}C for 600 seconds. The staining chamber was removed and slides were cooled for 600 seconds. Slides were thereafter rinsed thrice in 300 mL PBS for 5 minutes each. Hydrogen peroxide blocking reagent was placed on the sections and incubated in a humidified chamber at 36^{0}C for 10 minutes. Slides were thereafter rinsed with 300 mL PBS thrice for 120 seconds each.

A hundred microlitres of appropriately diluted serum bovine primary antibody such as Anti-IRS-1, leptin receptor antibody (LEPR) and Anti- PPAR-γ antibody was added to the sections on the slides for respective analyte and incubated in a humidified chamber at 36^{0}C for 30 minutes. Slides were thereafter washed with PBS thrice for 2 minutes each. A hundred microlitres of appropriately diluted Sav-HRP conjugates was added to the sections on the slides and incubated for 10 minutes at room temperature. Slides were rinsed with PBS thrice for 2 minutes each. Freshly prepared 3,3'-diaminobenzedine substrate solution (DAB) was applied on the slide to reveal the antibody staining colour. The slides were set aside for 5 minutes until the colour

intensity desired is attained. Slides were washed with PBS thrice for 2 minutes each. Slides were counterstained with hematoxylin for 30-60 seconds. Slides were washed with distilled water. Mounting solution, Dibutylphthalate Polysterene Xylene (DPX) was applied to the tissue sections on the slide and coverslip. The colour stain on the tissue sections was observed under ×10 and ×40 objective lens of the microscope. Photomicrographs of the sections were made.

3.22 Statistical analysis

Data were expressed as mean ±Standard Error of Mean (SEM) and the differences in mean were compared by analysis of variance (ANOVA) and Student's t-test where appropriate. $P<0.05$ was considered statistically significant. Graphpad prism 7.01 (Graphpad software, Inc, U.S.A.) was used to analyse the data.

CHAPTER FOUR

RESULTS

4.0. The results of all the analysis obtained in the study were presented in this chapter. Before presentation of the preliminary study, identification of *Citrus limon* plant, phytochemical analysis of the juice and then Gas Chromatography and Mass Spectrometry analysis of the juice results were presented prior to the results of the preliminary study. The preliminary study was conducted in non-pregnant female Wistar rats to assess body and organ weights, oetrous cycle, reproductive hormone levels, lipid profile and histological assessments of liver, ovarian and uterine tissues. This was done to ascertain and corroborates its effects in non pregnant states, the results observed then lead to its use in the main study, which is its use in pregnant states.

4.1. Identification of *Citrus limon* plant and fruit

Figure 4.1 shows the identification of *Citrus limon* plant, that is the leaf and the fruit that was obtained at Forestry Research Institute of Nigeria (FRIN) in Ibadan, Oyo state Nigeria. The juice of *Citrus limon* was used for the purpose of this study. The juice was also analysed for phytochemical and GC-MS screening in order to know the individual constituents of the juice. The analytical grade of the major constituents obtained from the screening was later used for the purpose of the study.

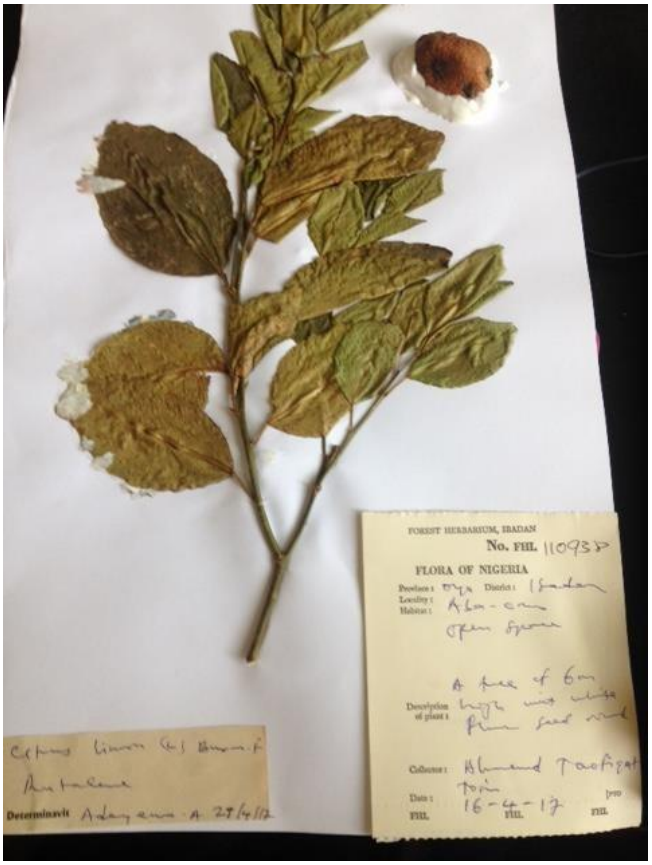


Figure 4.1 Identification of *Citrus limon* plant and fruit at Forestry Research Institute of Nigeria (FRIN). Voucher Number 110938

4.2 Phytochemical analysis of *Citrus limon* juice

Qualitative phytochemical analysis of *Citrus limon* juice (CLJ) discovered presence of saponins, flavonoids, alkaloids, terpenes, phenols and anthraquinone and absence of steroids, cardiac glycosides, tannin, resins and reducing sugars (Table 4.1).

Table 4.1: Phytochemical screening analysis of *Citrus limon* juice (CLJ)

S/N	Test	Observation
1.	Saponins	+
2.	Tannin	_
3.	Flavonoids	+
4.	Steroids	_
5.	Anthraquinone	+
6.	Cardiac glycosides	_
7.	Alkaloids	+
8.	Terpene	+
9.	Resins	_
10.	Reducing sugars	_
11.	Phenol	+
12.	Glycosides	_

KEY: + = Present and - = Absent

4.3 Gas Chromatography and Mass Spectrometry (GC-MS) analysis of *Citrus limon* juice.

Table 4.2 show abundance of chemical constituents with retention time of *Citrus limon* juice as extrapolated from GC-MS solution software and National Institute Standards and technology (NIST) library. Three main constituents were identified in the juice; 19.44% of Octadecanoic acid (stearic acid) at retention time of 40.177 mins; 25.81% of n- Hexadecanoic acid (palmitic acid) at retention time of 38.928 mins and 30.48% of 9-Octadecenoic acid (oleic acid) at retention time of 40.027 mins (Table 4.3). These values were extrapolated and retrieved from the values in GC-MS solution software and National Institute Standards and technology (NIST) library.

Table 4.2. Retention time and percentage of total constituents of *Citrus limon* juice.

Peak #	R.T. Min	First Scan	Max scan	Last scan	PK TY	Peak height	Corr. Area	Corr. %max	% of total
1.	5.975	77	113	127	rVB	5105511	27659093	11.30	3.444
2.	18.205	1648	1670	1696	rVB9	2505559	22715185	9.28	2.828
3.	38.928	4279	4308	4328	rVB	29909067	207260570	84.66	25.808
4.	40.027	4424	4448	4456	rBV3	30875256	244808400	100.00	30.483
5.	40.177	4456	4467	4470	rVV	38600156	156113810	63.77	19.439
6.	40.239	4470	4475	4478	rVV	16681233	44462830	18.16	5.536
7.	40.287	4478	4481	4484	rVV	17784805	26649277	10.89	3.318
8.	40.342	4484	4488	4493	rVV	10647346	20984452	8.57	2.613
9.	40.412	4493	4497	4502	rVV	7196473	15392888	6.29	1.917
10.	40.475	4502	4505	4516	rVV	4256065	9210807	3.76	1.147
11.	40.624	4519	4524	4542	rVB	3619567	9874862	4.03	1.230
12.	41.049	4573	4578	4609	rVB	7812379	17963145	7.34	2.237

Table 4.3: Nomenclature of individual constituents in *Citrus limon* juice.

Pk#	RT	Area%	Library/ID	Ref#	CAS#	Qual
1.	5.975	3.44	Hydrazine, 1,1-dimethyl-	280	000057-14-7	4
			Hydrazine, 1,1-dimethyl-	279	000057-14-7	4
			Urea	298	000057-13-6	4
2.	18.205	2.83	4-Morpholineacetonitrile	11068	005807-02-3	50
			2,5-Furandione, 3-methyl-4-propyl-	27426	016493-20-2	49
			Benzene, 1-fluoro-2-methoxy-	11299	000321-28-8	35
3.	38.928	25.81	n-Hexadecanoic acid	107549	000057-10-3	99
			Tetradecanoic acid	84455	000544-63-8	90
			Tridecanoic acid	72646	000638-53-9	86
4.	40.027	30.48	Oleic acid	129338	000112-80-1	99
			9-Octadecenoic acid, (E)-	129353	000112-79-8	99
			Cis-Vaccenic acid	129339	000506-17-2	99
5.	40.177	19.44	Octadecanoic acid	131262	000057-11-4	95
			Pentadecanoic acid	95855	001002-84-2	93
			Octadecanoic acid	131261	000057-11-4	80
6.	40.239	5.54	Azetidine, 1-benzoyl-3-ethenyl-	51005	118973-03-8	27
			Pyrazol-3-amine, 1-(4-methylphenyl)	50914	1000272-83-6	16
			2-Azetidinone,3-(1-methylethylidene)-1-phenyl-	51025	068695-52-3	14
7.	40.287	3.32	N-(2-Chloroethyl) benzamide	48211	026385-07-9	43
			Quinoline, N-benzoyl-1,2,3,4-tetra hydro-	91604	028748-92-7	10
			Thiazolo[3,2-a] [1,3,5]-triazin-6(7H)-one, 3,4 (2H)-dihydro-7-hydroxymethyl-7-methyl-3-phenyl-	124842	088696-71-3	9
8.	40.342	2.61	Valine, N-[2-oxo-4-phenylbutyryl]-	124961	1000211-36-9	22
			Benzyl alcohol, 5-methoxy-.alpha.-methyl-2-nitro-	59103	156785-62-5	9
			Pyrazole-3-carboxamide, N- (2-methoxyphenyl)-1-methyl-4-nitro	123815	292826-88-1	9
9.	40.412	1.92	9,12-Octodecadienoic acid (Z,Z)-	127649	000060-33-3	93
			9,12-Octadecadienoic acid, methylester, (E,E)-	139733	002566-97-4	87
			Methyl 9,12-heptadecadienoate	127645	1000336-36-2	83
10.	40.475	1.15	Ether, 3-hydroxy-2-butyl 1- (p-tolyl) ethyl-	67709	1000149-41-9	50
			Benzene, 1,4-dimethyl-2-(2-methylpropyl)-	32318	055669-88-0	47
			3-pyridinecarbonitrile, 1,4-dihydro-1-methyl	9354	019424-15-8	46
11.	40.624	1.23	3-Pyridinecarbonitrile, 1,4-dihydro-(4-Methylphenyl) methanol, neopentyl	5057	023974-91-6	58

			ether	54621	1000374-65-9	47
			(3-Methylphenyl) methanol, n-pentyl ether	54618	1000374-44-0	40
12.	41.049	2.24	Eicosanoic acid Eicosanoic acid n-Hexadecanoic acid	154909 154908 107549	000506-30-9 000506030-9 000057-10-3	97 96 94

4.4 Results of the preliminary assessment

4.4.1 *Citrus limon* juice on progressive body weight change and relative organ weights

A significant weight reduction was observed ($p < 0.05$) in 50% CLJ and 100% CLJ when compared with before CLJ administration (Figure 4.2). Also, a significant increase ($p < 0.05$) in the weight of ovary was obtained in 50% CLJ and 100% CLJ groups in comparison wirth control and a significant reduction ($p < 0.05$) in weight of liver in 75% CLJ group as compared with the control (Table 4.4).

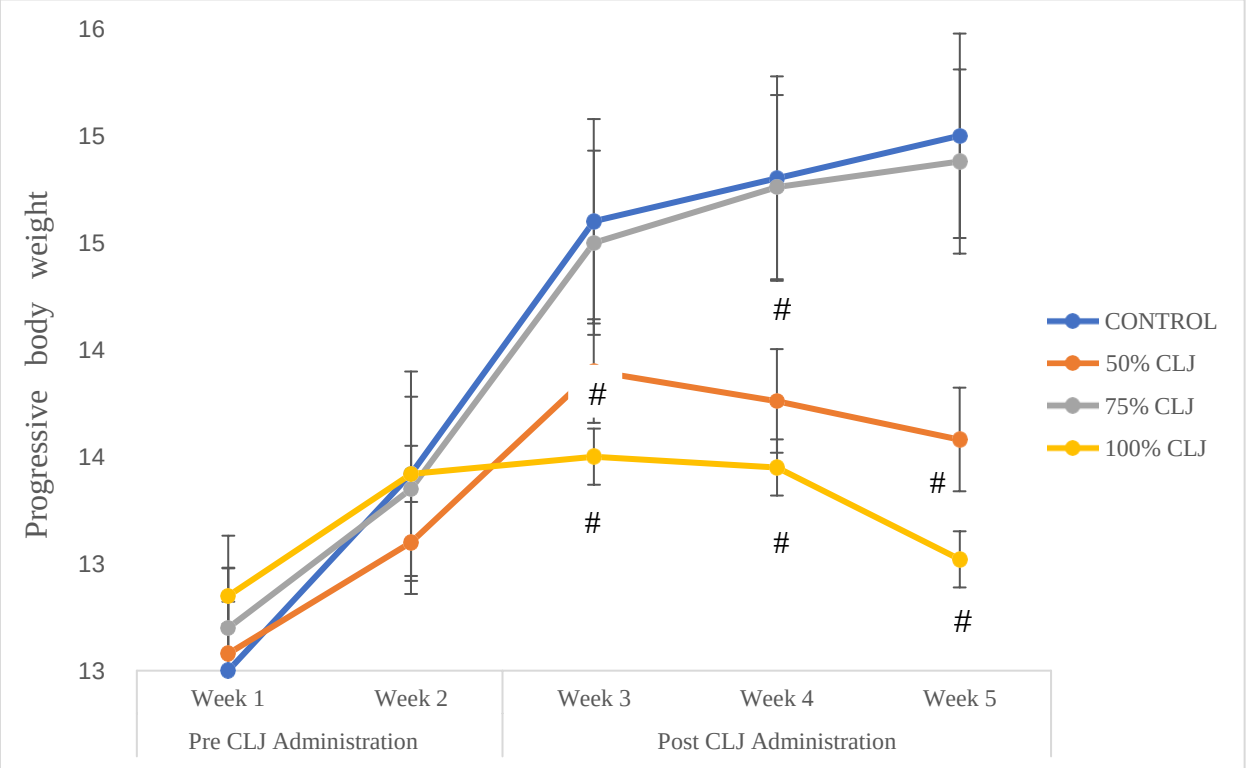


Figure 4.2. Effects of *Citrus limon* juice (CLJ) on mean progressive weight in non-pregnant female Wistar rats.

Data presented as mean± SEM, n=5, # = $p<0.05$ compared with pre-treatment period.

Table 4.4. Effects of *Citrus limon* juice (CLJ) on relative organ weights of non-pregnant female Wistar rats

GROUP	Ovary (%)	Uterus (%)	Liver (%)	Kidney (%)	Heart (%)
CONTROL	0.07±0.01	0.26±0.05	3.76±0.17	0.28±0.01	0.32±0.01
50% CLJ	0.10±0.01*	0.23±0.01	3.34±0.14	0.31±0.01	0.35±0.02
75% CLJ	0.09±0.00	0.17±0.01	3.23±0.02*	0.32±0.01	0.32±0.01
100% CLJ	0.11±0.01*	0.24±0.01	3.50±0.17	0.31±0.01	0.31±0.02

Data presented as mean± SEM, n=5, *p<0.05 compared with control

4.4.2. *Citrus limon* juice on oestrous cycle length and phase frequency

At post administration of CLJ, there was a significant reduction ($p < 0.05$) in the oestrus cycle length of 75% CLJ group when compared with pre CLJ administration (figure 4.3). *Citrus limon* juice also produced significant reduction in the proestrus and oestrus phase frequency across the groups when compared with pre CLJ administration (figure 4.4 and 4.5). Metestrus and diestrus frequency significantly increased ($p < 0.05$) in all the groups post administration of CLJ when compared with pre administration (figure 4.6 and 4.7).

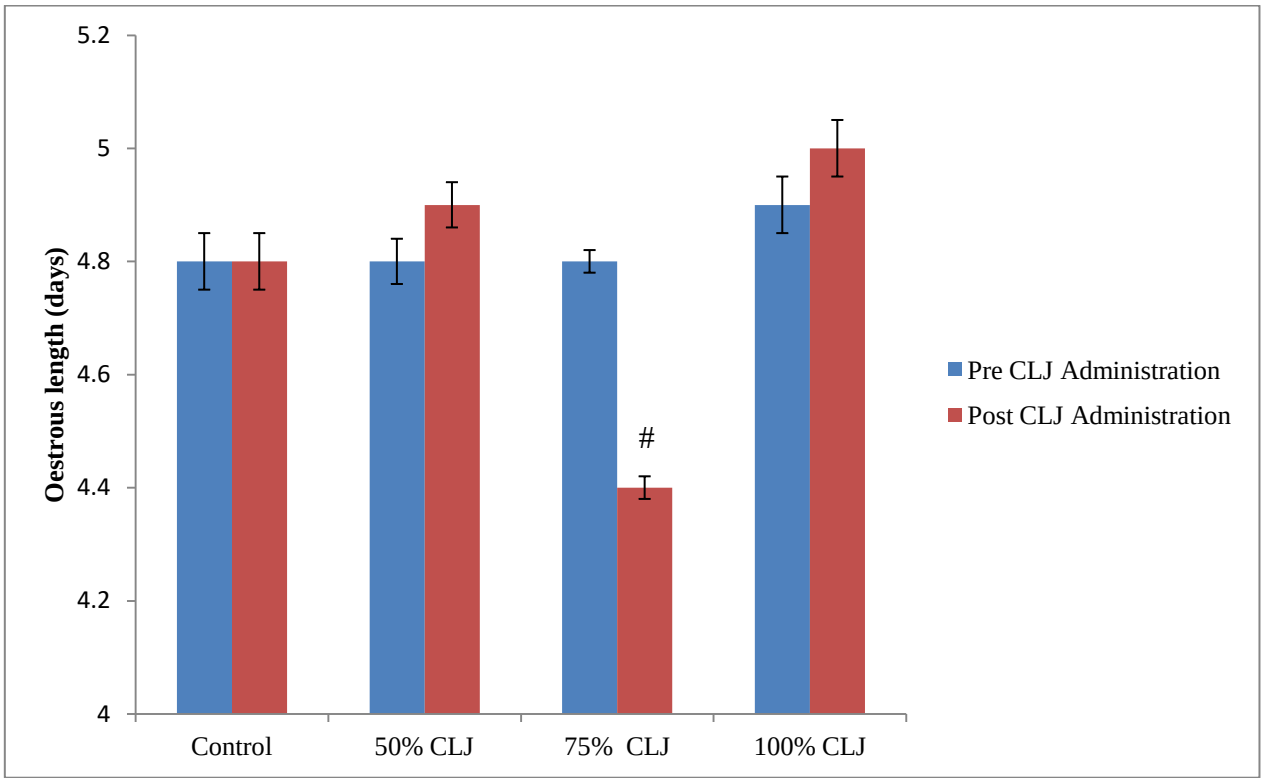


Figure 4.3. Effects of *Citrus limon* juice (CLJ) on oestrous cycle length in female Wistar rats. Data presented as mean± SEM, n=5, # = $p<0.05$ compared with pre administration of CLJ.

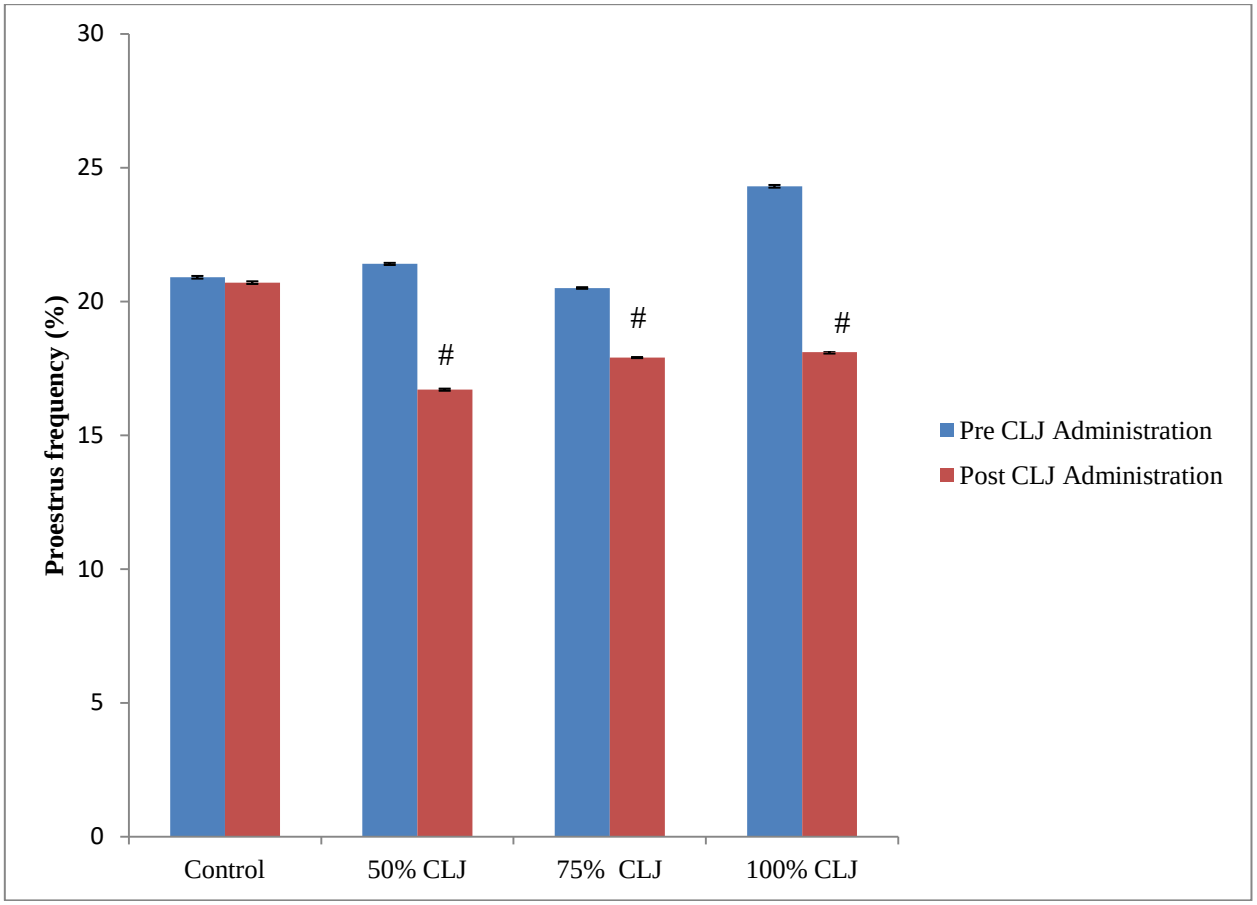


Figure 4.4. Effects of *Citrus limon* juice (CLJ) on frequency of the proestrus phase of the oestrous cycle in female Wistar rats.

Data presented as mean± SEM, n=5, # = $p<0.05$ compared with pre CLJ administration.

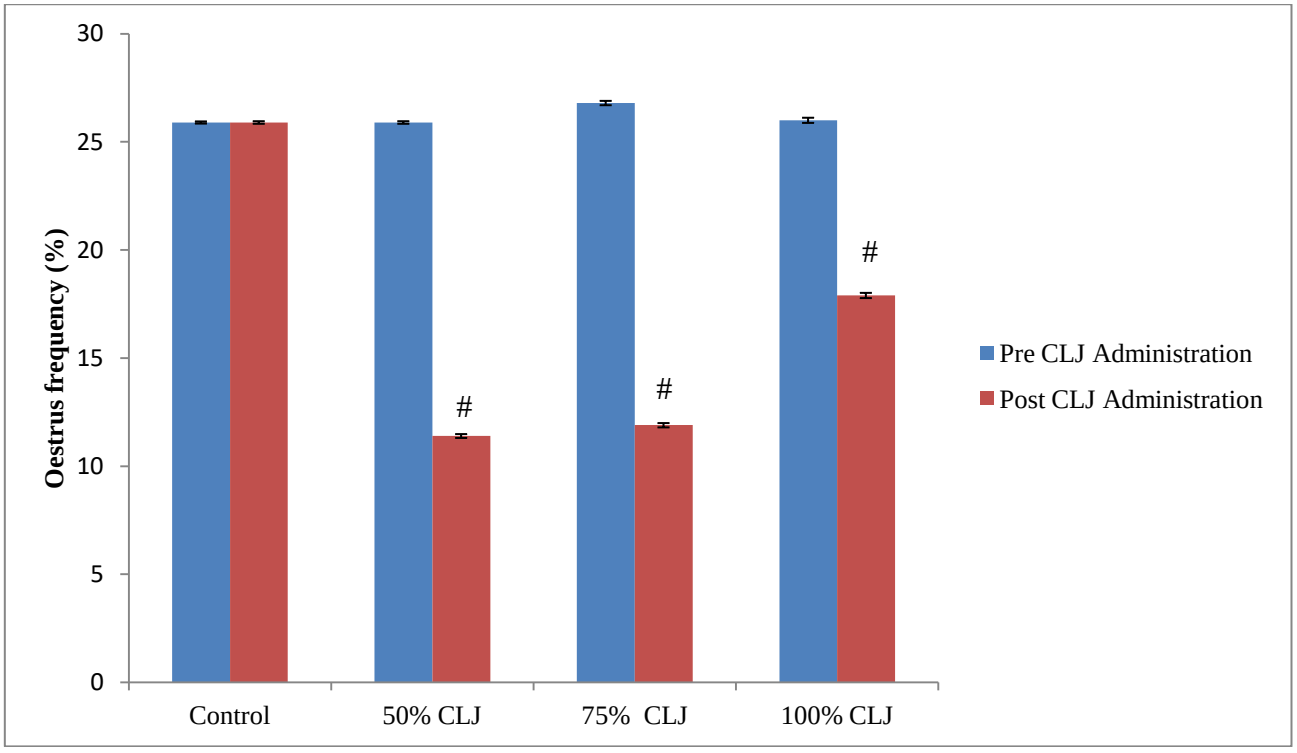


Figure 4.5. Effects of *Citrus limon* juice (CLJ) on frequency of the oestrus phase of the oestrous cycle in female Wistar rats.

Data presented as mean± SEM, n=5, # = $p<0.05$ compared with pre CLJ administration.

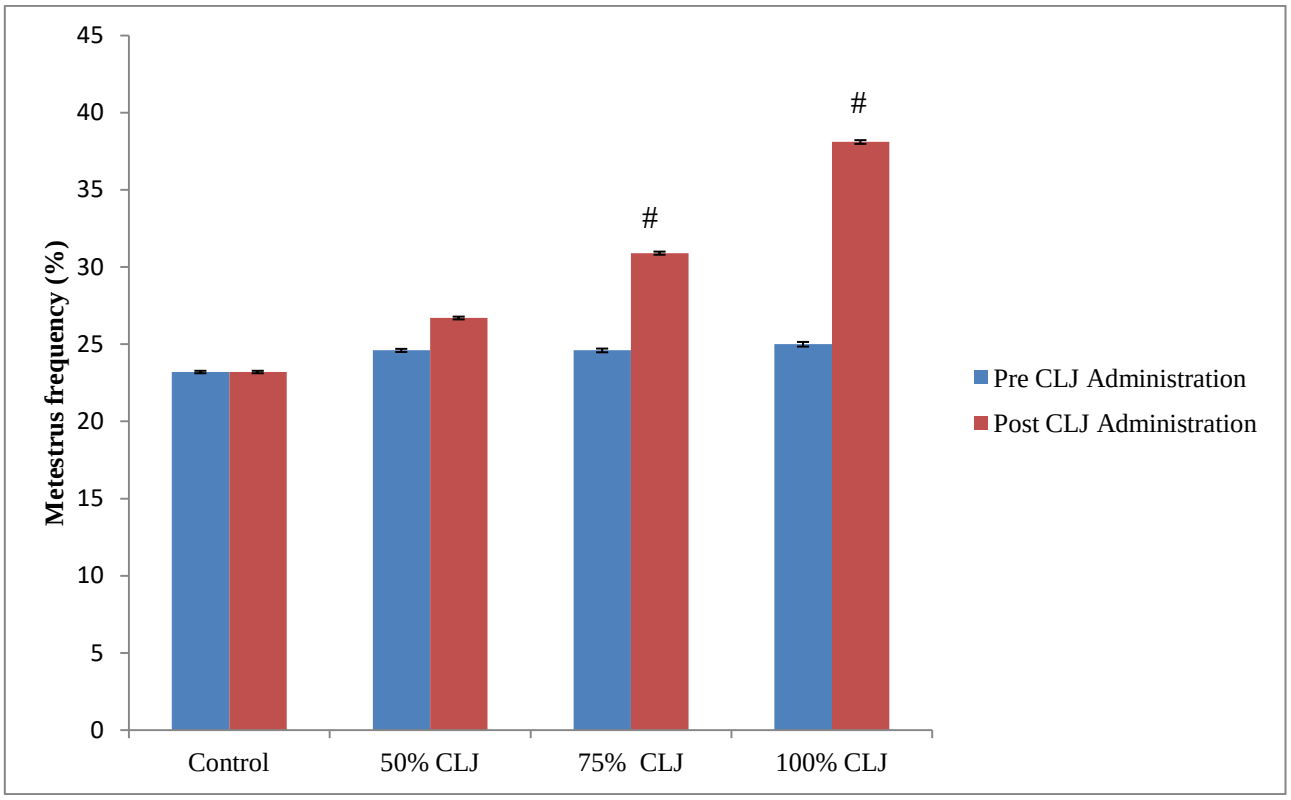


Figure 4.6. Effects of *Citrus limon* juice (CLJ) on frequency of the Metestrus phase of the oestrous cycle in female Wistar rats.

Data presented as mean± SEM, n=5, # = $p<0.05$ compared with pre CLJ administration.

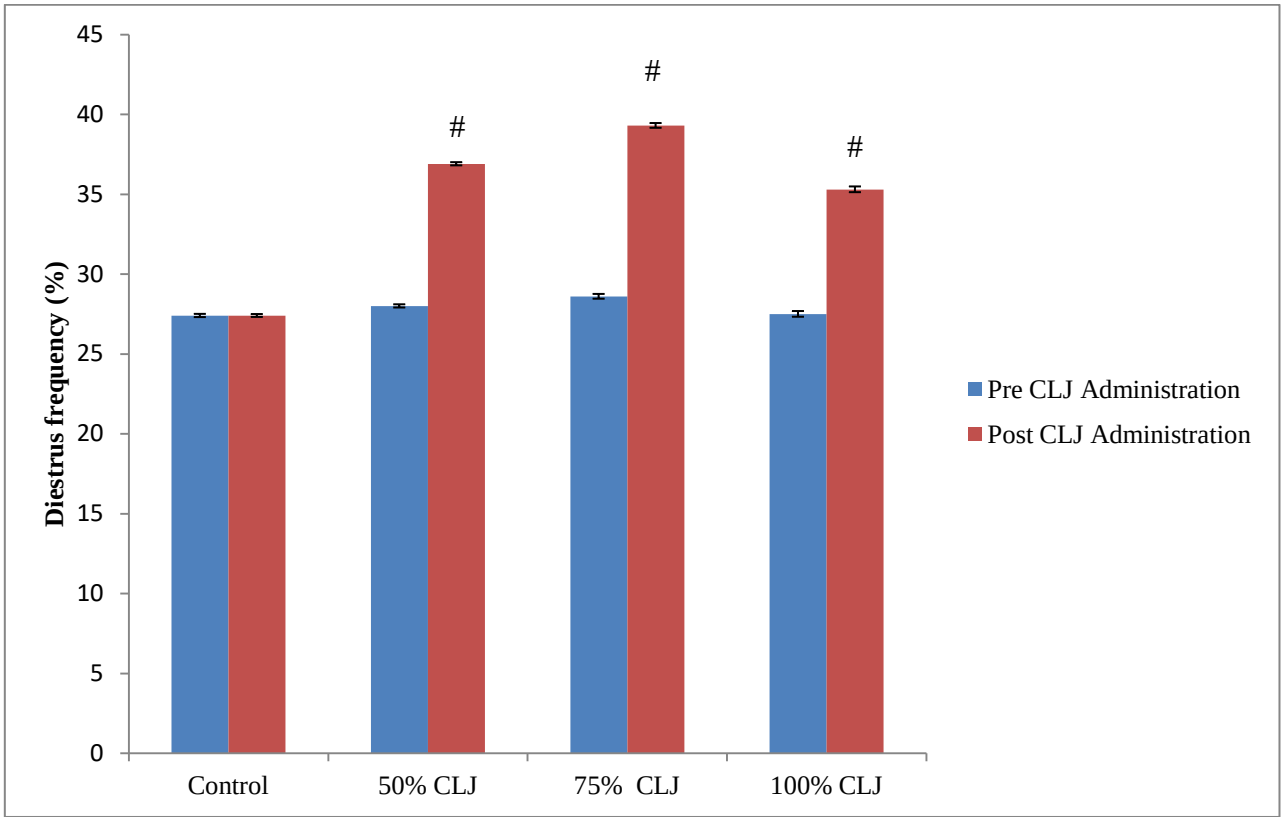


Figure 4.7. Effects of *Citrus limon* juice (CLJ) on frequency of the Diestrus phase of the oestrous cycle in female Wistar rats.

Data presented as mean± SEM, n=5, # = $p<0.05$ compared with pre CLJ administration.

4.4.3. *Citrus limon* juice on serum hormone conc. in non-pregnant female Wistar rats

Serum levels of luteinizing hormone increased significantly ($p < 0.05$) in 75% CLJ group in comparison with control. Oestradiol was significantly increased ($p < 0.05$) in 50% CLJ and 75% CLJ groups in comparison with control group (Table 4.5).

Table 4.5. Effects of *Citrus limon* juice (CLJ) on serum levels of luteinizing hormone, follicle stimulating hormone and oestradiol in non-pregnant female Wistar rats

GROUP	LH (mIU/mL)	FSH (mIU/mL)	Oestradiol (µg/mL)
CONTROL	2.70±0.02	2.28±0.01	20.24±0.01
50% CLJ	2.69±0.02	2.28±0.01	41.60±0.02*
75% CLJ	2.75±0.01*	2.29±0.02	34.68±0.02*
100% CLJ	2.69±0.02	2.28±0.01	26.33±0.01

Data presented as mean± SEM, n=5, *$p<0.05$ compared with control

4.4.4. *Citrus limon* juice on serum lipid profile in non-pregnant female Wistar rats

Total cholesterol ($p<0.05$) was increased significantly in 50% CLJ and 100% CLJ groups when compared with control (Table 4.6), however HDL-C reduced ($p<0.05$) in 75% CLJ and 100% CLJ groups as compared with control (Table 4.6). LDL-C and triglycerides reduced significantly ($p<0.05$) across the groups as compared with the control (Table 4.6).

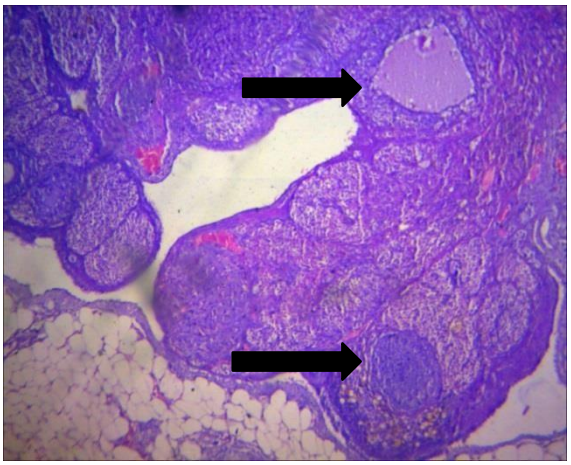
Table 4.6. Effects of *Citrus limon* juice (CLJ) on serum lipid profile in non-pregnant female Wistar rats

GROUP	TC (mg/dL)	HDL-C (mg/dL)	LDL-C (mg/dL)	TG (mg/dL)
CONTROL	95.55±0.05	43.09±0.01	224.32±0.02	326.44±0.02
50% CLJ	116.49±0.02*	39.41±0.01	71.62±0.01*	269.84±0.05*
75% CLJ	97.12±0.02	33.45±0.01*	60.58±0.01*	278.72±0.02*
100% CLJ	104.65±0.01*	35.05±0.01*	59.51±0.02*	290.49±0.02*

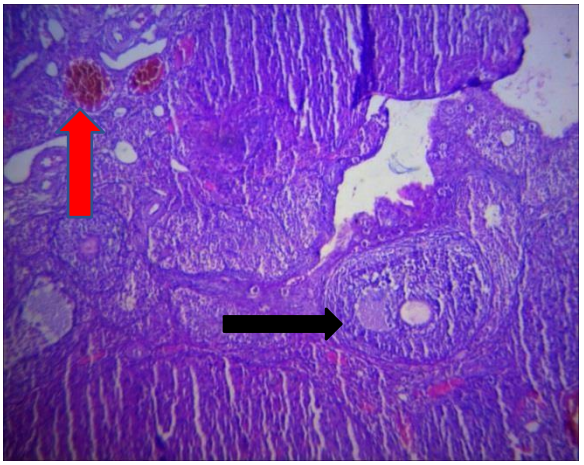
Data presented as mean± SEM, n=5, *p<0.05 compared with control. TC is Total Cholesterol; HDL-C is High Density Lipoprotein-Cholesterol; LDL-C is Low Density Lipoprotein-Cholesterol; TG is Triglycerides.

4.4.5. *Citrus limon* juice on histology of the ovary of non-pregnant female Wistar rats

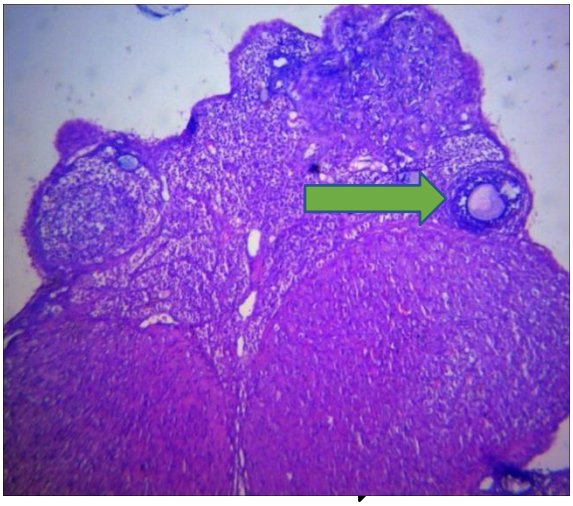
Control, 50% CLJ, 75% CLJ and 100% CLJ rats had normal ovarian stroma with follicles at different stages of maturation (plate 4.1). The ovarian section from the rats in 50% CLJ and 100% CLJ groups had mild congestion in the medulla (plate 4.1). There is no visible sign of lesion in all the groups.



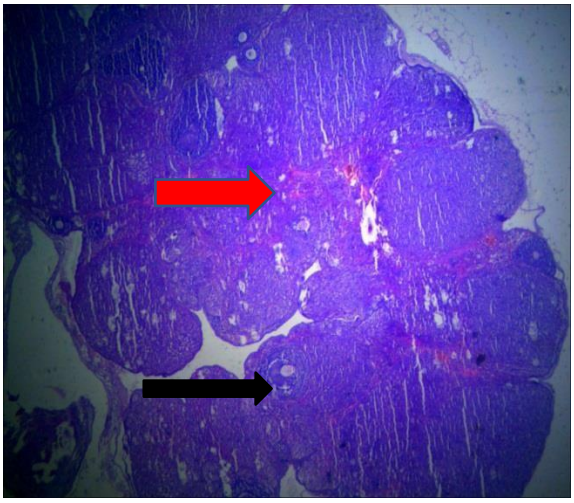
Control



50% CLJ



75% CLJ

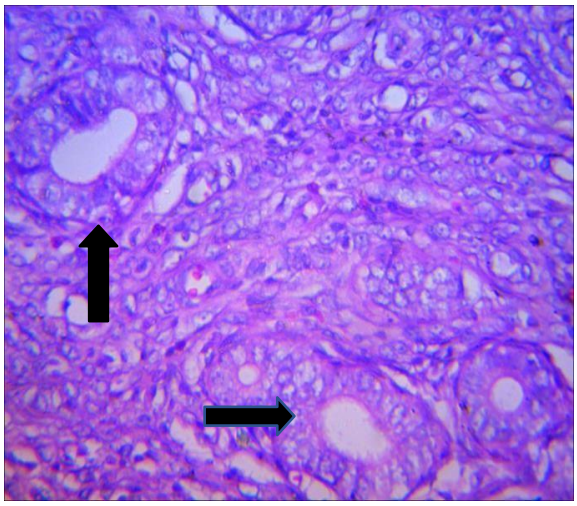


100% CLJ

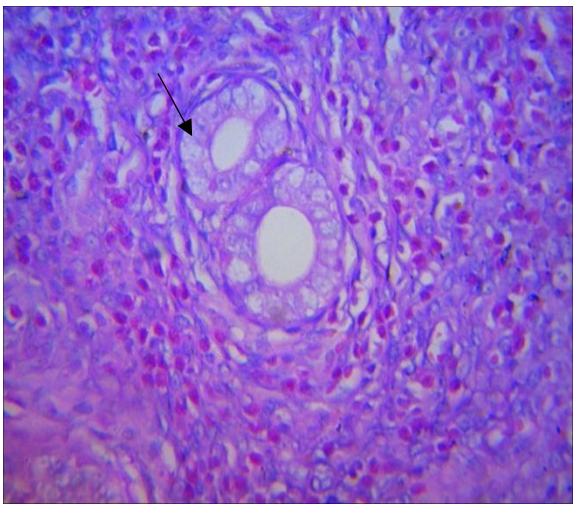
Plate 4.1: Photomicrographs of ovarian sections from control and *Citrus limon* juice treated non-pregnant rats showing the ovarian follicles at different stages of maturation (black arrow), mature follicle (green arrow) and congestion in the medulla (red arrow) (mag. X 100, H&E stain). Sections from the groups show no signs of lesion.

4.4.6. *Citrus limon* juice on histology of the uterus of non-pregnant female Wistar rats

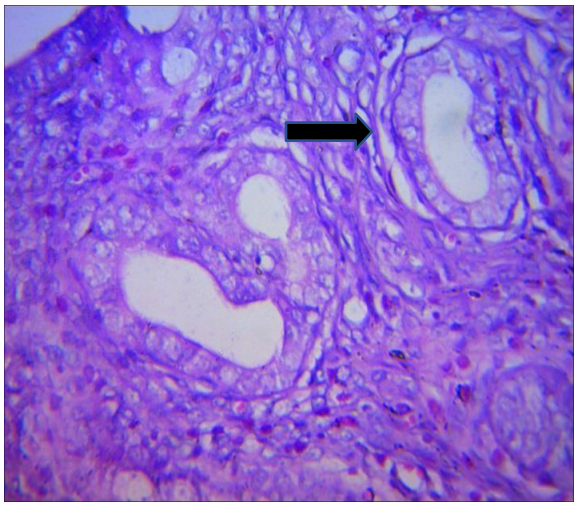
Rats in the control group had normal endometrium and uterine gland with no sign of inflammatory cells infiltration, surface epithelia cells was observed to be normal (plate 4.2). There is mild degeneration of glandular epithelia cells in 50% CLJ, 75% CLJ and 100% CLJ groups (plate 4.2).



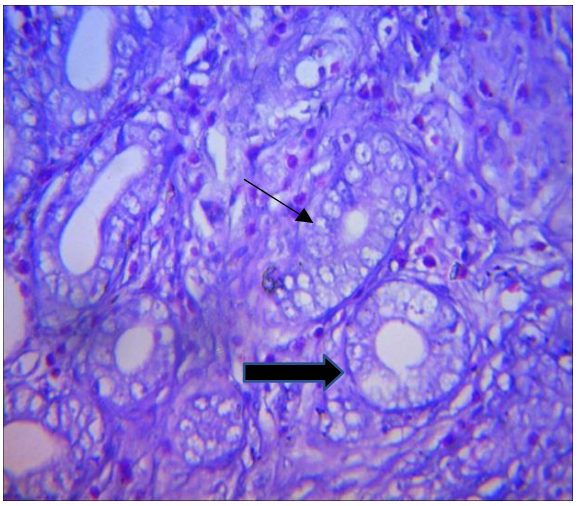
Control



50% CLJ



75% CLJ

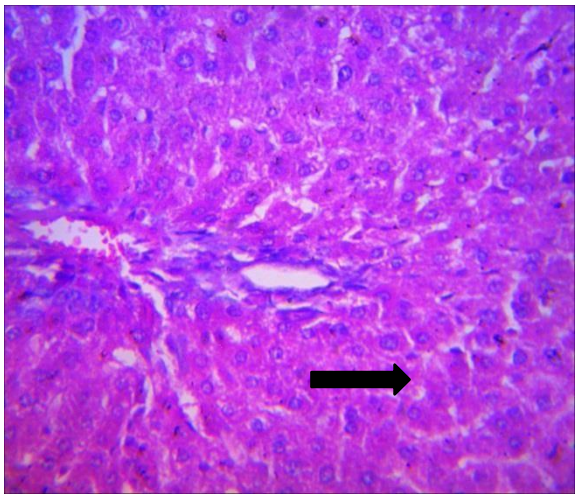


100% CLJ

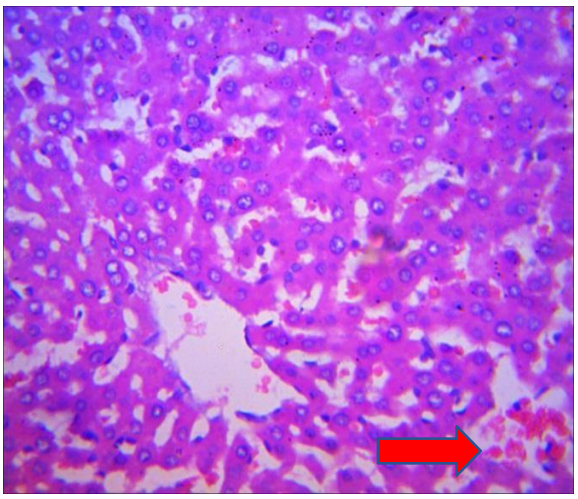
Plate 4.2: Photomicrographs of uterine sections from control and *Citrus limon* juice treated non- pregnant rats showing uterine glands (black arrow), degeneration glandular epithelia cells (slender arrow) (mag. X 400, H&E stain). Sections from the groups show no signs of lesion.

4.4.7. *Citrus limon* juice on histology of the liver of non-pregnant female Wistar rats

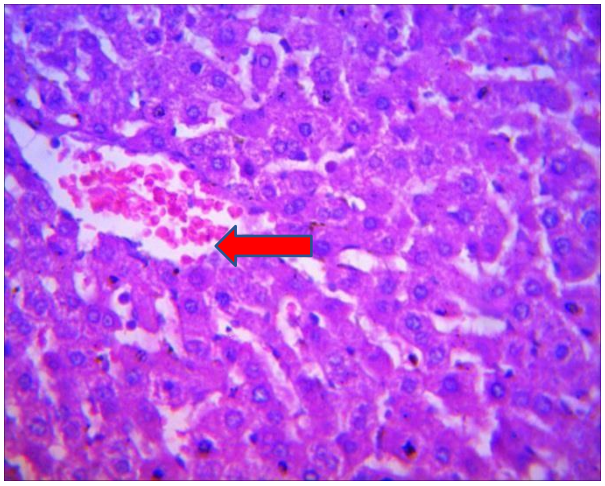
Rats in the control group had normal hepatocytes with no sign of infiltration of inflammatory cells (plate 4.3). Rats in 50% CLJ, 75% CLJ groups had mild diffuse vascular degeneration of the hepatocytes, while rats in 100% CLJ group had congestion of the portal vessels (plate 4.3).



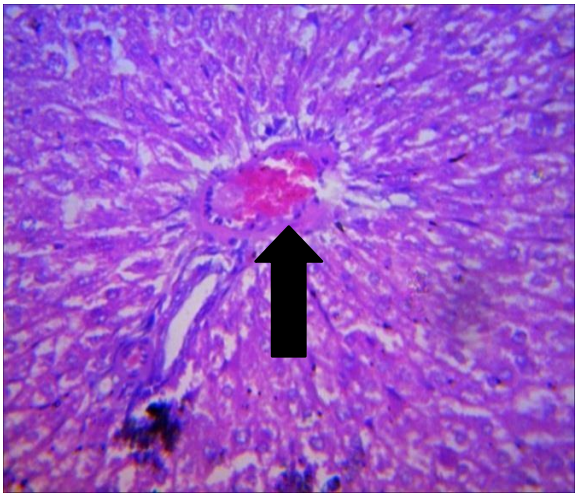
Control



50% CLJ



75% CLJ



100% CLJ

Plate 4.3: Photomicrographs of liver sections from control and *Citrus limon* juice treated non-pregnant rats showing hepatocytes (thin black arrow), mild diffuse vascular degeneration of the hepatocytes (thick black arrow) and congestion of the portal vessels (red arrow) (mag. X 400, H&E stain). Sections from the groups show no signs of lesion.

4.5. *Citrus limon* juice on maternal feed consumption in pregnant Wistar rat dams.

During the first and second week of administration, maternal feed consumption was reduced significantly ($p<0.05$) in *Citrus limon* juice (CLJ) and stearic acid (SA) groups when compared with control (Table 4.7). In week 3, the feed intake was reduced significantly ($p<0.05$) in stearic acid (SA) group in comparison with palmitic acid (PA) group (Table 4.7).

Table 4.7. Effects of *Citrus limon* juice (CLJ) and its major components on maternal feed consumption in pregnant Wistar rat dams.

GROUP	CONTROL	CLJ	OA	PA	SA
WEEK 1	81.57±1.53	63.14±1.10*	73.43±3.83	87.00±1.39	58.00±3.11*,c
WEEK 2	82.29±2.11	67.71±1.07*	73.71±1.82	83.71±1.95	58.43±3.64*,a,c
WEEK 3	81.83±1.45	80.33±1.06	84.83±1.73	89.67±1.22	71.00±1.73c

Data presented as mean± SEM, n=5, *p<0.05 in comparison to control, a=p<0.05 in comparison to CLJ, c=p<0.05 in comparison to PA. CLJ=*Citrus limon* juice; OA=Oleic Acid; PA=Palmitic Acid; SA=Stearic Acid.

4.6. *Citrus limon* juice on fasting blood glucose level in pregnant Wistar rat dams

Maternal blood glucose level significantly increased ($p<0.05$) in stearic acid (SA) group as compared to the control group (Figure 4.8).

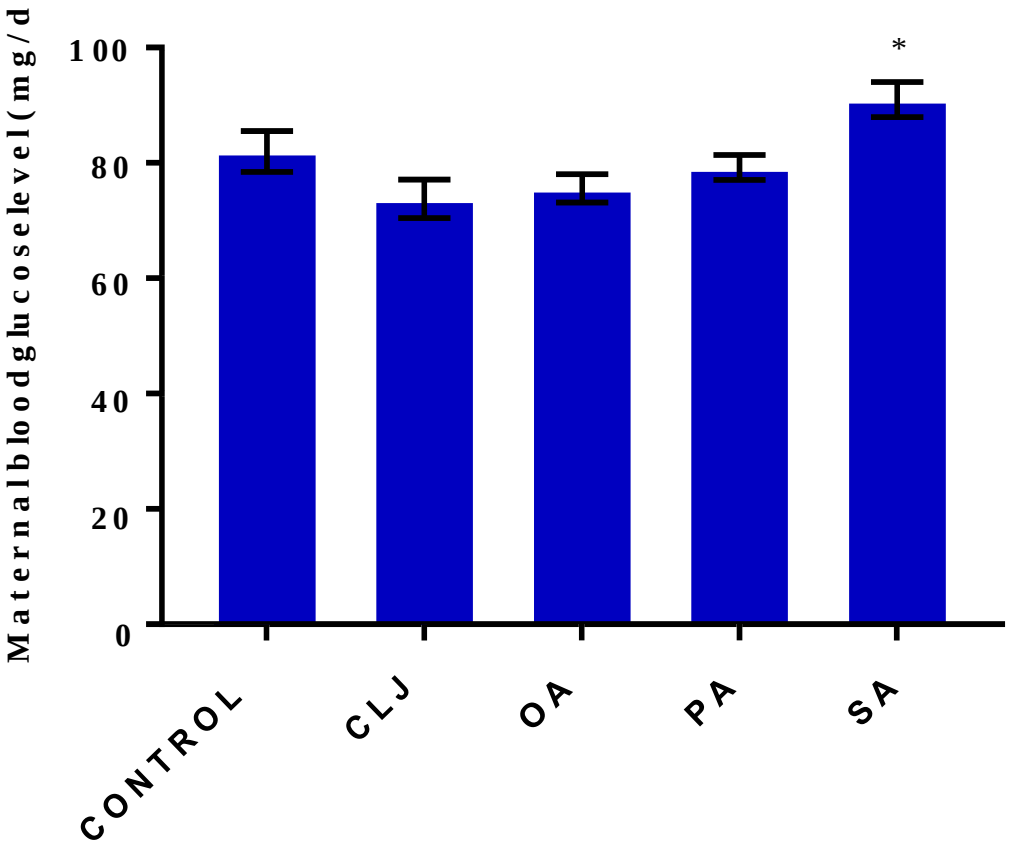


Figure 4.8. Effects of *Citrus limon* juice (CLJ) and its components on maternal blood glucose level in pregnant Wistar rat dams.

Data presented as mean± SEM, n=5, *p<0.05 compared with control. CLJ=*Citrus limon* juice; OA=oleic acid; PA=palmitic acid; SA=stearic acid.

4.7. *Citrus limon* juice on serum hormone levels in pregnant Wistar rat dam

Progesterone concentration was significantly reduced ($p<0.05$) in *Citrus limon* juice (CLJ) and palmitic acid (PA) groups when compared with control and increased in oleic acid (OA) and stearic acid (SA) groups as compared to CLJ and PA groups (Table 4.8). Oestrogen concentration was significantly reduced in palmitic acid (PA) group as compared with control.

Table 4.8. Effects of *Citrus limon* juice (CLJ) and its components on serum concentration of progesterone, oestrogen, leptin, chorionic gonadotropin and insulin in pregnant Wistar rat dams.

GROUP	Progesterone (ng/mL)	Leptin (ng/mL)	Oestrogen (ng/mL)	Chorionic gonadotropin (mIU/mL)	Insulin (mIU/mL)
CONTROL	114.57±7.57	4.10±0.41	3.66±0.38	3.55±0.43	1.13±0.09
CLJ	94.14±4.21*	4.65±0.14	3.16±1.05	4.13±1.06	1.13±0.05
OA	112.32±7.28[a,c]	3.99±0.16	2.03±0.91	2.82±0.89	1.08±0.03
PA	92.12±9.59*	4.39±0.16	1.81±1.07*	2.85±1.06	1.10±0.04
SA	112.91±3.31[a,c]	4.66±0.10	2.77±0.98	3.53±0.98	1.04±0.06

Data presented as mean± SEM, n=5, *=p<0.05 compared with control, a=p<0.05 compared with CLJ, c=p<0.05 compared with PA. CLJ=*Citrus limon* juice; OA=oleic acid; PA=palmitic acid; SA=stearic acid.

4.8. Effects of *Citrus limon* juice on lipid profile in pregnant Wistar rat dams

Total cholesterol was significantly increased ($p<0.05$) in *Citrus limon* juice (CLJ), oleic acid (OA) and stearic acid (SA) groups as compared to control. LDL-C was reduced significantly ($p<0.05$) across groups in comparison with control (Figure 4.9). HDL-C was reduced significantly ($p<0.05$) in CLJ, OA, and SA groups in comparison with control (Figure 4.9). Triglyceride was reduced significantly ($p<0.05$) in CLJ group in comparison with control and in OA group as compared with CLJ group (Figure 4.9).

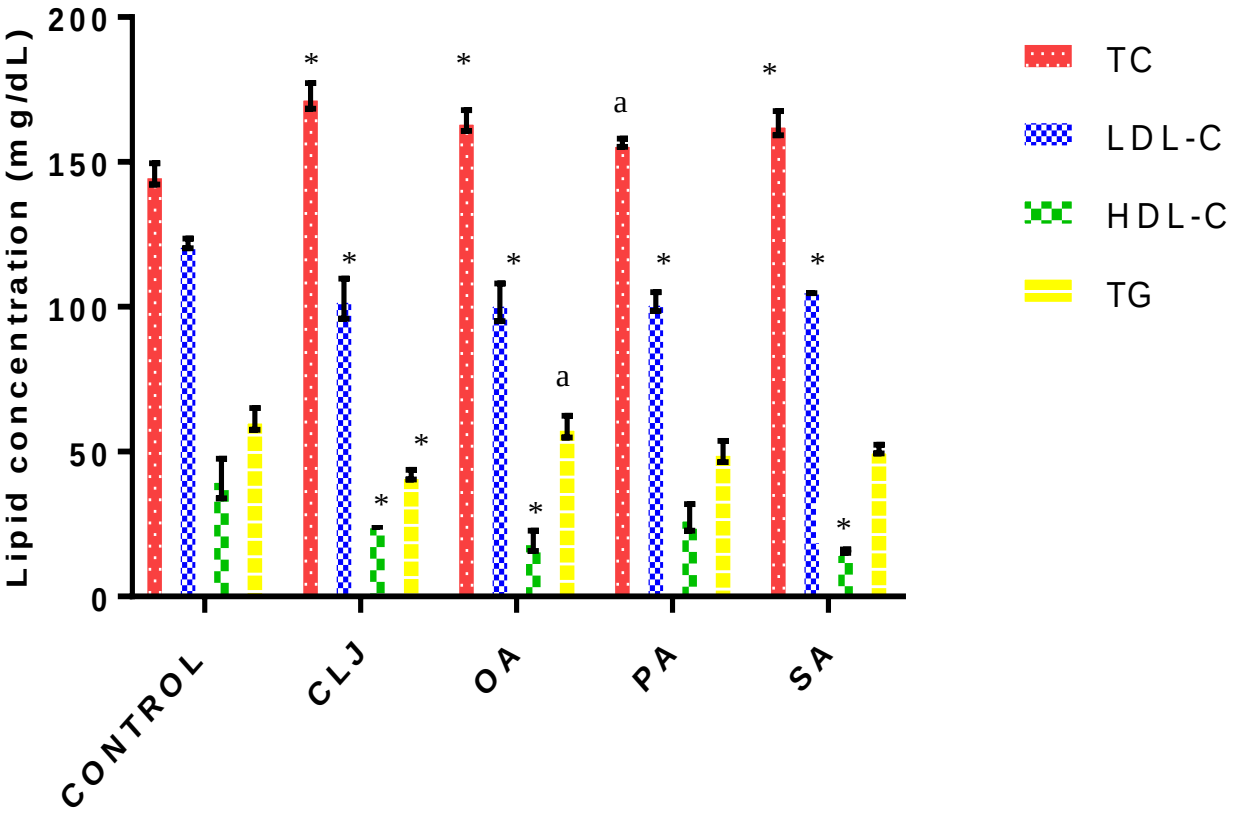


Figure 4.9. Effects of *Citrus limon* juice (CLJ) and its components on serum Total Cholesterol (TC), Low Density Lipoprotein- Cholesterol (LDL-C), High Density Lipoprotein-Cholesterol (HDL-C) and triglycerides in pregnant Wistar rat dams.

Data presented as mean± SEM, n=5, *p<0.05 compared with control, a=p<0.05 compared with CLJ. CLJ=*Citrus limon* juice; OA=oleic acid; PA=palmitic acid; SA=stearic acid.

4.9. *Citrus limon* juice and its major components on oxidative stress markers in pregnant Wistar rat dams

4.9.1. *Citrus limon* juice on malondialdehyde concentration

Malondialdehyde level was reduced ($p<0.05$) across groups in comparison with the control (figure 4.10). It was further reduced ($p<0.05$) in palmitic acid and stearic acid groups as compared to *Citrus limon* juice group (figure 4.10).

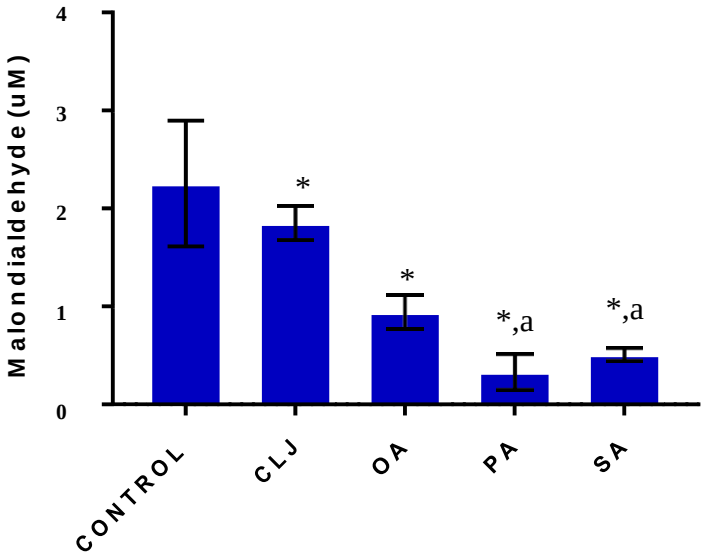


Figure 4.10. Effects of *Citrus limon* juice (CLJ) and its components on serum malondialdehyde level in pregnant Wistar rat dams.

Data presented as mean± SEM, n=5, *p<0.05 compared with control, a=p<0.05 compared with CLJ. CLJ=*Citrus limon* juice; OA=oleic acid; PA=palmitic acid; SA=stearic acid.

4.9.2. Effects of *Citrus limon* juice and its major components on maternal redox status

Superoxide dismutase level reduced ($p<0.05$) in oleic acid (OA) and palmitic acid (PA) groups as compared with control (Table 4.9), however it was increased ($p<0.05$) in stearic acid (SA) group as compared with oleic acid (OA) and palmitic acid (PA) groups (Table 4.9). Catalase enzyme was reduced ($p<0.05$) in oleic acid (OA) group in comparison with control (Table 4.9), its level was increased ($p<0.05$) in stearic acid (SA) group in comparison with oleic acid (OA) group (Table 4.9). Glutathione peroxidase level was increased ($p<0.05$) across the groups when compared with control (Table 4.9). Total antioxidant capacity increased ($p<0.05$) in palmitic acid (PA) rats in comparison with control rats (Table 4.9). *Citrus limon* juice and its major components administration does not affect total protein concentration across the groups as compared with control (Table 4.9).

Table 4.9. Effects of *Citrus limon* juice (CLJ) and its components on serum concentration of antioxidant enzymes, total antioxidant capacity and total protein in pregnant Wistar rat dams.

GROUP	SOD (U/mL)	CAT (Umol/mL/min)	GPX (U/L)	TAC (m/M)	TP (g/dL)
CONTROL	1.23±0.18	8.62±0.68	66.20±6.76	1.35±0.37	5.02±0.19
CLJ	1.11±0.08	7.94±0.69	89.32±10.41*	1.29±0.26	5.40±0.19
OA	0.71±0.09*	5.76±0.50*	80.69±6.25*	1.33±0.08	4.78±0.16
PA	0.79±0.05*	6.95±0.56	80.71±4.90*	1.93±0.18*	5.31±0.11
SA	1.35±0.11^{b,c}	8.65±0.32^{b}	82.19±5.27*	1.57±0.14	4.86±0.12

Data presented as mean± SEM, n=5, *=p<0.05 compared with control, b=p<0.05 compared with OA, c=p<0.05 compared with PA. CLJ=*Citrus limon* juice; OA=Oleic Acid; PA=Palmitic Acid; SA=Stearic Acid. SOD=Superoxide Dismutase. CAT=Catalase. GPX=Glutathione peroxidase. TAC=Total Antioxidant Capacity. TP=Total Protein.

4.10. Effects of maternal *Citrus limon* juice on male placental lipase concentration

Placental lipase concentration was reduced ($p<0.05$) in *Citrus limon* juice (CLJ) group in comparison with control, however, it was increased ($p<0.05$) in oleic acid (OA) group as compared with *Citrus limon* juice (CLJ) group (Figure 4.11). It was also increased ($p<0.05$) in stearic acid (SA) group in comparison with control, *Citrus limon* juice (CLJ), oleic acid (OA) and palmitic acid (PA) groups (Figure 4.11).

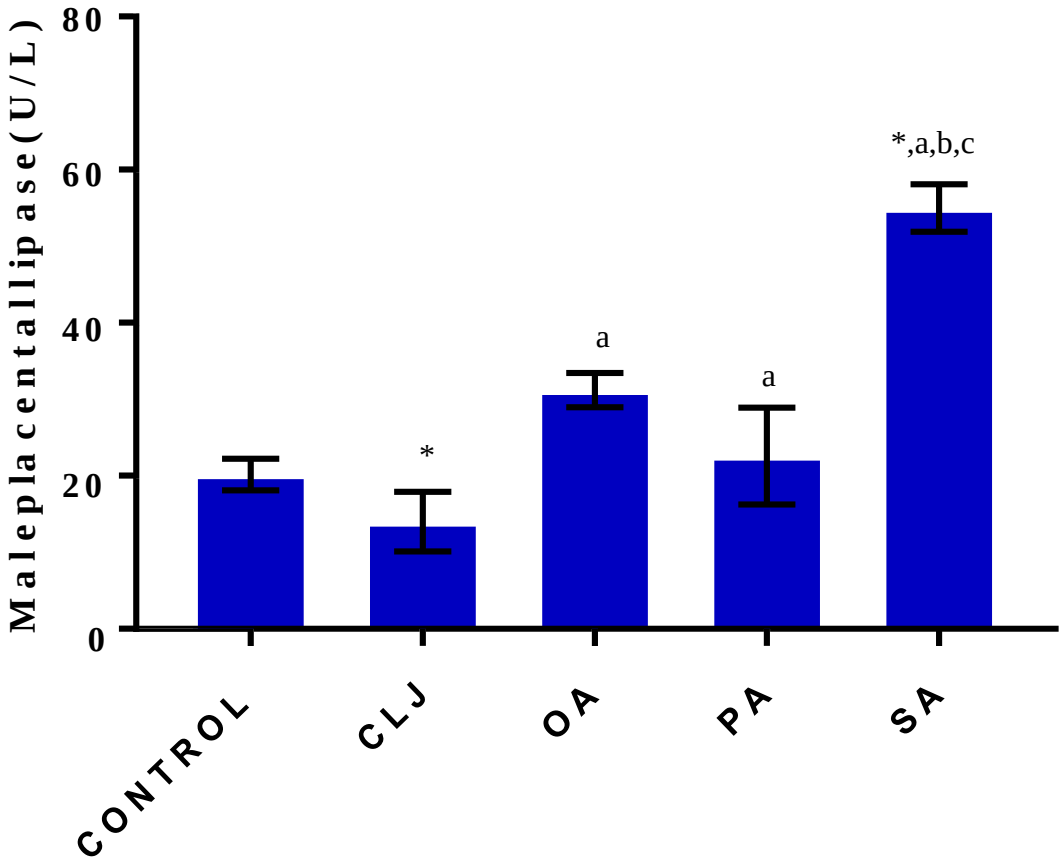


Figure 4.11. Effects of *Citrus limon* juice (CLJ) and its components on male placental lipase concentration in Wistar rats.

Data presented as mean± SEM, n=5, *p<0.05 in comparison to control, a=p<0.05 compared with CLJ, b=p<0.05 compared with OA, c=p<0.05 compared with PA. CLJ=*Citrus limon* juice; OA=oleic acid; PA=palmitic acid; SA=stearic acid.

4.11. Effects of maternal *Citrus limon* juice on male placental lactogen concentration

Placental lactogen was reduced ($p<0.05$) in *Citrus limon* juice (CLJ), Oleic Acid (OA), Palmitic Acid (PA) and Stearic Acid (SA) groups in comparison with control (Figure 4.12).

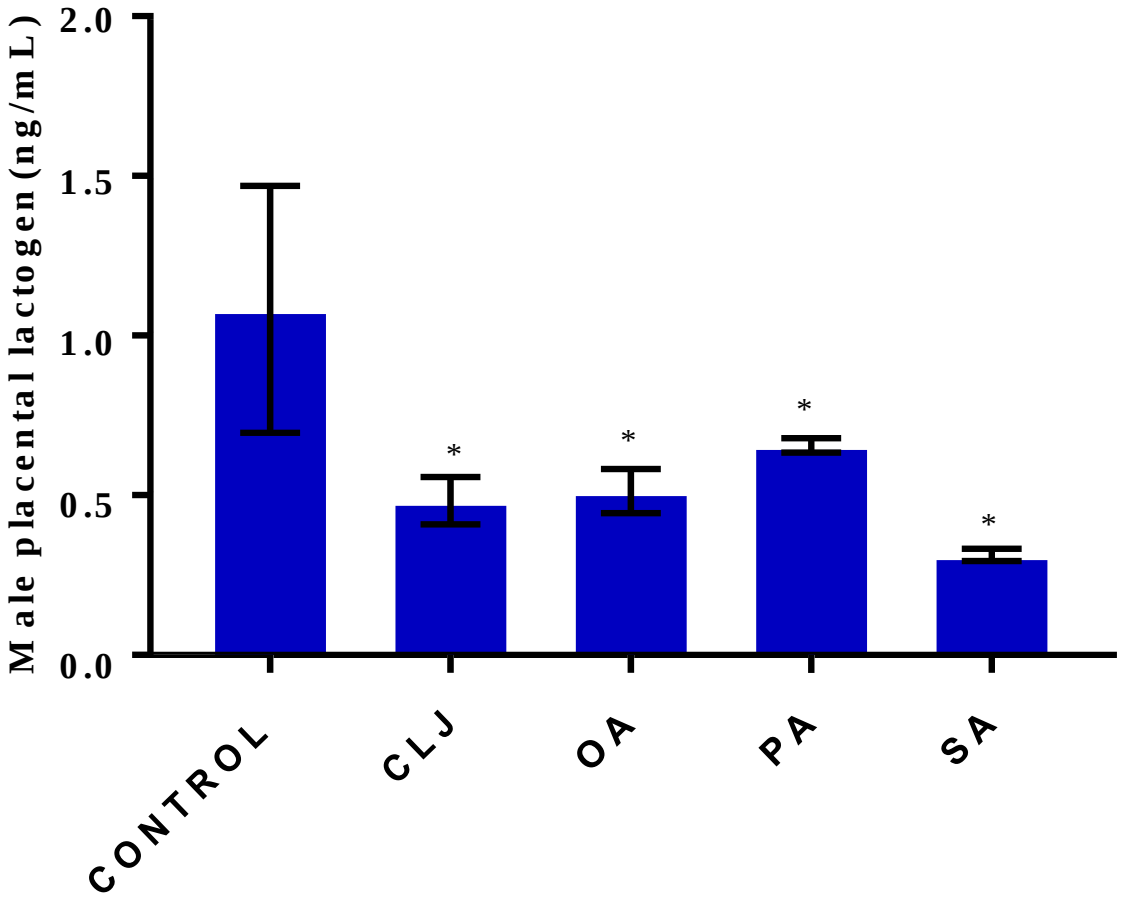


Figure 4.12. Effects of *Citrus limon* juice (CLJ) and its components on male placental lactogen level in Wistar rats.

Data presented as mean± SEM, n=5, *p<0.05 compared with control. CLJ=*Citrus limon* juice; OA=oleic acid; PA=palmitic acid; SA=stearic acid.

4.12. Effects of maternal *Citrus limon* juice on male placental GLUT-1 concentration

Placental GLUT-1 was reduced ($p<0.05$) in *Citrus limon* juice (CLJ), oleic acid (OA) and stearic acid (SA) groups in comparison with control but it was increased ($p<0.05$) in palmitic acid (PA) group as compared with control, *Citrus limon* juice (CLJ) and oleic acid (OA) groups (Figure 4.13).

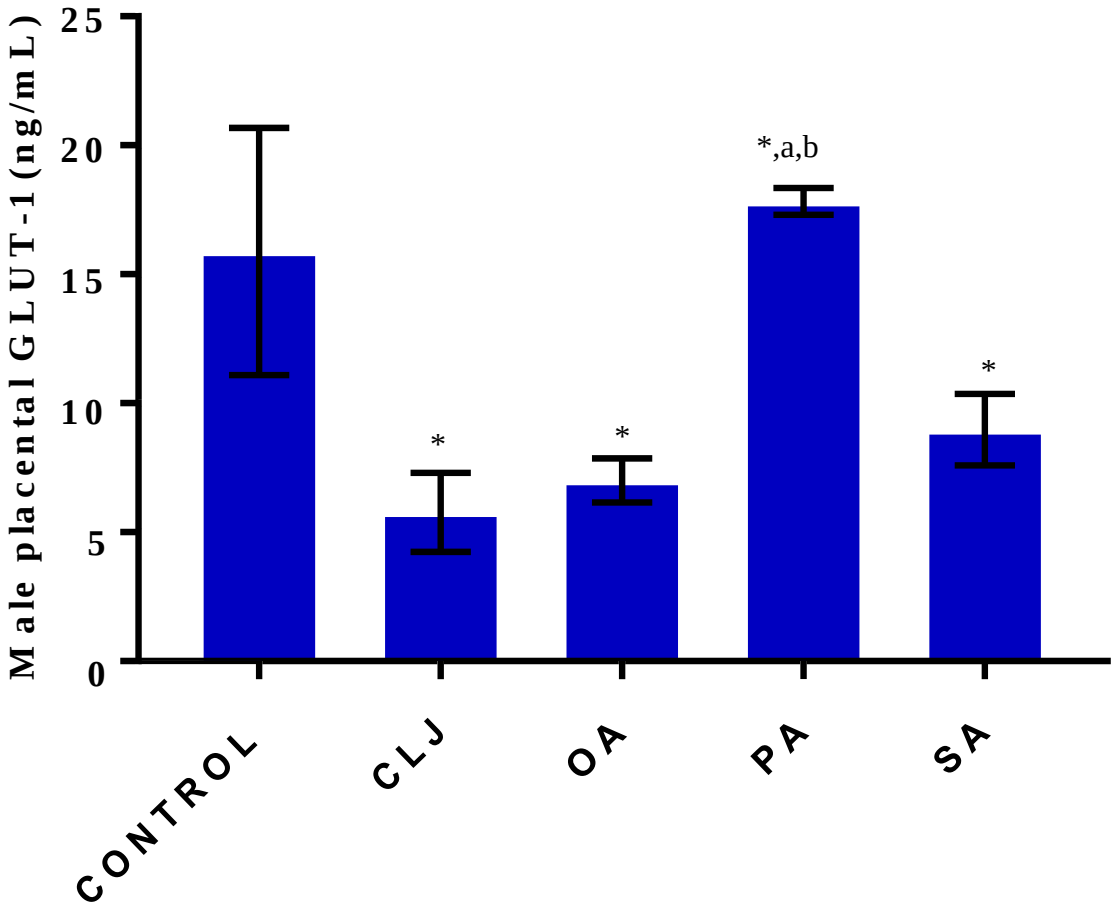


Figure 4.13. Effects of *Citrus limon* juice (CLJ) and its components on male placental GLUT- 1 concentration in Wistar rats.

Data presented as mean± SEM, n=5, *p<0.05 compared with control, a=p<0.05 compared with CLJ, b=p<0.05 compared with OA. CLJ=*Citrus limon* juice; OA=oleic acid; PA=palmitic acid; SA=stearic acid.

4.13. Effects of maternal *Citrus limon* juice on male placental FATP-1 concentration

Fatty Acid Transport Protein-1 (FATP-1) in the placenta was increased ($p<0.05$) in oleic acid (OA) group in comparison with control and *Citrus limon* juice (CLJ) groups (Figure 4.14), increased in palmitic acid (PA) group in comparison with control and CLJ group (Figure 4.14) and in stearic acid (SA) group in comparison with control, CLJ, OA and PA groups (Figure 4.18). It was however reduced in CLJ group as compared with control group (figure 4.14).

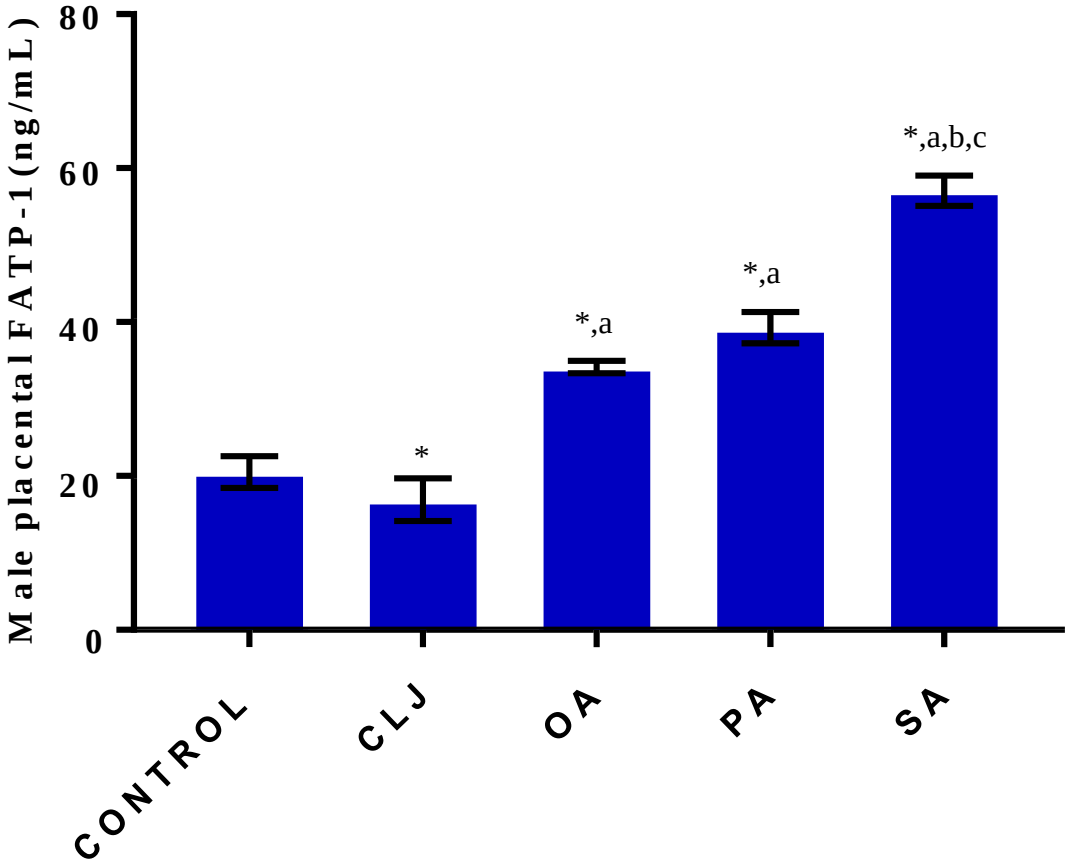


Figure 4.14. Effects of *Citrus limon* juice (CLJ) and its components on male placental FATP- 1 concentration in Wistar rats.

Data presented as mean± SEM, n=5, *p<0.05 compared with control, a=p<0.05 compared with CLJ, b=p<0.05 compared with OA, c=p<0.05 compared with PA. CLJ=*Citrus limon* juice; OA=Oleic Acid; PA=Palmitic Acid; SA=Stearic Acid.

4.14. Effects of maternal *Citrus limon* juice on male placental SNAAT-1 concentration

Placental Small Neutral Amino Acid Transporter-1 (SNAAT-1) was reduced ($p<0.05$) in *Citrus limon* juice (CLJ), oleic acid (OA) groups as compared with control (Figure 4.15). It was increased ($p<0.05$) in palmitic acid (PA) group in comparison with other groups (Figure 4.15).

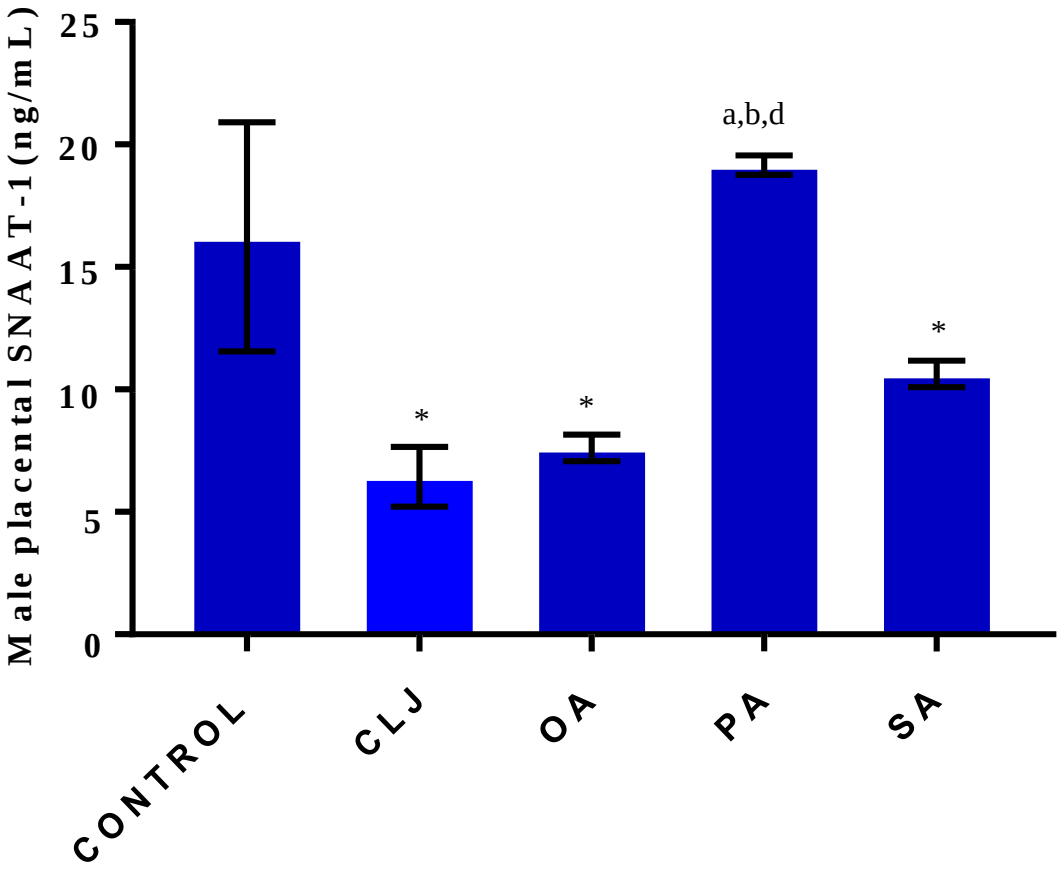


Figure 4.15. Effects of *Citrus limon* juice (CLJ) and its components on male placental SNAAT-1 concentration in Wistar rats.

Data presented as mean± SEM, n=5, *p<0.05 compared with control, a=p<0.05 compared with CLJ, b=p<0.05 compared with OA, c=p<0.05 compared with PA. CLJ=*Citrus limon* juice; OA=Oleic Acid; PA=Palmitic Acid; SA=Stearic Acid.

4.15. Effects of maternal *Citrus limon* juice on male placental lipid concentration

Male placental total cholesterol was reduced ($p<0.05$) across groups in comparison with the control and increased ($p<0.05$) in oleic acid (OA) group as compared with *Citrus limon* juice (CLJ), palmitic acid (PA) and stearic acid (SA) groups (Table 4.10). Male placental low density lipoprotein was reduced ($p<0.05$) across the groups in comparison with control and increased ($p<0.05$) in oleic acid (OA) group as compared with *Citrus limon* juice (CLJ), palmitic acid (PA) and stearic acid (SA) groups (Table 4.10). Male placental high density lipoprotein was increased ($p<0.05$) in stearic acid (SA) group in comparison with control, *Citrus limon* juice (CLJ), oleic acid (OA) and palmitic acid (PA) groups (Table 4.10). Male placental triglycerides was reduced ($p<0.05$) in *Citrus limon* juice (CLJ), oleic acid (OA) and stearic acid (SA) groups as compared with control (Table 4.10), but it was increased ($p<0.05$) in palmitic acid (PA) group in comparison with control, *Citrus limon* juice (CLJ) and oleic acid (OA) groups (Table 4.10).

Table 4.10. Effects of maternal *Citrus limon* juice (CLJ) and its major components on male placental lipid concentration in Wistar rats.

GROUPS	TC (mg/dL)	LDL-C (mg/dL)	HDL-C (mg/dL)	TG (mg/dL)
CONTROL	253.92±0.10	239.29±0.10	2.20±0.22	62.15±2.66
CLJ	162.90±0.09*	151.86±0.09*	0.98±0.29	50.26±3.36*
OA	196.04±0.08*,a	186.67±0.08*,a	0.24±0.05	46.45±0.93*
PA	137.23±0.12*,a,b	117.44±0.12*,a,b	0.38±0.17	84.05±3.99*,a,b
SA	187.17±0.10*,a,b,c	166.57±0.10*,a,b,c	10.51±0.37*,a,b,c	50.49±1.69*,c

Data presented as mean± SEM, n=5, *p<0.05 compared with control, a=p<0.05 compared with CLJ, b=p<0.05 compared with OA, c=p<0.05 compared with PA. CLJ=*Citrus limon* juice; OA=oleic acid; PA=palmitic acid; SA=stearic acid. TC=Total Cholesterol. LDL-C=Low Density Lipoprotein-Cholesterol. HDL-C=High Density Lipoprotein-Cholesterol. TG=Triglyceride.

4.16. Maternal *Citrus limon* juice on male placental oxidative stress markers in Wistar rats

4.16.1. Maternal *Citrus limon* juice on male placental malondialdehyde

Male placental malondialdehyde was increased ($p<0.05$) in *Citrus limon* juice (CLJ) group as compared with control (Figure 4.16), it was reduced ($p<0.05$) in oleic acid (OA) and stearic acid (SA) groups as compared with CLJ group (Figure 4.16), but was increased ($p<0.05$) in palmitic acid (PA) group in comparison with oleic acid (OA) group (Figure 4.16).

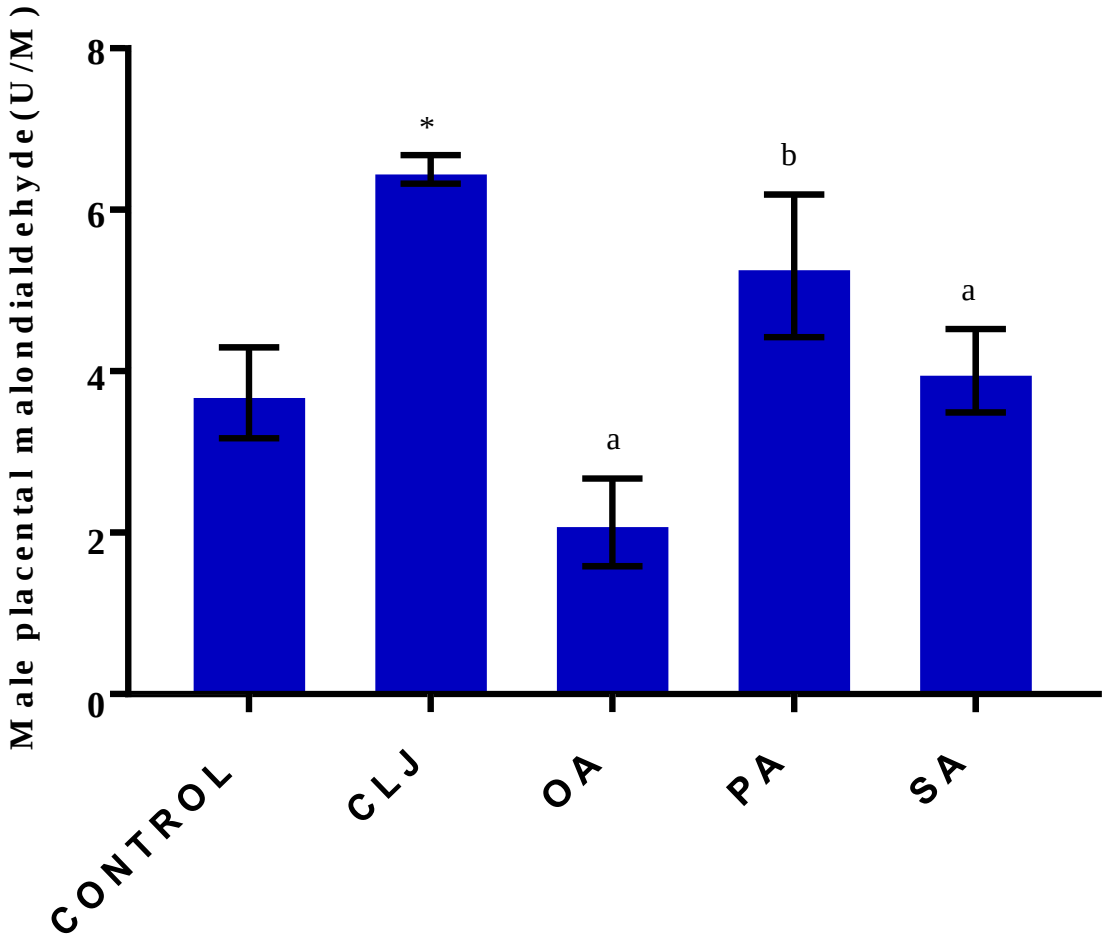


Figure 4.16. Effects of *Citrus limon* juice (CLJ) and its major components on male placental malondialdehyde concentration in Wistar rats.

Data presented as mean± SEM, n=5. CLJ=*Citrus limon* juice; OA=Oleic Acid; PA=Palmitic Acid; SA=Stearic Acid.

4.16.2. Effects of maternal *Citrus limon* juice on male placental redox status

Superoxide dismutase was increased ($p<0.05$) in *Citrus limon* juice (CLJ) group as compared with control (Table 4.11) but was reduced ($p<0.05$) in oleic acid (OA), palmitic acid (PA) and stearic acid (SA) groups as compared with *Citrus limon* juice (CLJ) group (Table 4.11). Catalase was decreased ($p<0.05$) in *Citrus limon* juice (CLJ) group as compared with control (Table 4.11) but was increased ($p<0.05$) in palmitic acid (PA) group in comparison with *Citrus limon* juice (CLJ) group and in stearic acid (SA) group as compared with *Citrus limon* juice (CLJ) and oleic acid (OA) groups (Table 4.11). Glutathione peroxidase was increased ($p<0.05$) in palmitic acid (PA) and stearic acid (SA) groups in comparison with control (Table 4.11). Total antioxidant capacity was reduced ($p<0.05$) in oleic acid (OA) and palmitic acid (PA) groups in comparison to *Citrus limon* juice (CLJ) (Table 4.11). Total protein was reduced ($p<0.05$) in oleic acid (OA) group in comparison to control group (Table 4.11).

Table 4.11. Effects of *Citrus limon* juice (CLJ) and its major components on male placental antioxidant enzymes, total antioxidant capacity and total protein in Wistar rat.

GROUP	SOD (U/mL)	CAT (Umol/mL/min)	GPX (U/L)	TAC (m/M)	TP (g/dL)
CONTROL	0.51±0.06	22.78±0.02	8.74±2.16	8.72±0.35	3.61±0.18
CLJ	1.12±0.17*	14.22±2.83*	10.64±0.92	10.05±0.39	3.07±0.04
OA	0.40±0.05[a]	18.16±0.46	10.39±1.52	7.34±0.62[a]	2.73±0.24*
PA	0.34±0.08[a]	22.98±1.06[a]	15.73±2.34*	7.29±0.95[a]	3.29±0.34
SA	0.13±0.02[a]	24.56±0.32[a,b]	16.30±2.42*	8.79±0.65	2.93±0.04

Data presented as mean± SEM, n=5, *=$p<0.05$ compared with control, a=$p<0.05$ compared with CLJ, b=$p<0.05$ compared with OA. CLJ=*Citrus limon* juice; OA=Oleic Acid; PA=Palmitic Acid; SA=Stearic Acid. SOD=Superoxide Dismutase. CAT=Catalase. GPX=Glutathione peroxidase. TAC=Total Antioxidant Capacity. TP=Total Protein.

4.17. Effects of maternal *Citrus limon* juice on male pup and placental morphometric indices

A significant reduction ($p<0.05$) in abdominal circumference and crown-rump length were observed across the groups in comparison to control group (Table 4.12). Placental thickness was increased ($p<0.05$) in *Citrus limon* juice (CLJ) group in comparison to control (Table 4.13).

Table 4.12. Effects of maternal *Citrus limon* juice (CLJ) and its major components on male pup morphometric indices in Wistar rats.

GROUPS	FW (g)	HC (mm)	AC (mm)	CRL (mm)	AGD*i* (mmg^3)
CONTROL	2.50±0.08	10.56±0.20	13.58±0.32	74.71±4.90	1.84±0.10
CLJ	2.14±0.01	10.76±0.47	12.53±0.33*	59.60±1.10*	1.81±0.11
OA	2.24±0.09	11.16±0.26	12.50±0.29*	57.00±2.42*	1.88±0.12
PA	2.34±0.04	11.11±0.54	12.20±0.19*	57.75±0.61*	1.72±0.07
SA	2.07±0.04	11.59±0.69	12.47±0.39*	57.00±2.55*	1.77±0.08

Data presented as mean± SEM, n=5, *p<0.05 compared with control. CLJ=*Citrus limon* juice; OA=Oleic Acid; PA=Palmitic Acid; SA=Stearic Acid. FW=Foetal Weight; HC=Head Circumference; AC=Abdominal Circumference; CRL=Crown-Rump Length; AGD*i*=Anogenital Distance index

Table 4.13. Effects of maternal *Citrus limon* juice (CLJ) and its major components on male placental morphometric indices in Wistar rats

GROUPS	LS	FW (g)	PW (g)	PT (mm)	PV (mm^3)	PCSA (cm^2)	PC	FPR
CONTROL	7±0.36	2.29±0.08	0.40±0.01	2.29±0.17	0.50±0.02	2.08±0.05	0.18±0.01	5.78±0.21
CLJ	7±0.47	2.14±0.01	0.46±0.03	3.29±0.09*	0.46±0.02	2.19±0.06	0.21±0.01	5.07±0.30
OA	8±0.25	2.24±0.09	0.42±0.02	2.58±0.14	0.53±0.03	2.20±0.05	0.19±0.01	5.34±0.20
PA	8±0.20	2.34±0.04	0.42±0.02	2.58±0.13	0.49±0.02	2.10±0.03	0.18±0.01	5.83±0.29
SA	7±0.38	2.07±0.04	0.41±0.02	2.26±0.14	0.48±0.03	2.18±0.05	0.20±0.01	5.26±0.21

Data presented as mean ± SEM. n = 5. *p<0.05 as compared with control group. CLJ = *Citrus limon* Juice. OA = Oleic Acid. PA = Palmitic Acid. SA = Stearic Acid. LS = Litter Size. FW = Foetal Weight. PW = Placental Weight. PT = Placental Thickness, PV = Placental Volume. PCSA = Placental Chorionic Surface Area. PC = Placental Coefficient. FPR = Foeto-Placental Ratio.

4.18. Effects of maternal *Citrus limon* juice on histology of the liver of male pups

Photomicrographs of hepatic tissue in all the groups show hepatocytes that are immature and are separated by primitive sinusoids (Plate 4.4). The portal triads are different due to the non- appearance of connective tissues, also mild vascular congestion of the vessels was observed (Plate 4.4).

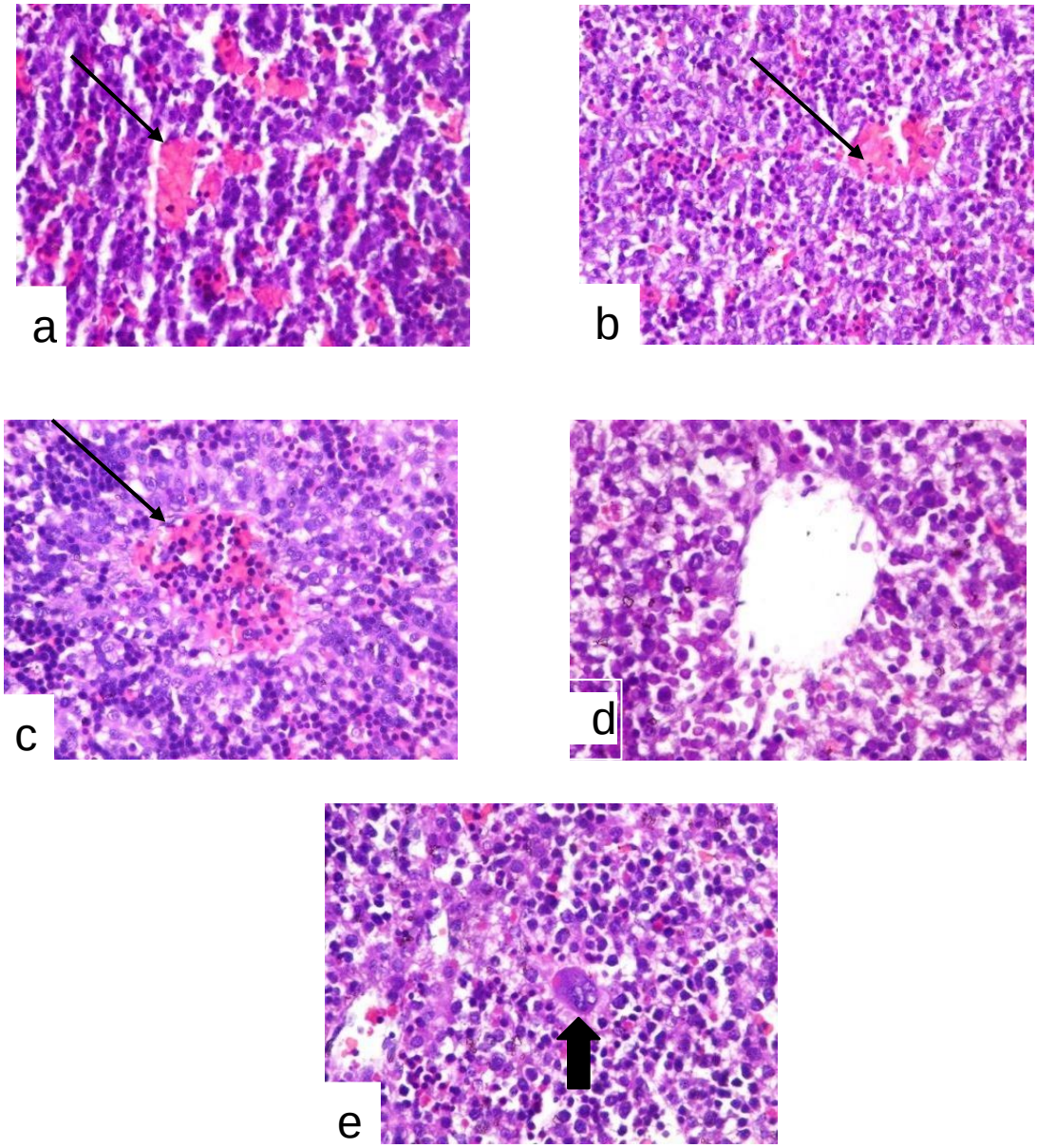


Plate 4.4: Photomicrographs of liver sections from male pups from control, *Citrus limon* juice and its major components treated pregnant Wistar rat dams. Tissues were stained with H&E and presented at ×400 magnification. a) Control: Photomicrograph shows immature hepatocytes which are separated by primitive sinusoids. There is mild sinusoidal congestion (slender arrow). b) CLJ: Disorganized cords of immature hepatocytes that are separated by primitive sinusoids were observed in the photomicrograph. The portal triads are quite different due to the absence of peripheral connective tissue. No significant lesion seen. c) OA: The features seen is as that of CLJ. d) PA: The features seen is as that of CLJ. e) SA: megakaryocytes (thick black arrow) were observed in the photomicrograph.

4.19. Effects of maternal *Citrus limon* juice on histology of the heart of male pups

Photomicrographs of cardiac tissue in all the groups show numerous mitotically active cardiomyocytes with primitive features (Plate 4.5). The atrioventricular, aortic and pulmonary valves observed are immature (Plate 4.5). Inflammatory cells are observed in *Citrus limon* juice (CLJ), oleic acid (OA), palmitic acid (PA) and stearic acid (SA) groups (Plate 4.5).

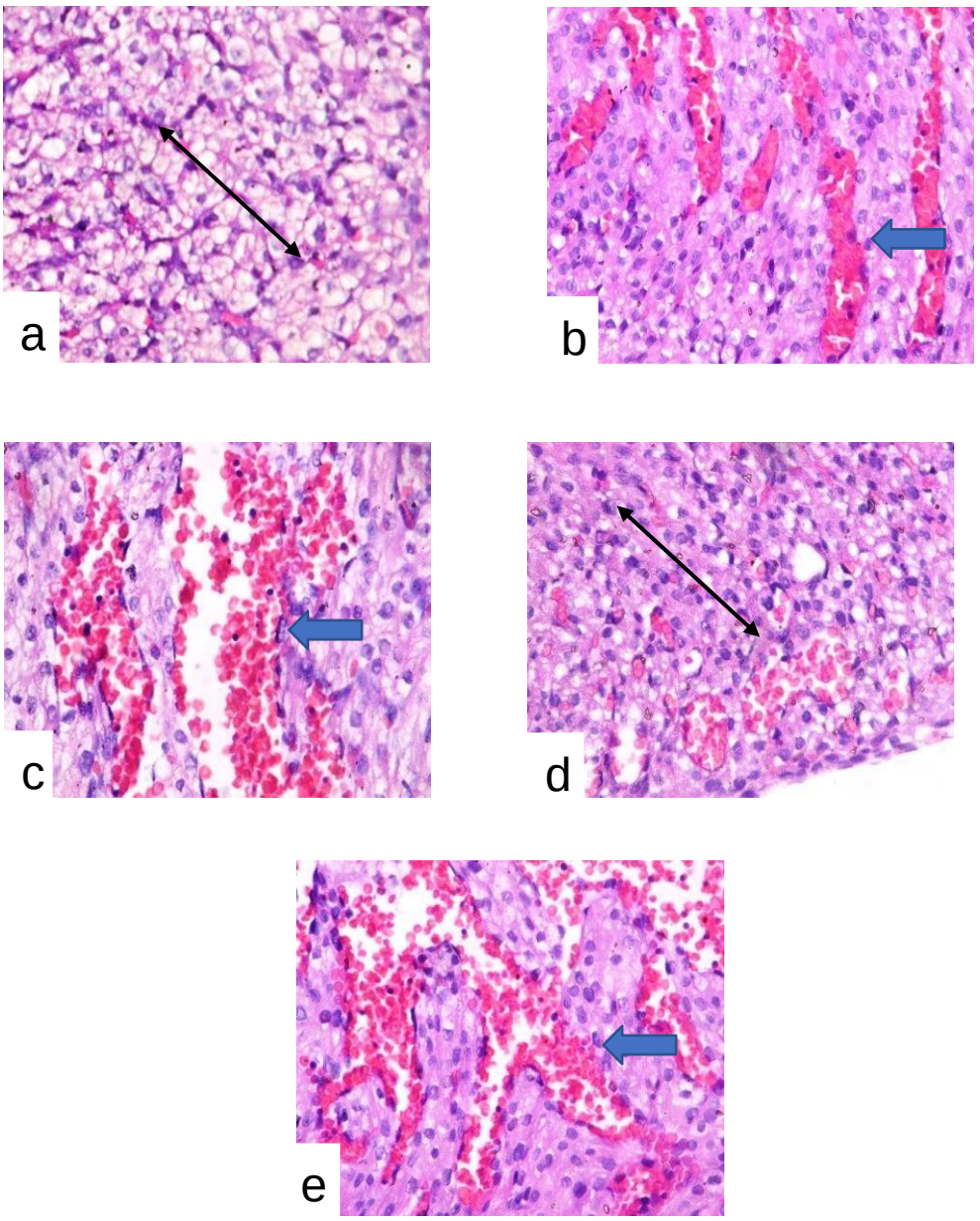


Plate 4.5: Photomicrographs of heart sections from male pups from control, *Citrus limon* juice and its major components treated pregnant Wistar rat dams. Tissues were stained with H&E and presented at ×400 magnification. Photomicrographs of cardiac tissue show myofibrils, numerous mitotically active cardiomyocytes with primitive features (spanned arrow). The atrioventricular, aortic and pulmonary valves formed are not mature. There is infiltration of inflammatory cells (blue arrow).

4.20. Effects of maternal *Citrus limon* juice on histology of the kidney of male pups

Photomicrographs of renal tissue show nephrons that are not mature in the superficial subcapsular zone (Plate 4.6). The cortex is distinct and contains basophilic subcapsular nephrogenic, juxtamedullary and the intercortical zone.

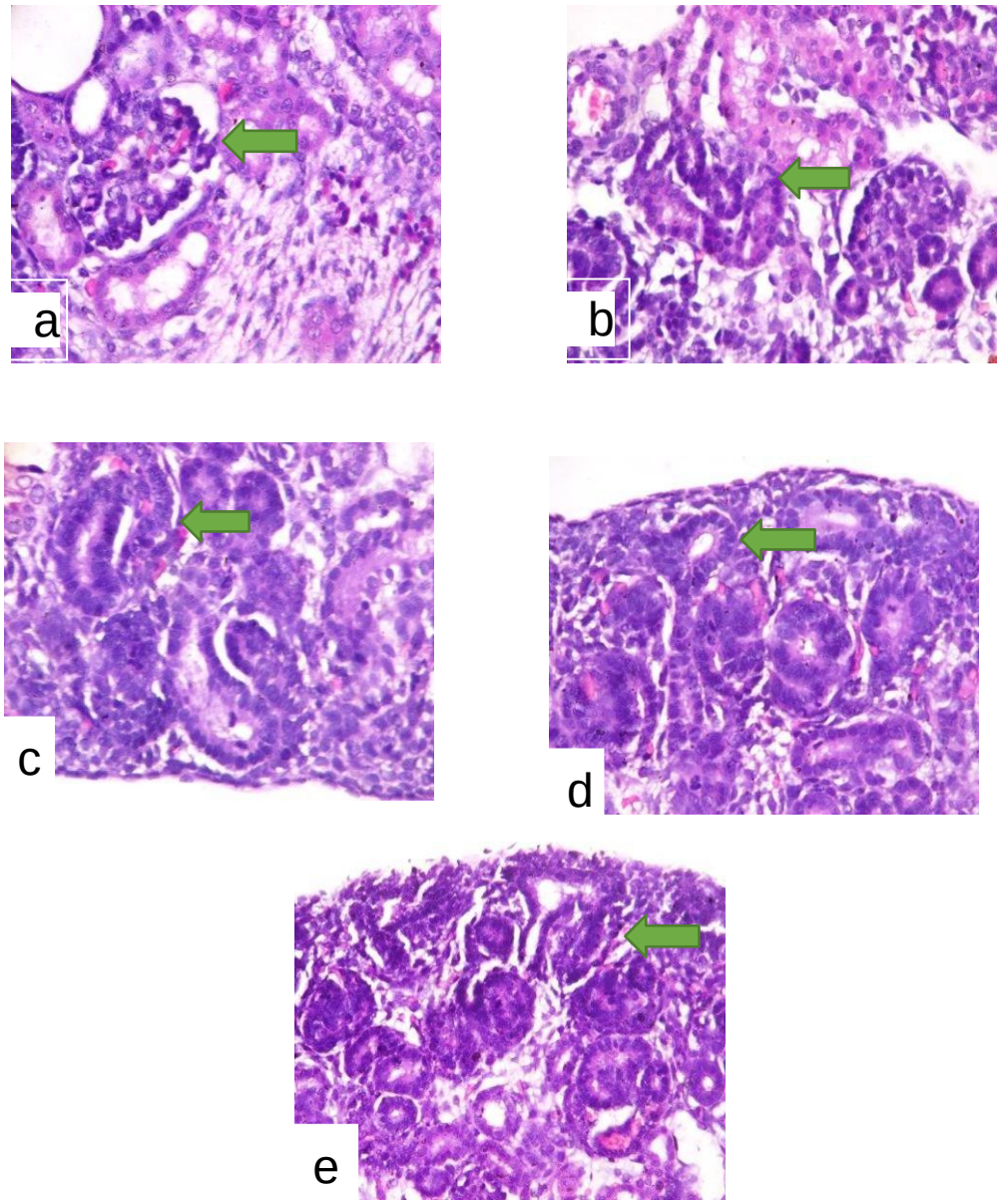


Plate 4.6: Photomicrographs of kidney sections from male pups from control, *Citrus limon* juice and its major components treated pregnant Wistar rat dams. Tissues were stained with H&E and presented at ×400 magnification. Photomicrographs of renal tissue in all the groups show immature nephrons in the superficial subcapsular zone. The cortex was observed to be distinct and contains basophilic subcapsular nephrogenic, juxtamedullary and the intercortical zone.

4.21. Effects of maternal *Citrus limon* juice on histology of the placenta of male pups

Photomicrographs of rats in control group show normal labyrinth zone, the decidual cells appear normal with moderate fat deposits (Plate 4.7). Photomicrographs of placental tissue in CLJ group show oedematous basal zone with moderate vascular congestion (Plate 4.7). Rats in OA group show presence of moderate glycogen cells and moderate fat deposits in the basal intervilous spaces (Plate 4.7). Photomicrographs of placental tissue in PA group show moderate vascular congestions in the labyrinth zone, presence of fat deposit (Plate 4.7). Placental tissue of SA group shows mild oedema in the basal layer (Plate 4.7).

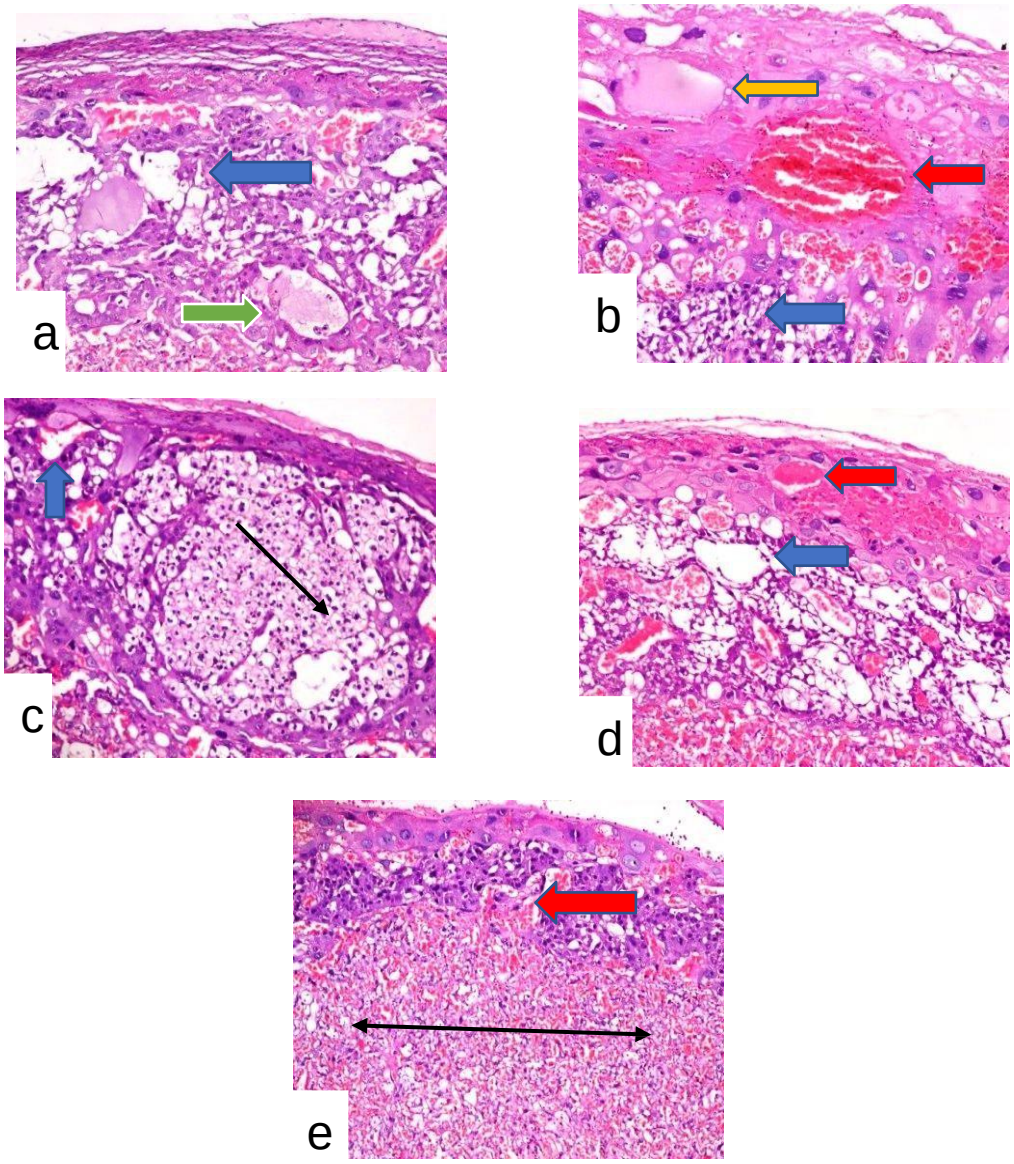


Plate 4.7: Photomicrographs of placenta sections from male pups of control, *Citrus limon* juice and its major components treated pregnant Wistar rat dams. Tissues were stained with H&E and presented at ×100 magnification. a) Control: Photomicrographs of placental tissue show normal labyrinth zone. The decidual cells appear normal with moderate fat deposits (blue arrow). There are degenerating glycogen cells (green arrow). b) CLJ: Photomicrographs of placental tissue show normal labyrinth zone. The basal zone is oedematous (yellow arrow) with moderate vascular congestion (red arrow). c) OA: Photomicrographs of placental tissue show moderate presence of glycogen cells (slender arrow), the basal intervilous spaces has moderate fat deposits (blue arrow). d) PA: Photomicrographs of placental tissue show moderate vascular congestions in the labyrinth zone (red arrow), fat deposit is in abundant (blue arrow). e) SA: Photomicrographs of placental tissue show normal labyrinth zone (spanned arrow). There is mild oedema in the basal layer (red arrow).

4.22. Maternal *Citrus limon* juice on male placental expression of insulin, leptin receptors and peroxisome proliferating-activating receptor gamma.

4.22.1. Maternal *Citrus limon* juice on expression of insulin receptor-1 (IRS-1) in male placenta

The IRS1 positive nuclei in *Citrus limon* juice (CLJ) and palmitic acid (PA) groups was strongly expressed in comparison with control (figure 4.17), but the expression was low in oleic acid (OA) and stearic acid (SA) groups in comparison with control (Figure4.17).

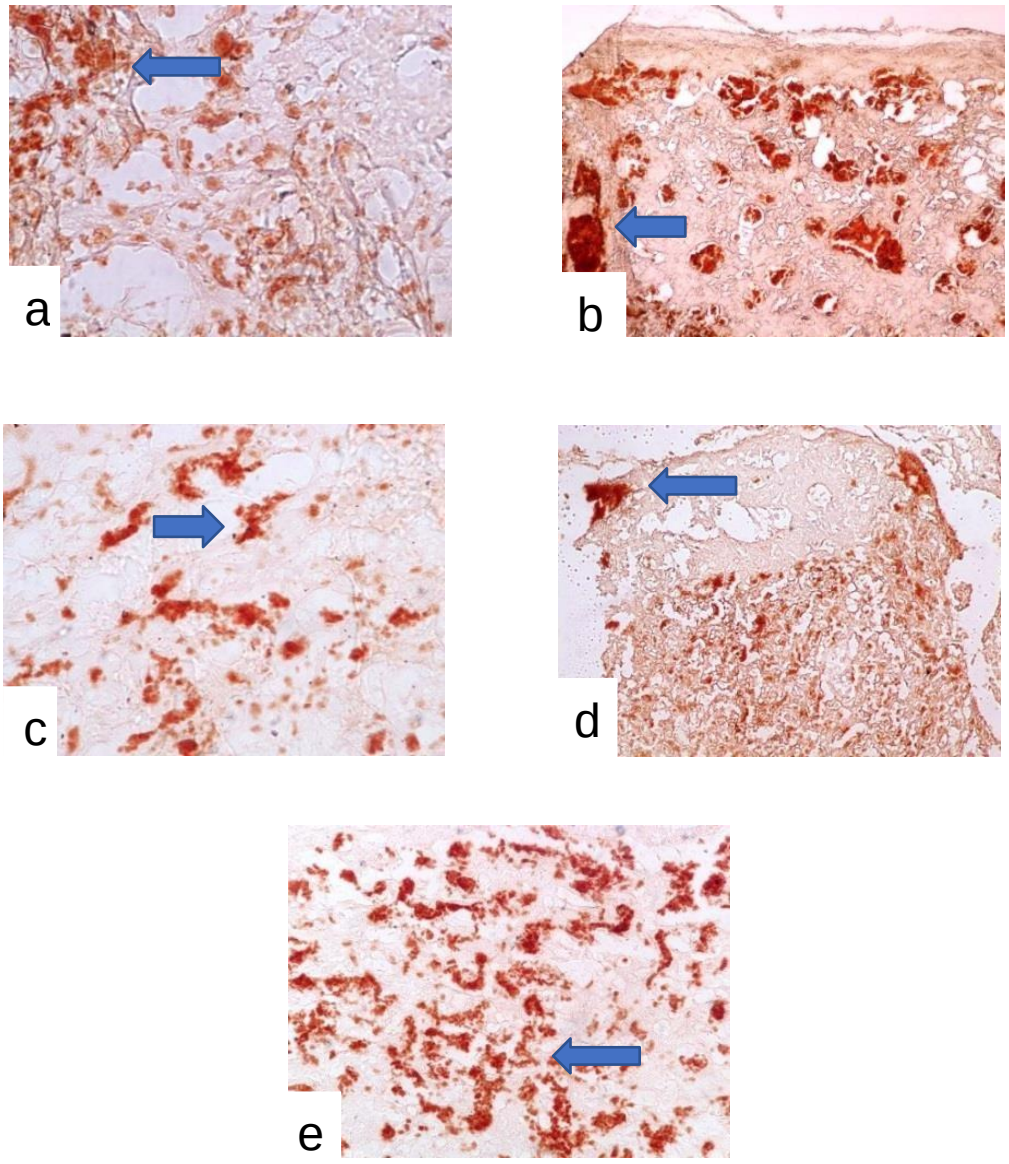


Plate 4.8: Qualitative immunohistochemistry of IRS-1 expression in male placenta sections from control, *Citrus limon* juice and its major components treated pregnant Wistar rat dams. Intensity of expressed protein (blue arrow). a=control, b=CLJ, c=OA, d=PA, e=SA. Tissues were stained with 3,3'-diaminobenzidine (DAB) and presented at ×100 magnification.

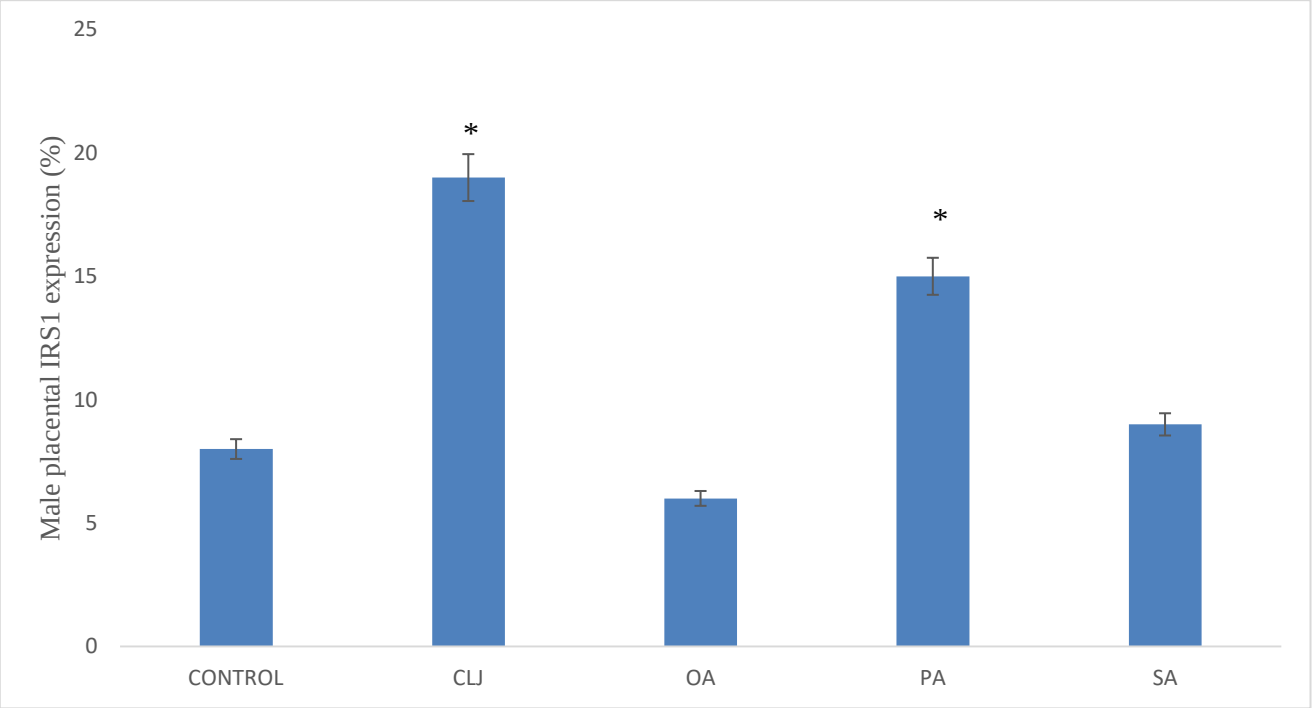


Figure 4.17. Quantitative immunohistochemistry of IRS-1 expression in the male placenta of control, *Citrus limon* juice (CLJ) and its components treated pregnant Wistar rat dams. *p<0.05 compared with control group, as generated from ImageJ 1.46 software and Student's t-test. CLJ=*Citrus limon* juice; OA=Oleic Acid; PA=Palmitic Acid; SA=Stearic Acid.

4.22.2. Maternal *Citrus limon* juice on expression of leptin receptor (LEPR) in male placenta

The expression of LEPR positive nuclei in stearic acid (SA) group was highly expressed in comparison with control (Figure 4.18), but the expression was low in palmitic acid (PA) group in comparison with control (Figure 4.18).

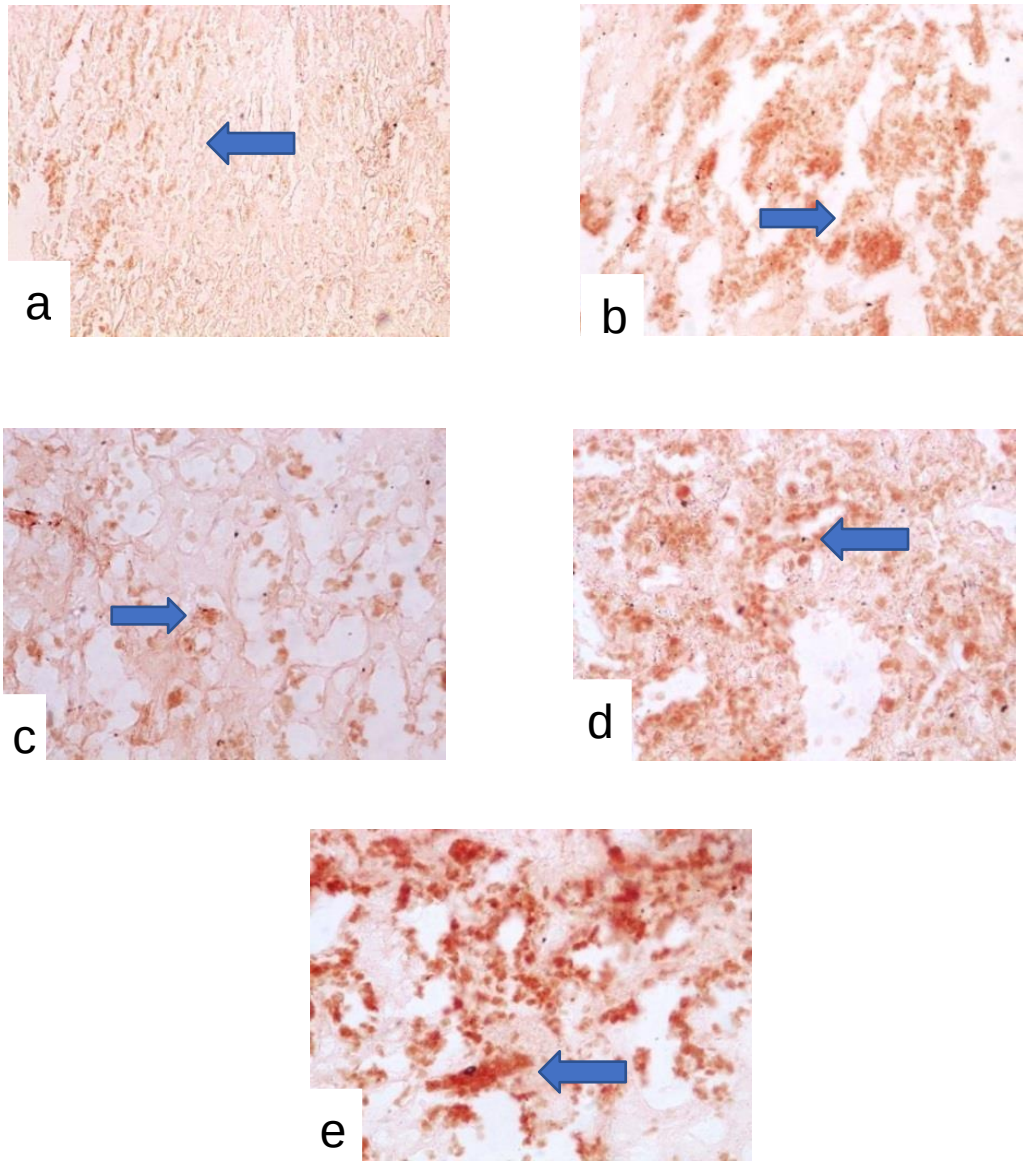


Plate 4.9: Qualitative immunohistochemistry of LEPR expression in male placenta sections from control, *Citrus limon* juice and its major components treated pregnant Wistar rat dams. Intensity of expressed protein (blue arrow). a=control, b=CLJ, c=OA, d=PA, e=SA. Tissues were stained with 3,3'-diaminobenzidine (DAB) and presented at ×100 magnification.

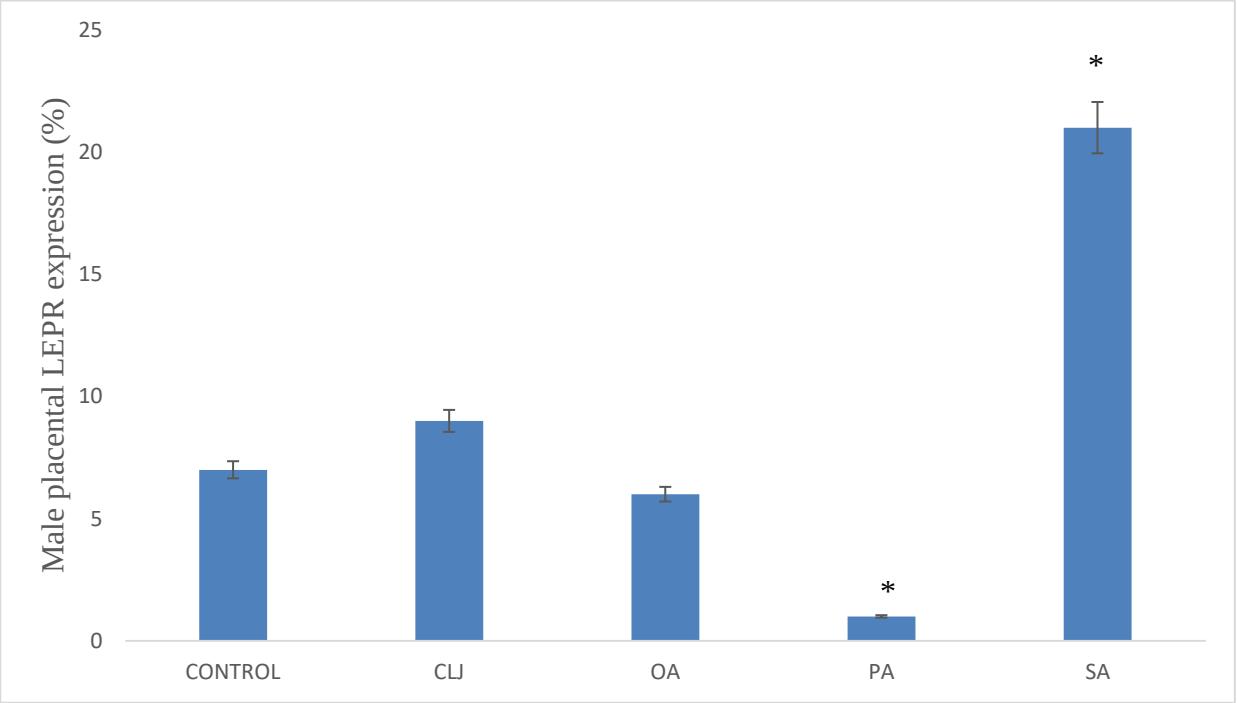


Figure 4.18. Quantitative immunohistochemistry of LEPR expression in the male placenta of control, *Citrus limon* juice (CLJ) and its components treated pregnant Wistar rat dams. *p<0.05 compared with control as generated from ImageJ 1.46 software and Student's t-test. CLJ=*Citrus limon* juice; OA=Oleic Acid; PA=Palmitic Acid; SA=Stearic Acid.

4.22.3. Effects of maternal *Citrus limon* juice on expression of peroxisome proliferating activating receptor gamma (PPAR-γ) in male placenta

The expression of PPAR-γ positive nuclei in palmitic acid (PA) and stearic acid (SA) groups was highly expressed in comparison with control (Figure 4.19) and the expression was low in *Citrus limon* juice (CLJ) and oleic acid (OA) groups in comparison with control (Figure 4.19).

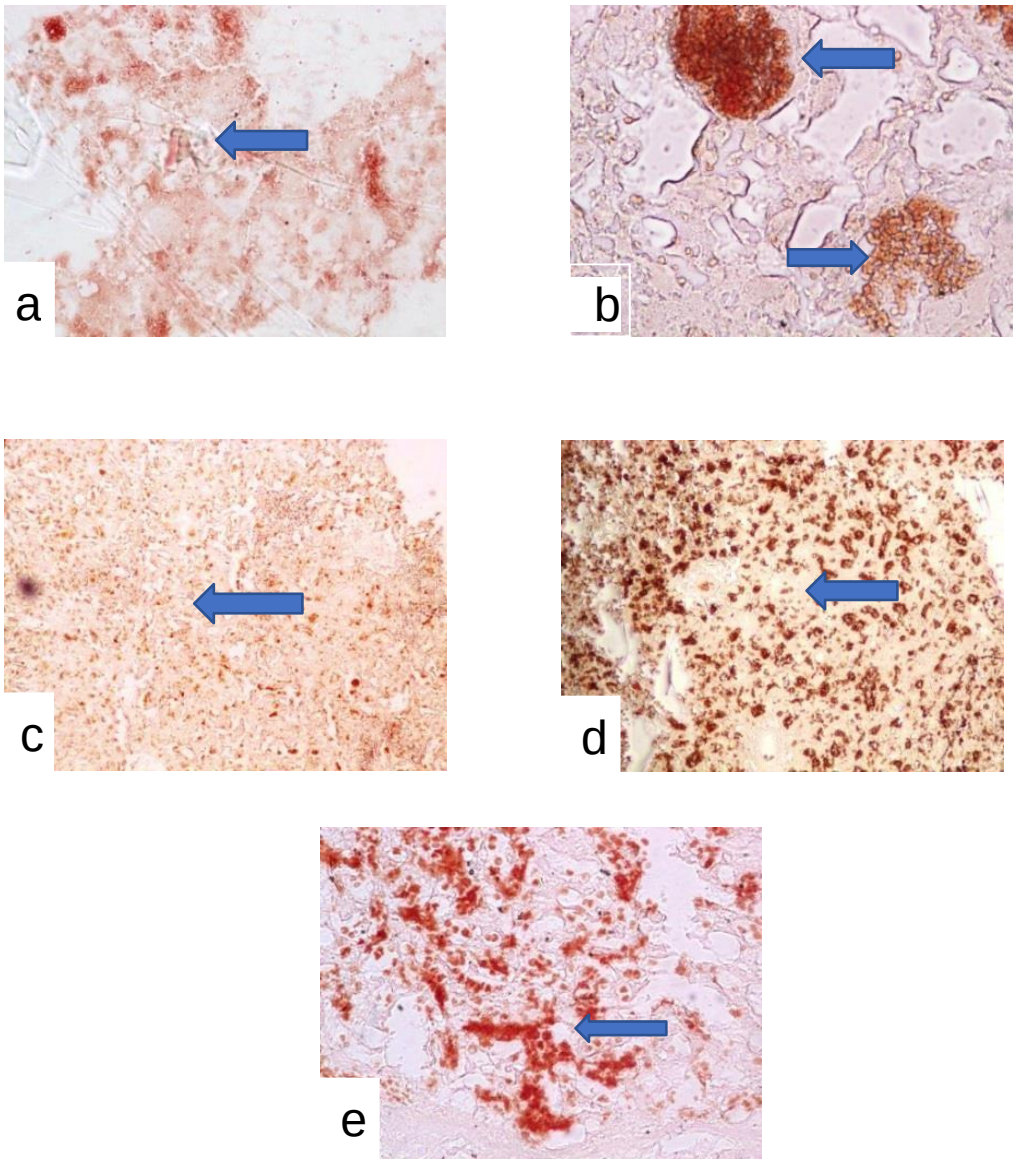


Plate 4.10: Qualitative immunohistochemistry of PPAR-γ expression in male placenta sections from control, *Citrus limon* juice and its major components treated pregnant Wistar rat dams. Intensity of expressed protein (blue arrow). a=control, b=CLJ, c=OA, d=PA, e=SA. Tissues were stained with 3,3'-diaminobenzidine (DAB) and presented at ×100 magnification.

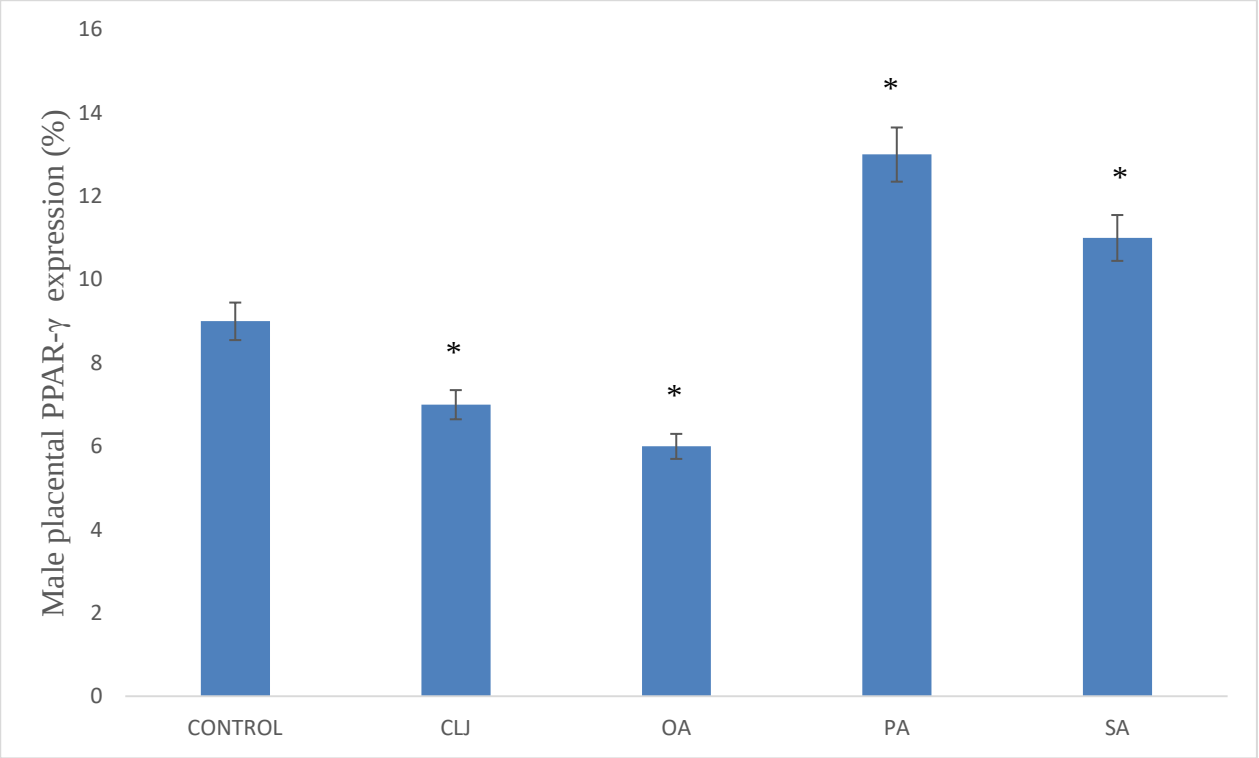


Figure 4.19. Quantitative immunohistochemistry of PPAR-γ expression in the male placenta of control, *Citrus limon* juice (CLJ) and its components in Wistar rats. *p<0.05 compared with control as generated from ImageJ 1.46 software and Student's t-test. CLJ=*Citrus limon* juice; OA=Oleic Acid; PA=Palmitic Acid; SA=Stearic Acid.

4.23. Effects of maternal *Citrus limon* juice on female placental lipase concentration

Female placental lipase was increased ($p<0.05$) in oleic acid (OA) group in comparison with control, palmitic acid (PA) and stearic acid (SA) groups (Figure 4.20).

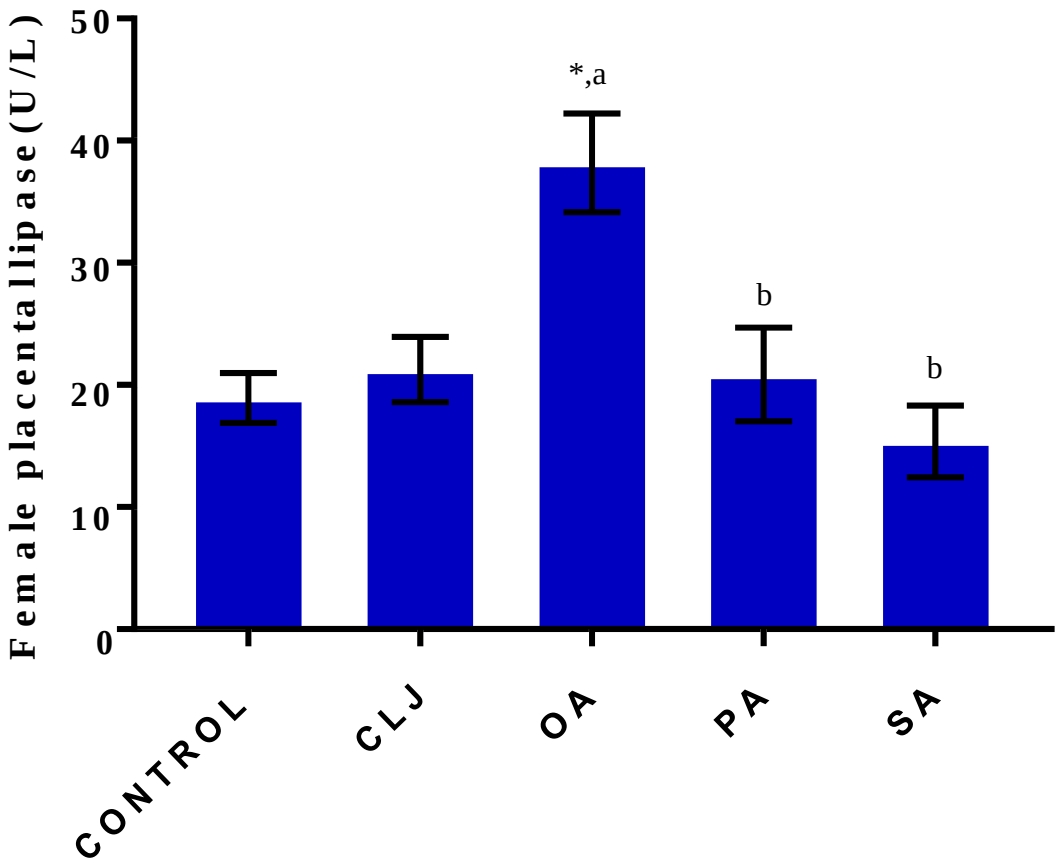


Figure 4.20. Effects of *Citrus limon* juice (CLJ) and its components on female placental lipase in Wistar rats.

Data presented as mean± SEM, n=5, *p<0.05 compared with control, b=p<0.05 compared with OA. CLJ=*Citrus limon* juice; OA=Oleic Acid; PA=Palmitic Acid; SA=Stearic Acid.

4.24. Effects of maternal *Citrus limon* juice on female placental lactogen concentration

Female placental lactogen level was reduced ($p<0.05$) in *Citrus limon* juice (CLJ) group in comparison with control (Figure 4.21), it was increased ($p<0.05$) in oleic acid (OA) group in comparison with *Citrus limon* juice (CLJ) group but reduced ($p<0.05$) in palmitic acid (PA) group in comparison to oleic acid (OA) group (Figure 4.21).

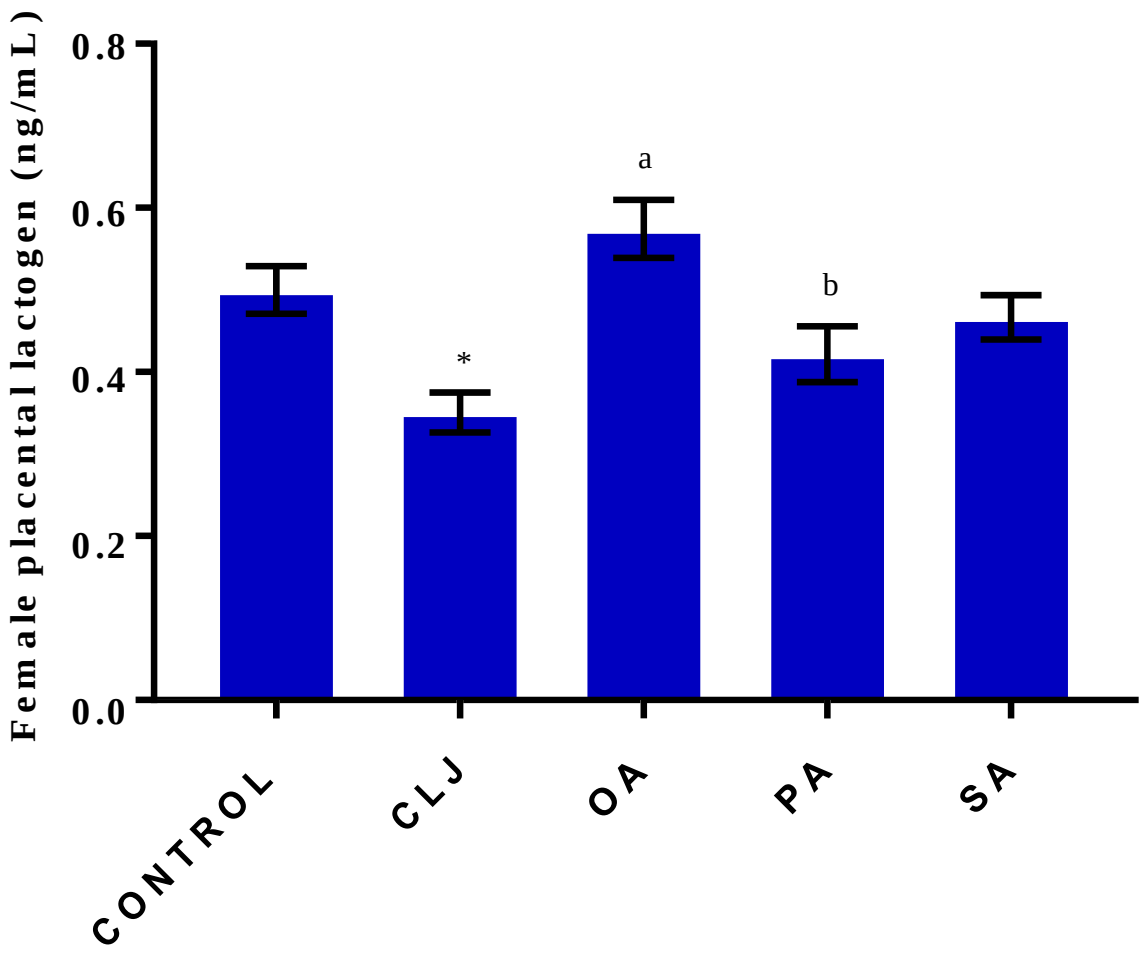


Figure 4.21. Effects of *Citrus limon* juice (CLJ) and its components on female placental lactogen concentration in Wistar rats.

Data presented as mean± SEM, n=5, *p<0.05 compared with control, a=p<0.05 compared with CLJ, b=p<0.05 compared with OA. CLJ=*Citrus limon* juice; OA=Oleic Acid; PA=Palmitic Acid; SA=Stearic Acid.

4.25. Effects of maternal *Citrus limon* juice on female placental GLUT-1 concentration

Female placental GLUT-1 was reduced ($p<0.05$) in *Citrus limon* juice (CLJ), oleic acid (OA) and stearic acid (SA) groups in comparison with control (Figure 4.22), it was also reduced ($p<0.05$) in oleic acid (OA) group as compared with *Citrus limon* juice (CLJ) group and stearic acid (SA) group in comparison with *Citrus limon* juice (CLJ) and oleic acid (OA) groups (Figure 4.22). The level was however increased ($p<0.05$) in palmitic acid (PA) group in comparison with oleic acid OA group (Figure 4.22).

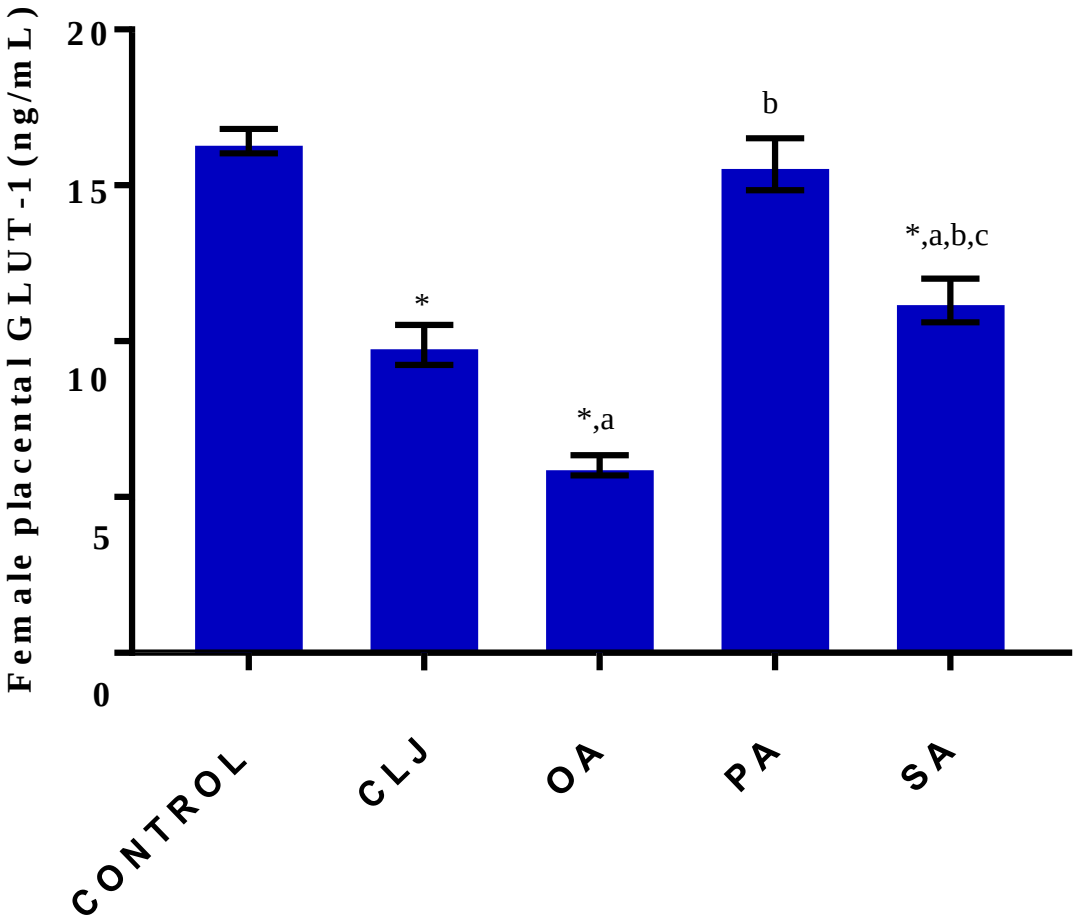


Figure 4.22. *Citrus limon* juice (CLJ) and its components on female placental GLUT-1 concentration in Wistar rats.

Data presented as mean± SEM, n=5, *p<0.05 compared with control, a=p<0.05 compared with CLJ, b=p<0.05 compared with OA, c=p<0.05 compared with PA. CLJ=*Citrus limon* juice; OA=Oleic Acid; PA=Palmitic Acid; SA=Stearic Acid.

4.26. Effects of maternal *Citrus limon* juice on female placental FATP-1 concentration

Fatty Acid Transport Protein-1 (FATP-1) in female placenta was increased ($p<0.05$) in oleic acid (OA) and palmitic acid (PA) groups in comparison with control (Figure 4.23). However, it was reduced ($p<0.05$) in stearic acid (SA) group in comparison with *Citrus limon* juice (CLJ), oleic acid (OA) and palmitic acid (PA) groups (Figure 4.23).

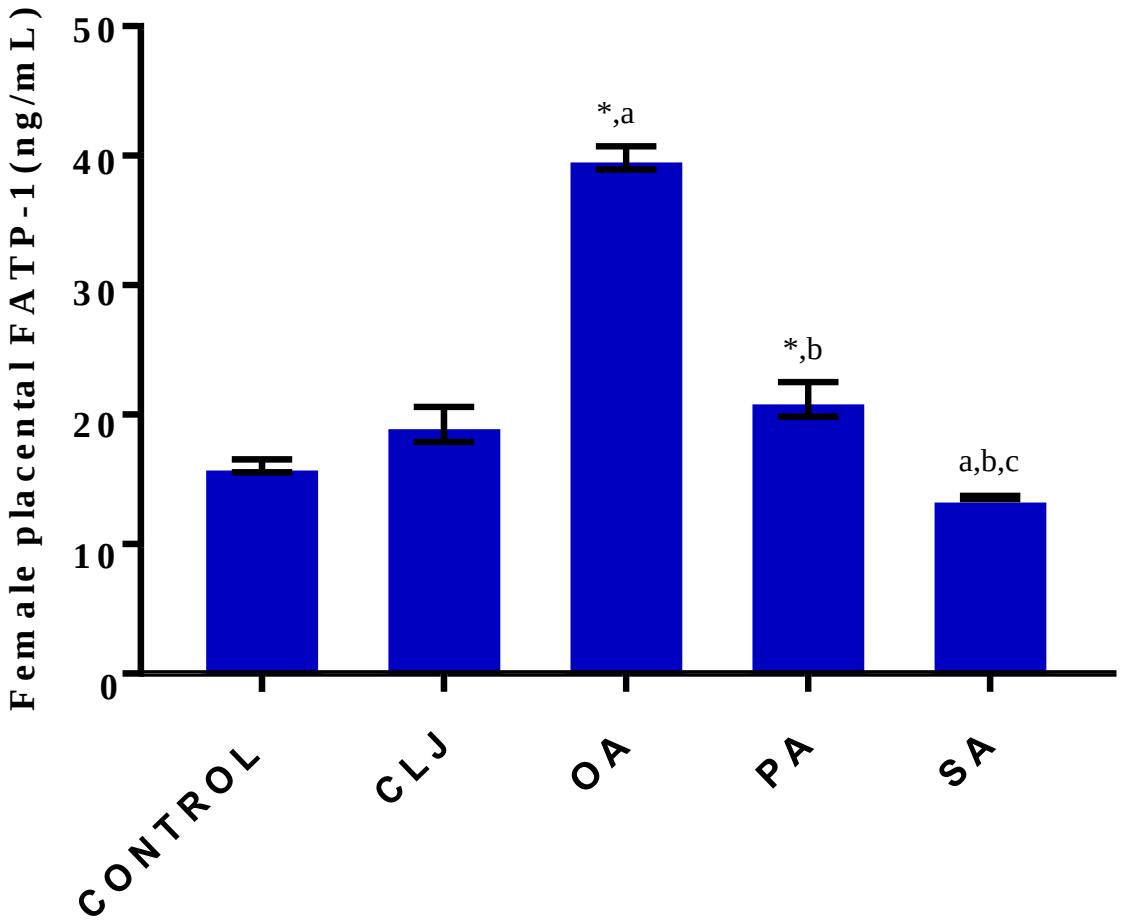


Figure 4.23. *Citrus limon* juice (CLJ) and its components on female placental FATP- 1 concentration in Wistar rats.

Data presented as mean± SEM, n=5, *p<0.05 compared with control, a=p<0.05 compared with CLJ, b=p<0.05 compared with OA, c=p<0.05 compared with PA. CLJ=*Citrus limon* juice; OA=oleic acid; PA=palmitic acid; SA=stearic acid.

4.27. Effects of maternal *Citrus limon* juice on female placental SNAAT-1 concentration

Female placental Small Neutral Amino Acid Transport Protein-1 (SNAAT-1) was reduced ($p<0.05$) in *Citrus limon* juice (CLJ), oleic acid (OA) and stearic acid (SA) groups in comparison with control (Figure 4.24), but was increased ($p<0.05$) in palmitic acid (PA) group in comparison with oleic acid (OA) group (Figure 4.24).

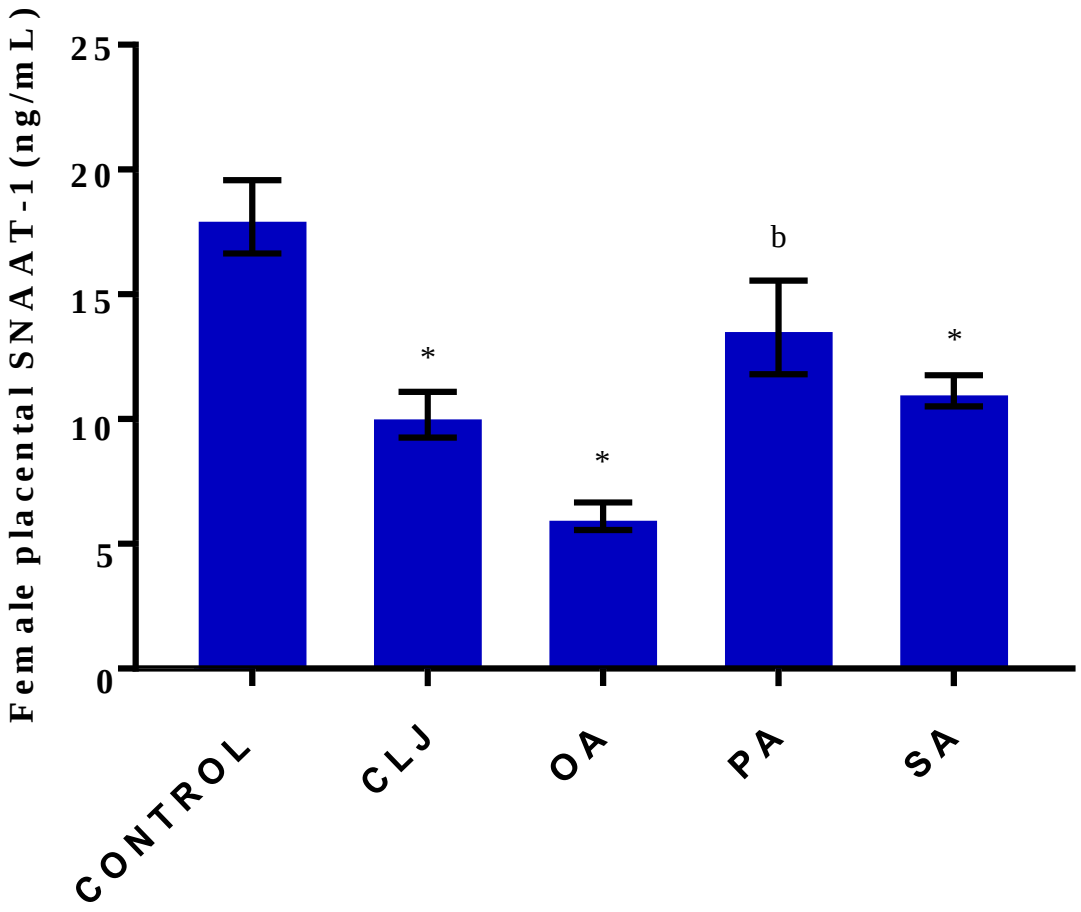


Figure 4.24. Effects of *Citrus limon* juice (CLJ) and its components on female placental SNAT-1 concentration in Wistar rats.

Data presented as mean± SEM, n=5, *p<0.05 compared with control, b=p<0.05 compared with OA. CLJ=*Citrus limon* juice; OA=Oleic Acid; PA=Palmitic Acid; SA=Stearic Acid.

4.28. Effects of maternal *Citrus limon* juice and its major components on female placental lipid concentration

Female placental total cholesterol was reduced ($p<0.05$) across groups in comparison with control (Table 4.14). Female placental low density lipoprotein was reduced ($p<0.05$) across the groups in comparison with control and increased ($p<0.05$) in stearic acid (SA) group in comparison with oleic acid (OA) and palmitic acid (PA) groups (Table 4.14). Female placental high density lipoprotein was reduced ($p<0.05$) across the groups as compared to control (Table 4.14). Female placental triglycerides was increased ($p<0.05$) in *Citrus limon* juice (CLJ), oleic acid (OA) and palmitic acid (PA) groups in comparison with control (Table 4.12), but it was reduced ($p<0.05$) in stearic acid (SA) group in comparison with control, *Citrus limon* juice (CLJ), oleic acid (OA) and palmitic acid (PA) groups (Table 4.14).

Table 4.14. Effects of maternal *Citrus limon* juice (CLJ) and its major components on female placental lipid concentration in Wistar rats.

GROUPS	TC (mg/dL)	LDL-C (mg/dL)	HDL-C (mg/dL)	TG (mg/dL)
CONTROL	252.52±0.02	236.42±0.02	2.46±0.59	68.21±1.49
CLJ	235.92±0.01*	216.92±0.06*	0.67±0.11*	81.67±0.10*
OA	163.37±0.01*,a	145.30±0.01*,a	1.37±0.29*,a	84.59±0.16*,a
PA	142.83±0.06*,a,b	128.46±0.06*,a,b	0.31±0.08*,b	71.58±0.25*,a,b
SA	221.71±0.06*,a,b,c	211.09±0.06*,a,b,c	1.24±0.19*,a,c	46.89±0.08*,a,c

Data presented as mean± SEM, n=5, *p<0.05 compared with control, a=p<0.05 compared with CLJ, b=p<0.05 compared with OA, c=p<0.05 compared with PA. CLJ=*Citrus limon* juice; OA=oleic acid; PA=palmitic acid; SA=stearic acid.

4.29. Effects of maternal *Citrus limon* juice and its major components on female placental oxidative stress markers in Wistar rats

4.29.1. Effects of maternal *Citrus limon* juice on female placental malondialdehyde

Female placental malondialdehyde concentration was increased ($p<0.05$) in *Citrus limon* juice (CLJ) group in comparison with control (Figure 4.25) but reduced ($p<0.05$) in oleic acid (OA), palmitic acid (PA) and stearic acid (SA) groups in comparison with *Citrus limon*juice(CLJ)group(Figure4.25).

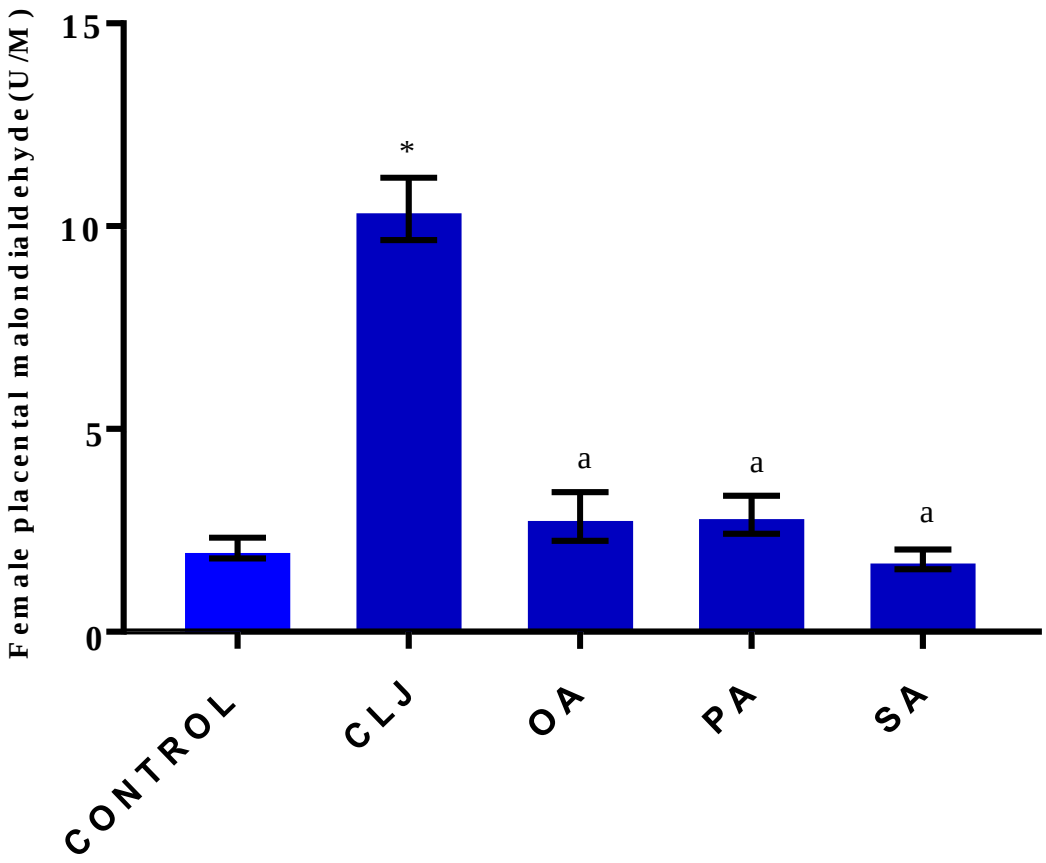


Figure 4.25. Effects of *Citrus limon* juice (CLJ) and its components on female placental malondialdehyde concentration in Wistar rats.

Data presented as mean± SEM, n=5, *p<0.05 compared with control, a=p<0.05 compared with CLJ. CLJ=*Citrus limon* juice; OA=Oleic Acid; PA=Palmitic Acid; SA=Stearic Acid.

4.29.2. Effects of maternal *Citrus limon* juice and its major components on female placental redox status

Superoxide dismutase concentration was reduced ($p<0.05$) across groups as compared to control (Table 4.15). Catalase concentration was increased ($p<0.05$) in palmitic acid (PA) and stearic acid (SA) groups in comparison with control group (Table 4.15). Glutathione peroxidase was increased ($p<0.05$) in stearic acid (SA) group in comparison with control, *Citrus limon* juice (CLJ), oleic acid (OA) and palmitic acid (PA) groups (Table 4.15). Non- significant change was observed in total antioxidant capacity across the groups in comparison with control (Table 4.15). Total protein was increased ($p<0.05$) in *Citrus limon* juice (CLJ) group in comparison with control while the concentration was reduced ($p<0.05$) in stearic acid (SA) group in comparison with *Citrus limon* juice (CLJ) group (Table 4.15).

Table 4.15. Effects of *Citrus limon* juice (CLJ) and its major components on female placental activities of antioxidant enzymes, total antioxidant capacity and total protein in Wistar rat.

GROUP	SOD (U/mL)	CAT (Umol/mL/min)	GPX (U/L)	TAC (m/M)	TP (g/dL)
CONTROL	1.06±0.21	16.57±2.34	8.87±1.82	7.58±0.92	2.84±0.19
CLJ	0.47±0.09*	20.09±0.46*	11.90±2.12	6.98±0.41	3.46±0.03*
OA	0.49±0.04*	18.92±1.77*	10.58±1.84*	6.41±0.80	3.06±0.10
PA	0.35±0.02*	23.16±0.20*[a,b]	12.76±1.39*	6.85±0.31	3.21±0.05
SA	0.61±0.11*	22.67±0.18*	47.65±1.17*,[a,b,c]	7.65±0.18	2.82±0.19[a]

Data presented as mean± SEM, n=5, *=p<0.05 compared with control, a=p<0.05 compared with CLJ, b=p<0.05 compared with OA, c=p<0.05 compared with PA. CLJ=*Citrus limon* juice; OA=Oleic Acid; PA=Palmitic Acid; SA=Stearic Acid. SOD=Superoxide Dismutase. CAT=Catalase. GPX=Glutathione peroxidase. TAC=Total Antioxidant Capacity. TP=Total Protein.

4.30. Effects of maternal *Citrus limon* juice and its major components on female pups and placental morphometric indices

Foetal weight was significantly reduced ($p<0.05$) in *Citrus limon* juice (CLJ) and stearic acid (SA) groups in comparison with control (Table 4.16). Crown-rump length was reduced ($p<0.05$) across groups in comparison with control (Table 4.16). There was increase ($p<0.05$) in placental thickness in *Citrus limon* juice (CLJ) and oleic acid (OA) groups in comparison with control (Table 4.17). Foeto-placental ratio was reduced ($p<0.05$) in *Citrus limon* juice (CLJ) group in comparison with control group (Table 4.17).

Table 4.16. Effects of maternal *Citrus limon* juice (CLJ) and its major components on female pup morphometric indices in Wistar rats.

GROUPS	FW (g)	HC (mm)	AC (mm)	CRL (mm)	AGD*i* (mmg^3)
CONTROL	2.42±0.09	10.45±0.19	13.26±0.23	74.50±3.06	1.00±0.09
CLJ	2.11±0.04*	10.87±0.17	12.65±0.36	59.17±1.69*	1.02±0.11
OA	2.32±0.09	10.99±0.18	12.13±0.34	54.90±1.77*	1.04±0.08
PA	2.27±0.06	11.35±0.70	12.39±0.30	59.10±1.65*	1.04±0.08
SA	2.07±0.03*	11.30±0.52	12.34±0.35	54.70±1.96*	1.06±0.08

Data presented as mean± SEM, n=5, *p<0.05 compared with control. CLJ=*Citrus limon* juice; OA=Oleic Acid; PA=Palmitic Acid; SA=Stearic Acid. FW=Foetal Weight; HC=Head Circumference; AC=Abdominal Circumference; CRL=Crown-Rump Length; AGD*i*=Anogenital Distance index

Table 4.17. Effects of maternal *Citrus limon* juice (CLJ) and its major components on female placental morphometric indices in Wistar rats.

GROUPS	LS	FW (g)	PW (g)	PT (mm)	PV (mm^3)	PCSA (cm^2)	PC	FPR
CONTROL	7±0.36	2.42±0.09	0.41±0.02	2.22±0.19	0.49±0.02	2.13±0.05	0.18±0.01	6.17±0.37
CLJ	7±0.47	2.11±0.04*	0.45±0.03	3.59±0.07*	0.44±0.02	2.01±0.05	0.21±0.01	4.84±0.17*
OA	8±0.25	2.32±0.09	0.46±0.02	2.88±0.21*	0.51±0.02	2.16±0.05	0.21±0.01	5.19±0.27
PA	8±0.20	2.27±0.06	0.44±0.02	2.54±0.15	0.51±0.03	2.19±0.07	0.20±0.01	5.47±0.29
SA	7±0.38	2.07±0.03*	0.36±0.02	2.14±0.13	0.49±0.02	2.05±0.08	0.17±0.01	5.97±0.18

Data presented as mean ± SEM. n = 5. *p<0.05 when compared with control. CLJ = *Citrus limon* Juice. OA = Oleic Acid. PA = Palmitic Acid. SA= Stearic Acid. LS = Litter Size. FW = Foetal Weight. PW = Placental Weight. PT = Placental Thickness, PV = Placental Volume. PCSA = Placental Chorionic Surface Area. PC = Placental Coefficient. FPR = Foeto-Placental Ratio.

4.31. Effects of maternal *Citrus limon* juice and its major components on histology of the liver of female pups

Photomicrographs of hepatic tissue across the groups show immature hepatocytes that are separated by primitive sinusoids (Plate 4.11). There is mild presence of megakaryocytes in CLJ and OA groups (Plate 4.11).

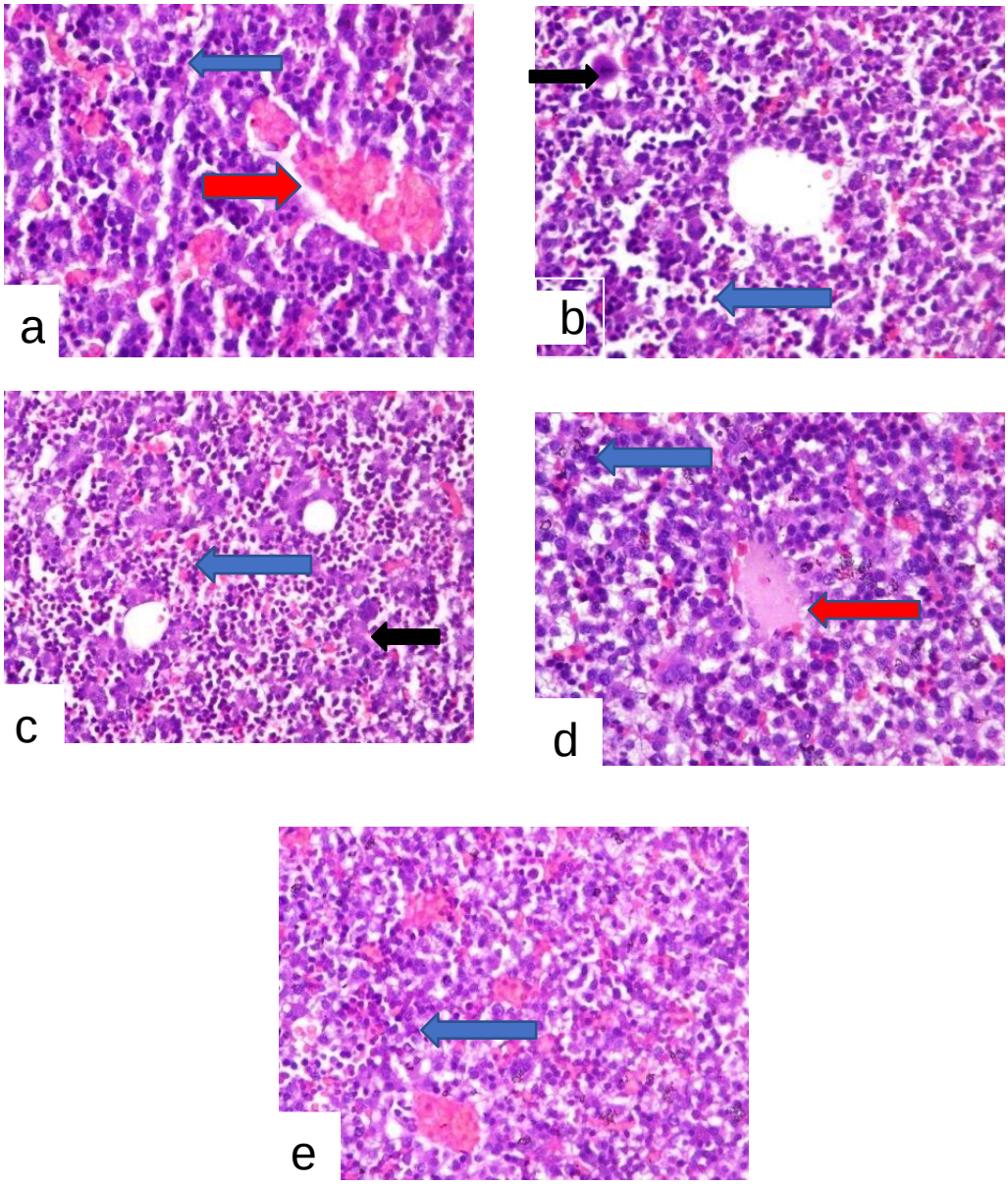


Plate 4.11: Photomicrographs of liver sections from female pups of control, *Citrus limon* juice and its major components treated pregnant Wistar rat dams. Tissues were stained with H&E and presented at ×400 magnification. a) Control: Photomicrographs of hepatic tissue shows immature hepatocytes that are separated by primitive sinusoids (blue arrow) and mild sinusoidal congestion (red arrow). b) CLJ: Immature hepatocytes (blue arrow) and mild presence of megakaryocytes (black arrow) are observed. c) OA: Immature hepatocytes (blue arrow) and mild presence of megakaryocytes (black arrow) are observed. d) PA: Mild sinusoidal congestion (red arrow) was observed. e) SA: The portal triads are different due to the nonappearance of peripheral connective tissue.

4.32. Effects of maternal *Citrus limon* juice and its major composition on histology of the heart of female pups

Photomicrographs of cardiac tissue in all the groups show myofibrils and mitotically active cardiomyocytes with primitive features (Plate 4.12). Inflammatory cells are observed in oleic acid (OA), palmitic acid (PA) and stearic acid (SA) groups (Plate 4.12).

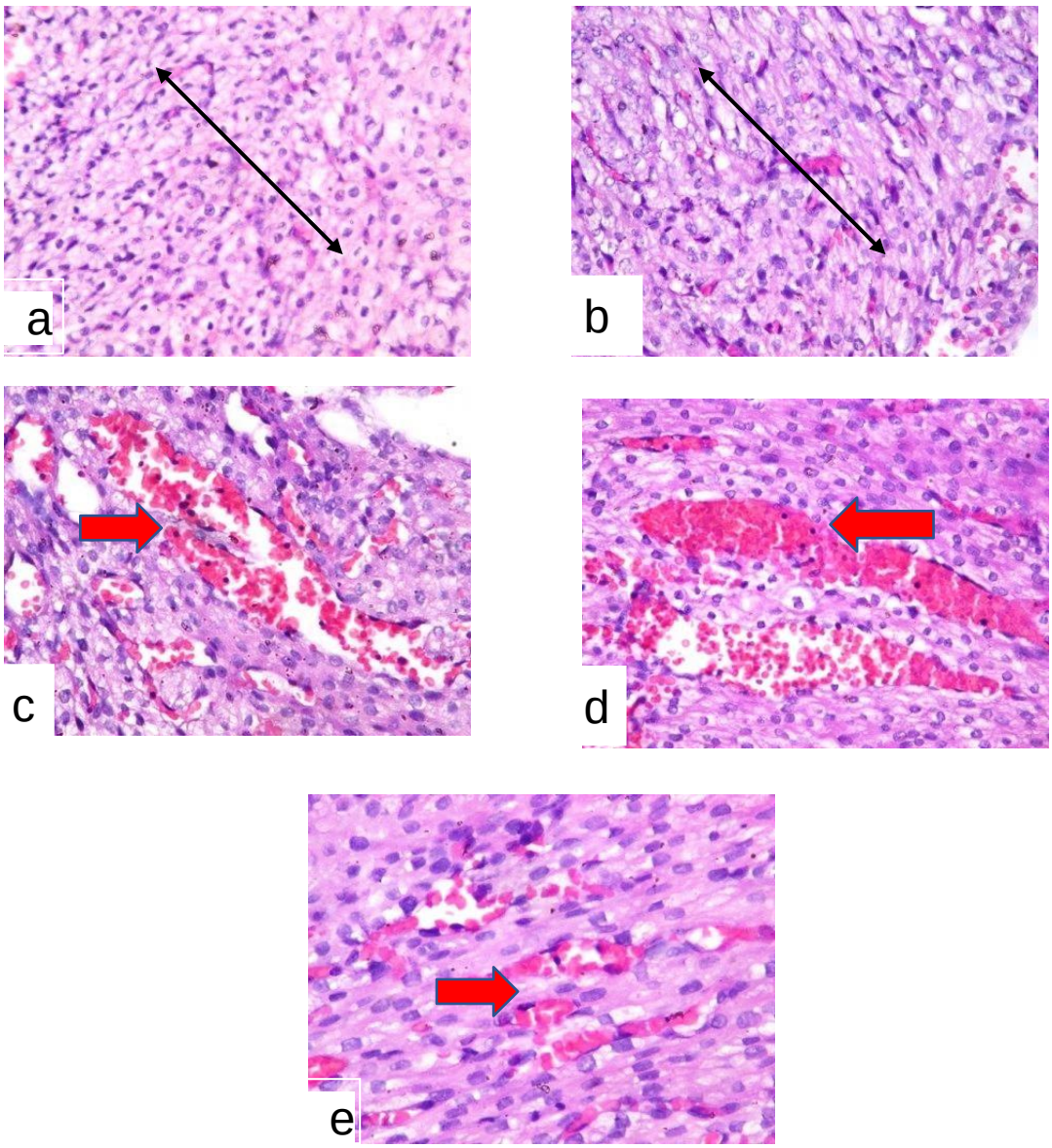


Plate 4.12: Photomicrographs of heart sections from female pups of control, *Citrus limon* juice and its major components treated pregnant Wistar rat dams. Tissues were stained with H&E and presented at ×400 magnification. a) Control: Photomicrographs show cardiac tissue with myofibrils and numerous mitotically active cardiomyocytes with primitive features (spanned arrow). b) CLJ: Photomicrographs show cardiac tissue with myofibrils and numerous mitotically active cardiomyocytes with primitive features (spanned arrow). c), d) and e) OA, PA and SA rats' cardiac tissues show infiltration by inflammatory cells and vascular congestion (red arrow).

4.33. Effects of maternal *Citrus limon* juice and its major components on histology of the kidney of female pups

Photomicrographs of renal tissue in all the groups show numerous immature nephrons admixed in loose connective tissue; also the cortex is observed to be distinct and contains basophilic subcapsular nephrogenic, juxtamedullary and intercortical zone (Plate 4.13).

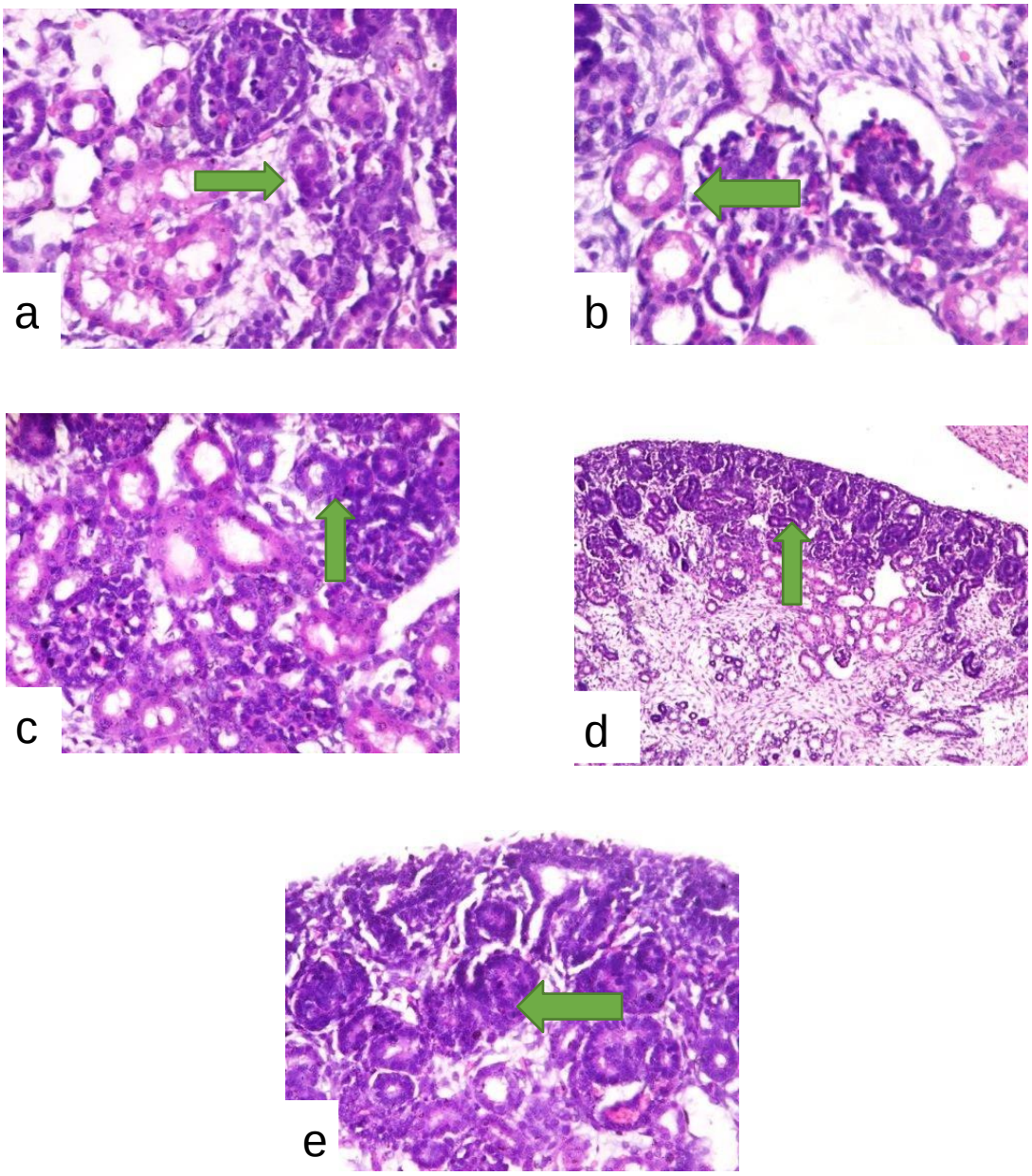


Plate 4.13: Photomicrographs of kidney sections from female pups of control, *Citrus limon* juice and its major components treated pregnant Wistar rat dams. Tissues were stained with H&E and presented at ×400 magnification. Photomicrographs of renal tissue in all the groups show numerous immature nephrons admixed in loose connective tissue, the cortex is distinct and contains basophilic subcapsular nephrogenic, juxtamedullary and intercortical zone (green arrow).

4.34. Effects of maternal *Citrus limon* juice and its major components on histology of the placenta of female pups

Photomicrographs of placental tissue of the control rats show normal labyrinth zone and a basal layer with moderate vascular congestions as well as few fats deposits (Plate 4.14). Placental tissue from *Citrus limon* juice (CLJ) rats show labyrinth zone with severe vascular congestion (Plate 4.14). Placental tissue from oleic acid (OA) rats shows basal layer with moderate fat deposit and oedematous labyrinth zone with inflammatory cells (Plate 4.14). Placental tissue from OA group shows basal layer with moderate infiltration of inflammatory cells vascular congestion, the labyrinth zone appears oedematous with mild fat deposit and hyperplastic decidual cells. Placental tissue from stearic acid (SA) group show basal layer with moderate oedema and vascular congestions and the labyrinth zone with mild fat deposit (Plate 4.14).

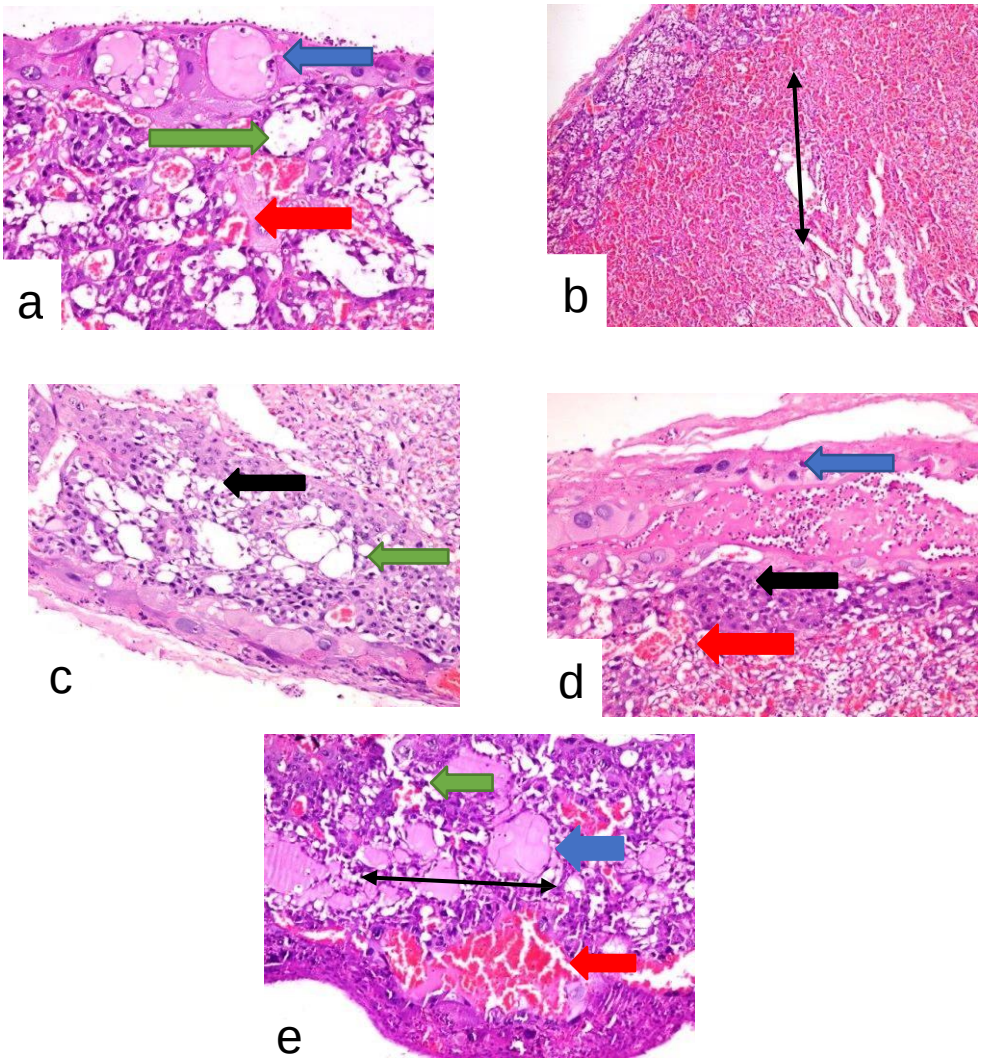


Plate 4.14: Photomicrographs of placenta sections from female pups of control, *Citrus limon* juice and its major components treated pregnant Wistar rat dams. Tissues were stained with H&E and presented at ×100 magnification. a) Control: Photomicrographs of placental tissue show basal layer with moderate vascular congestions (red arrow), oedema (blue arrow) and fats deposits (green arrow). The decidual cells appear hyperplastic (blue arrow). b) CLJ: Placental tissue show labyrinth zone with severe vascular congestion (spanned arrow). c) OA: Placental tissue show basal layer with moderate fat deposit (green arrow), and oedema in the labyrinth zone with infiltration by the inflammatory cells (black arrow). d) PA: Placental tissue shows infiltration of inflammatory cells (black arrow) and moderate vascular congestions in the basal layer. The labyrinth zone appears oedematous (blue arrow) with mild fat deposit and the decidual cells appear hyperplastic. e) SA: Placental tissue show basal layer with moderate oedema and moderate vascular congestions (red arrow). The labyrinth zone (span arrow) appears moderately oedematous (blue arrow) with mild fat deposit (green arrow). The decidual cells appear hyperplastic.

4.35. Effects of maternal *Citrus limon* juice and its major components on female placental expression of insulin, leptin receptors and peroxisome proliferating-activating receptor gamma

4.35.1. Effects of maternal *Citrus limon* juice and its major components on expression of insulin receptor (IRS-1) in female placenta

The expression of IRS-1 positive nuclei was low in *Citrus limon* juice (CLJ), oleic acid (OA) and stearic acid (SA) groups in comparison with control (Figure 4.26).

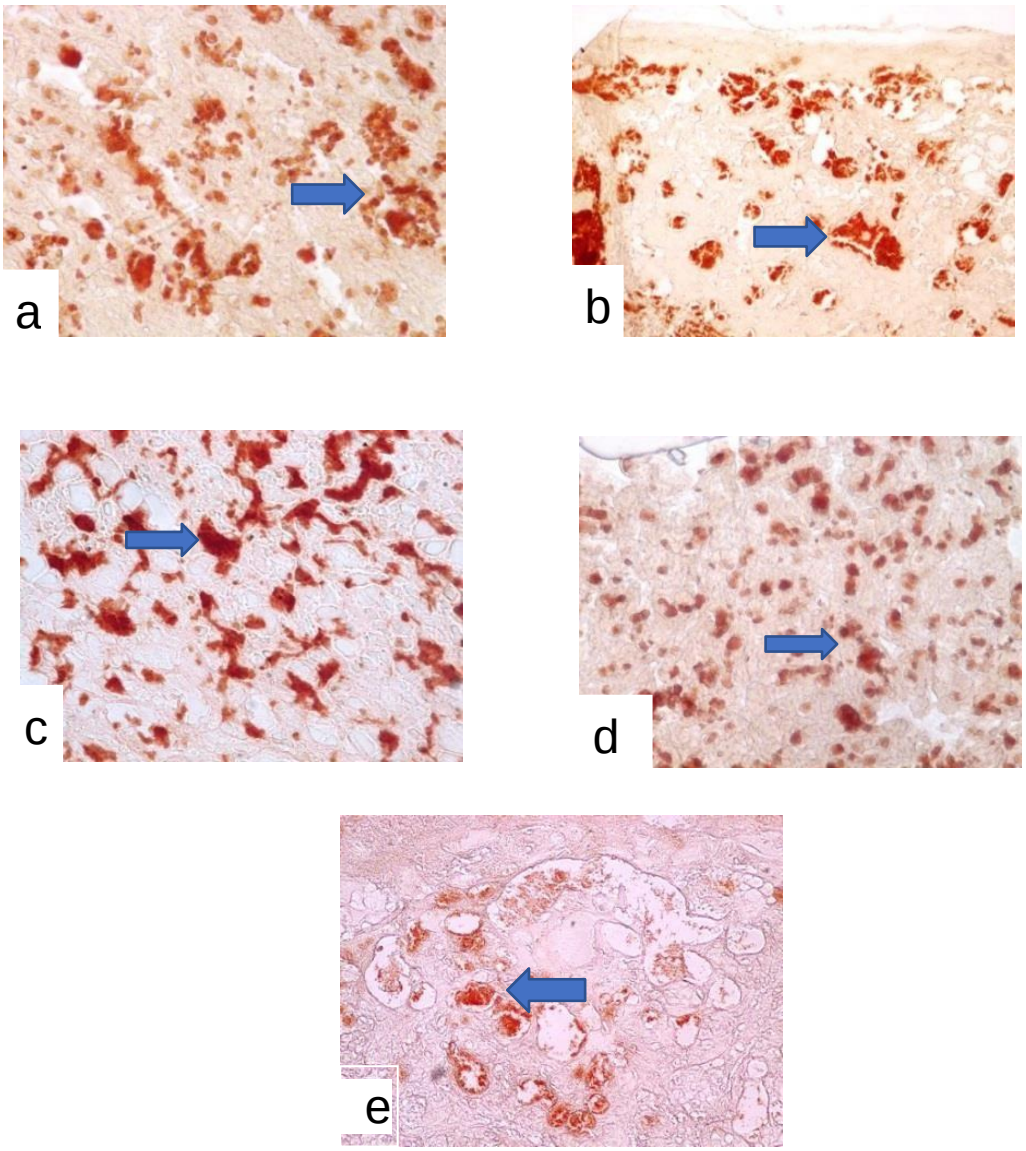


Plate 4.15: Qualitative immunohistochemistry of IRS-1 expression in female placenta sections from control, *Citrus limon* juice and its major components treated pregnant Wistar rat dams. Intensity of expressed protein (blue arrow). a=control, b=CLJ, c=OA, d=PA, e=SA. Tissues were stained with 3,3'-diaminobenzidine (DAB) and presented at ×100 magnification.

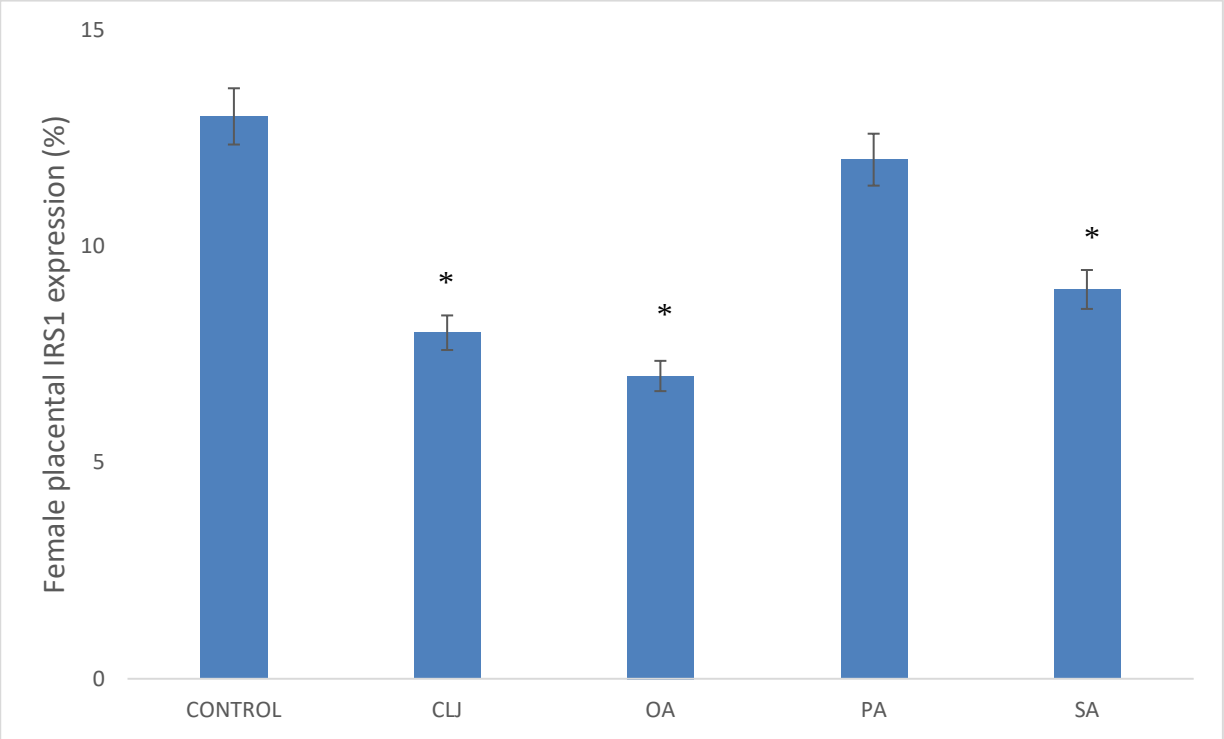


Figure 4.26. Quantitative immunohistochemistry of IRS-1 expression in the female placenta of control, *Citrus limon* juice (CLJ) and its major components treated pregnant Wistar rat dams.

*p<0.05 compared with control as generated from ImageJ 1.46 software and Student's t-test. CLJ=*Citrus limon* juice; OA=Oleic Acid; PA=Palmitic Acid; SA=Stearic Acid.

4.35.2. Effects of maternal *Citrus limon* juice and its major components on expression of leptin receptor (LEPR) in female placenta

The expression of LEPR positive nuclei was high in *Citrus limon* juice (CLJ) group in comparison with control although it was low in oleic acid (OA), palmitic acid (PA) and stearic acid (SA) groups in comparison with control (Figure 4.27).

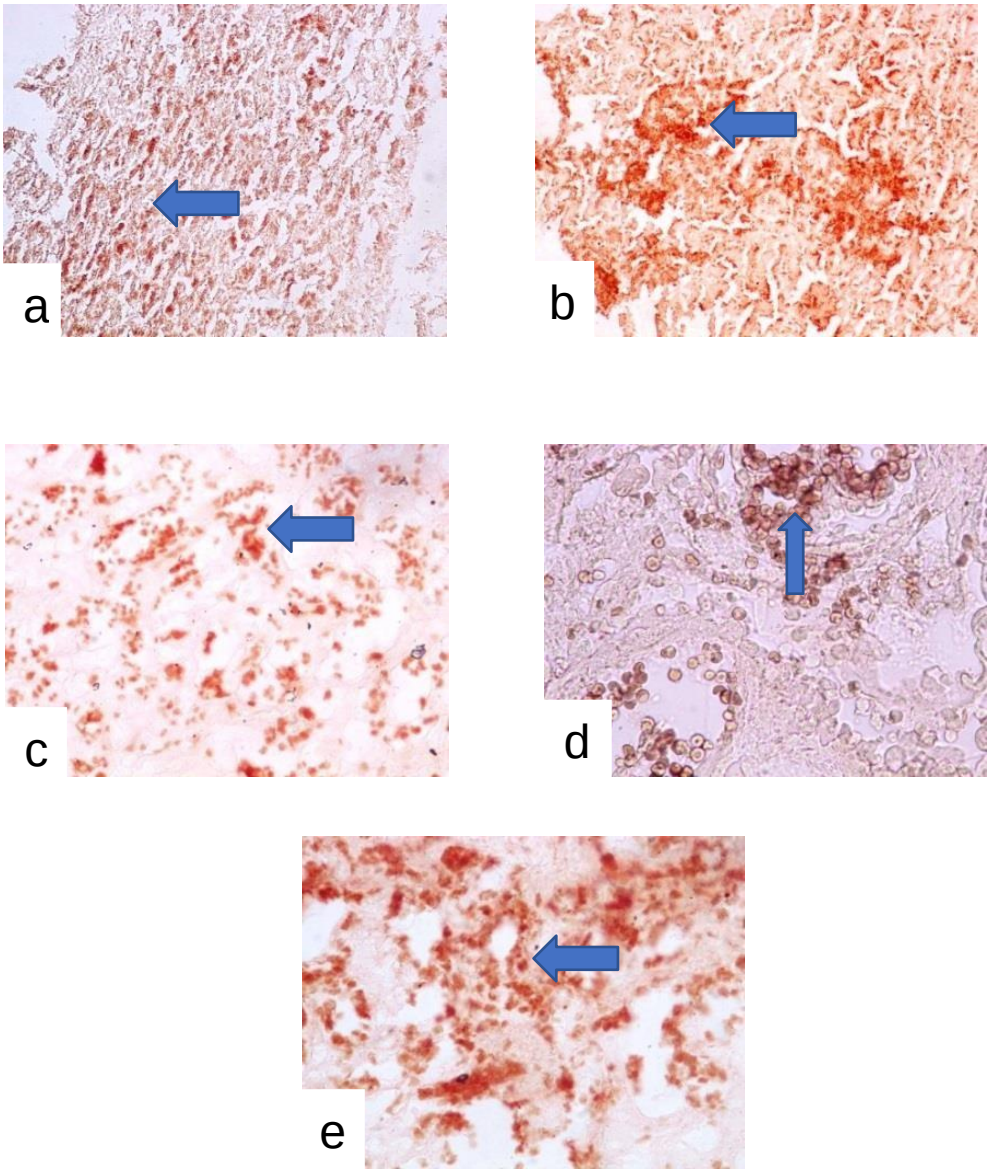


Plate 4.16: Qualitative immunohistochemistry of LEPR expression in female placenta sections from control, *Citrus limon* juice and its major components treated pregnant Wistar rat dams. Intensity of expressed protein (blue arrow). a=control, b=CLJ, c=OA, d=PA, e=SA. Tissues were stained with 3,3'-diaminobenzidine (DAB) and presented at ×100 magnification.

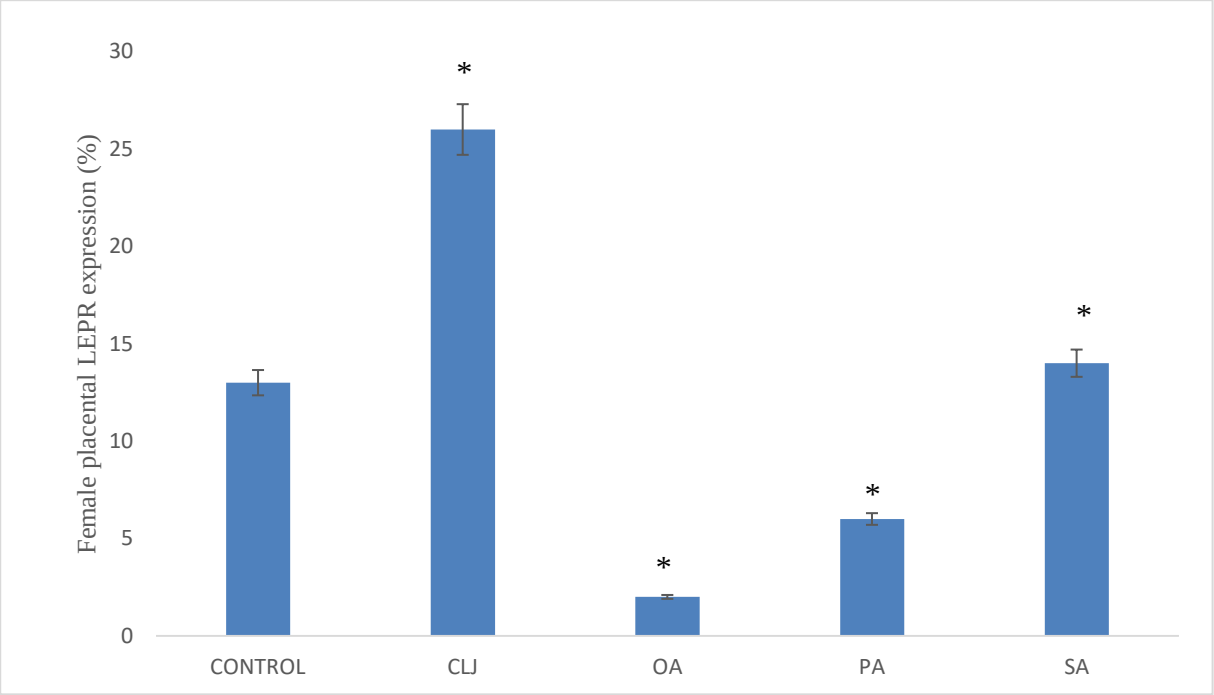


Figure 4.27. Quantitative immunohistochemistry of LEPR expression in the female placenta of control, *Citrus limon* juice (CLJ) and its major components treated pregnant Wistar rat dams.

*$p<0.05$ compared with control as generated from ImageJ 1.46 software and Student's t-test. CLJ=*Citrus limon* juice; OA=Oleic Acid; PA=Palmitic Acid; SA=Stearic Acid.

4.35.3. Effects of maternal *Citrus limon* juice on expression of peroxisome proliferating activating receptor gamma (PPAR-γ) in female placenta

The expression of PPAR-γ positive nuclei was high in palmitic acid (PA) and stearic acid (SA) groups in comparison with control but was low in *Citrus limon* juice (CLJ) and oleic acid (OA) groups in comparison with control (Figure 4.28).

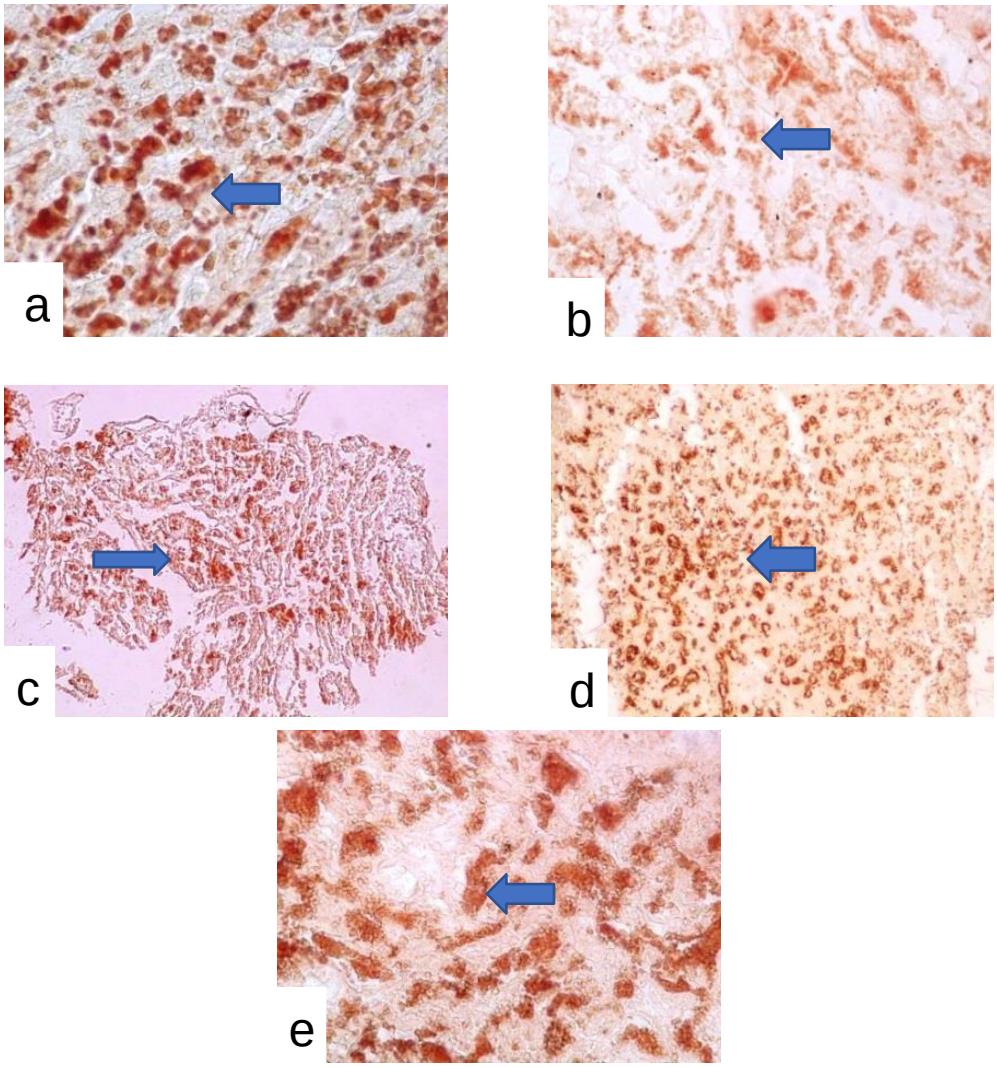


Plate 4.17: Qualitative immunohistochemistry of PPAR-γ expression in female placenta sections from control, *Citrus limon* juice and its major components treated pregnant Wistar rat dams. Intensity of expressed protein (blue arrow). a=control, b=CLJ, c=OA, d=PA, e=SA. Tissues were stained with 3,3'-diaminobenzidine (DAB) and presented at ×100 magnification.

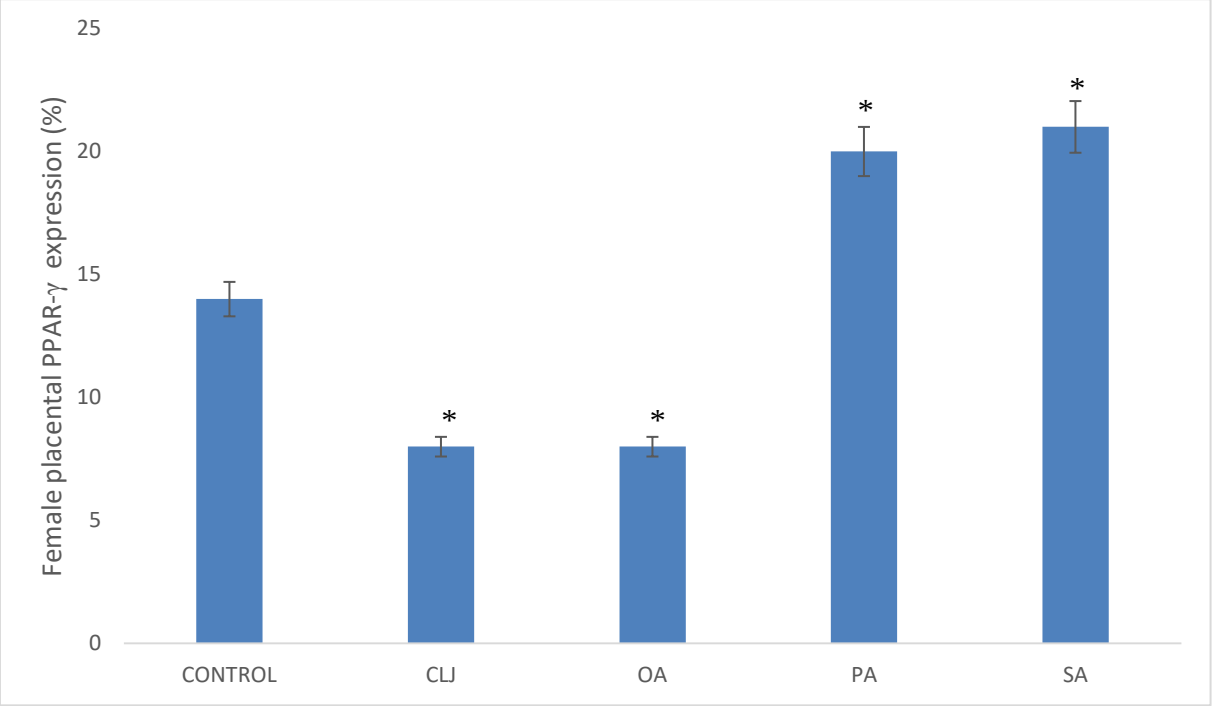


Figure 4.28. Quantitative immunohistochemistry of PPAR-γ expression in the female placenta of control, *Citrus limon* juice (CLJ) and its components treated pregnant Wistar rat dams.

*p<0.05 compared with control as generated from ImageJ 1.46 software and Student's t-test. CLJ=*Citrus limon* juice; OA=Oleic Acid; PA=Palmitic Acid; SA=Stearic Acid.

CHAPTER FIVE

DISCUSSION

5.1. Phytochemical Screening and GC-MS Analysis of *Citrus limon* juice (CLJ)

Qualitative phytochemical screening of *Citrus limon* juice (CLJ) observed the presence of saponins, flavonoids, alkaloids, terpenes, phenols and anthraquinone and the absence of steroids, cardiac glycosides, tannin, resins and reducing sugars (Table 4.1). The result of the present study corroborates the findings of Oguwike and Onubueze (2013). The compounds found in the juice has been shown to be involved in its biological activity, for instance terpenes and alkaloids are used in the prevention and treatments of cancers such as breast and prostatic cancer (Rauf *et al.,* 2014), flavonoids has also been observed to possesses anti-inflammatory, anti viral, antioxidant and hypolipidemic functions (Samundy *et al.,* 2016).

GC-MS analysis of *Citrus limon* juice revealed presence of 12 compounds of which 9-octadecenoic acid (oleic acid), n-Hexadecanoic acid (palmitic acid) and octadecanoic acid (stearic acid) are the major components obtained in the juice, in terms of percentage composition as extrapolated and retrieved from the values in GC-MS solution software and National Institute Standards and technology (NIST) library. The effects of maternal administration of *Citrus limon* juice, oleic, palmitic and stearic acids was thereafter assessed on placental efficiency and foetal morphometry in Wistar rats.

5.2. Effects of *Citrus limon* juice on reproductive functions in non-pregnant female Wistar rats

The preliminary study (study one) was designed to examine the effects of *Citrus limon* juice (CLJ) on reproductive indices in rats, the doses were selected in varying percentage of CLJ in distilled water to simulate how women of reproductive age takes the juice for the maintenance of weight purpose. The outcome of the study shows that administration of Citrus *limon* juice in non-pregnant Wistar rats, resulted in significant reduction in body weight and lipid profile as well as mild disruption of reproductive hormone and oestrus cycle. Its ability to reduce body weight and lipid profile corroborates its use as a hypolipideamic agent for weight reduction and mainainance (Khan *et al*., 2010; Olukanni *et al.,* 2013; Samundy *et al*., 2016). However, lipids being a major nutrients essential for optimal placental and foetal growth and development, its significant reduction in the mother and placenta could serve as an insult in utero thereby impacting on the optimal development of the foetus, consequently leading to development of diseases in adulthood, this is term developmental programming of adult diseases (Fowden *et al.,* 2006).

Administration of CLJ resulted into significant reduction in the oestrous cycle length in 75% CLJ group when compared to pre CLJ administration. Evaluation of the oestrus cycle in animals is a useful indicator in determining the integrity of the hypothalamic-pituitary-ovarian axis (Goldman *et al*., 2007). Proestrus is the phase of the oestrus cycle through which animals are procreative, during this phase ovarian follicle mature and uterine lining proliferates while ovulation occurs in the oestrus phase of oestrous cycle during which female animals are receptive to male animals (Kim *et al*., 2016). Treatment with *Citrus limon* juice produced significant reduction in the proestrus and oestrus phase frequency across the groups when compared with pre CLJ administration. Metestrus and diestrus phases are characterised by the activities of the corpus luteum which produces progesterone (Hirshfield, 1985). There was significant increase in frequency of metestrus and diestrus in all the groups post administration of CLJ when compared with pre administration. Increase in the frequency of metestrus and diestrus phase in animals is associated with lack of receptive behaviour (Kim *et al*., 2016). In addition, oestrous cycle ensues under the influence of follicle stimulating hormone, luteinizing hormone and oestrogen (Kim *et al*., 2016).

Although the concentration of luteinizing hormone and oestrogen increased in some of the groups, the changes observed in the oestrous cycle phases in the study may indicate sub-optimal tissue uptake of these hormones. Thus, the increase in frequency of the phases as well as reduction in the oestrous cycle days noted in the study likely suggests reduction in reproductive capability.

5.3. Effects of *Citrus limon* juice and its major components on maternal feed consumption, glucose concentration, hormones, lipid profile and oxidative stress markers

Maternal feed consumption was observed to be reduced in *Citrus limon* juice and stearic acid groups in the present study; the reduction corroborates its use for weight management. Consumption of low calorie diet as well as fatty acid rich foods such as *Citrus limon* juice has been reported to tend to be filling and induce satiety (Szalay, 2018). Hence the reduction in the feed intake observed in the study, suggesting stearic acid as the component of the juice that may be implicated to this effect. Maternal internal environment/ nutrition is associated with foetal and placental growth. Foetal glucose is derived from the mother exclusively, placental lactogen as well as transplacental glucose transport determines glucose delivery to the foetus (Kalhan and Parimi, 2000; Fowden *et al.,* 2006; Stuat *et al.,* 2012), although the blood glucose increased in the maternal circulation in this study, however placental lactogen and GLUT-1 concentration as well as expression of insulin receptor (IRS-1) was reduced in the study, this may predispose the foetus into developing hypoglycaemia, hypoglyceaemia has been reported to act as an insult in *utero* thus leading to foetal programming of adult diseases in later life (Fowden *et al.,* 2006). Pregnancy hormones such as progesterone, human chorionic gonadotropin, oestrogen and metabolic hormone like insulin and leptin are required throughout pregnancy in order to maintain optimal foetal growth and development (Gude *et al.,* 2004). The significant reduction that was observed in the level of progesterone and oestradiol in the study and other factors mentioned above could alter nutrient delivery and metabolism in the developing foetus.

Lipids are essential nutrient that regulate normal pregnancy homeostasis (Gude *et al.*, 2004). Maternal lipid profile was reduced in the study; this corroborates the reports that show that women of reproductive age (pregnant women inclusive) consume *Citrus limon* juice, due to its hypolipidemic property (Samundy *et al.*, 2016; Lixandru, 2016). Thus, the juice may be a potential insult which may trigger sub-optimal intrauterine environment in the developing foetus, consequently leading to foetal programming of adult diseases. Malondialdehyde is generated as a result of lipid peroxidation due to the activities of free radicals resulting into cell/tissue damage. The reduction in malondialdehyde and antioxidant enzymes observed in this study shows little or no maternal oxidation of lipids.

These findings corroborate the results of previous study which shows *Citrus limon* juice to possess anti-oxidant properties (Khan *et al*., 2010). The antioxidant property may be associated with flavonoid as well as terpene composition in the juice.

5.4. Effects of maternal administration of *Citrus limon* juice and its major components on male placental efficiency and foetal morphometry.

Due to the crucial intrauterine development of an individual, placenta has been an organ of interest and curiosity (Anjankar *et al.*, 2014). Placenta is critical for foetal growth and development because it provides an interface between mother and foetus through transport of nutrient substrates (Myatt, 2006; Fowden *et al.*, 2009). These substrates such as glucose, amino acids, fatty acids and cholesterol are essential for optimal foetal growth (Brett *et al.*, 2014). In a typical pregnancy condition, maternal triglycerides increase gradually throughout gestation and are substrates for placental lipase which is associated with free fatty acids release for transport to the foetus (Dutta-Roy, 2000; Gauster *et al.*, 2007). Fatty acids transport across the placenta is perilous for foetal growth, most especially in the last trimester of pregnancy because fat accumulation increases exponentially during this period (Larque *et al.*, 2013) and the transport is regulated by maternal triglycerides, placental lipase as well as fatty acid transporters such as FATP-1 (Schaiff *et al.*, 2005).

However, in this study maternal triglycerides and placental lipase reduced in CLJ group thereby affecting the transplacental delivery of free fatty acids to the foetus, thus impacting on optimal growth and development of the foetus. This may have resulted into foetal microsomia which is evident with the reduction in abdominal circumference and crown-rump length observed in the *Citrus limon* juice group. In addition, placental lactogen determines glucose delivery to the foetus (Stuat *et al.,* 2012), since glucose is a major macronutrient required for optimal placental and foetal growth, glucose delivery to the foetus is regulated by transplacental transporters such as GLUT-1, of which reduced concentration could impact negatively on optimal foetal growth (Brett *et al.*,2014). The observed reduction of placental lactogen and GLUT-1 in this study may predispose the foetus into developing hypoglycaemia, which has been reported to have long term health consequences (Fowden *et al.,* 2006).

Amino acids are essential for optimal foetal and placental growth because it is required for synthesis of protein and nucleic acids (Gude *et al.*, 2004). Their transplacental transport is dependent on transport proteins such as system A, an example of which is Small Neutral Amino Acid Transporter-1 (SNAAT-1) (Jansson, 2001). The observed reduction in the concentration of the placental amino acid transporter may have impeded placental efficiency thus the changes observed in the birth morphometric indices in the male pup contributed to the poor foetal outcomes in the male offspring.
Birth morphometric indices such as crown-rump length, abdominal circumference and birth weight as well as placental morphometric indices such as placental thickness are indication of intrauterine environment/nutrition and overall health of a foetus in *utero* (Shastry and Bhat, 2015). The observed reductions may have resulted into foetal microsomia which is evident with the reduction in abdominal circumference and crown-rump length as well as increase in placental thickness observed in the male pups. Hence, the changes in the quantity of nutrients provided to the male pups as a result of placental phenotype alteration may result into developing metabolic, reproductive and cardiovascular diseases in later life (Fowden *et al.,* 2009). The result of the male placental oxidative stress response suggests that increase in placental oxidation of lipids in CLJ group led to cell/tissue damage, which impacted negatively on the placental efficiency. Anti-oxidants enzymes were increased in order to mop up the effect of lipid peroxidation that was observed in the male placenta. These results

corroborate the findings of previous study that shows antioxidant property of the juice (Olukanni *et al.*, 2013).

In sum, administration of *Citrus limon* juice and its major components, resulted in reduction of placental lipase, lactogen and lipid profile which are associated with alterations in placenta and foetal morphometry in the offspring. Also reduction in the key transporters of nutrient observed in the study such as glucose, fatty acids and reduced receptor expression of these nutrient transporters may also have contributed to reduce placental efficiency, resulting in the changes observed in the birth morphometry of the foetus, which contributed to the poor pregnancy outcome in the foetus. Vaughan *et al.,* (2008) reported that reduced placenta efficiency has been linked to upsurge in the incidence of intrauterine growth restriction, consequently resulting into long-term consequences such as metabolic, reproductive and cardiovascular diseases in later life.
The placenta regulates transfer of nutrient by either morphological or functional adaptations so as to ensure optimal growth of the foetus (Sandovici *et al.,* 2012). These adaptations arise in response to maternal or foetal signals that eventually leads to alterations in placental efficiency (Fowden *et al.,* 2009). Placental efficiency is representation of placental adaptation, failure of placenta to adapt to intrauterine environment may results into foetal programming, which eventually upshot the risk of cardiovascular and metabolic disease in adulthood (Hayward *et al.*, 2016).

5.5. Effects of maternal administration of *Citrus limon* juice and its major components on female placental efficiency and foetal morphometry

Placental functions have been reported to be affected by adverse maternal conditions, thereby resulting into modifications in its efficiency (Tarrade *et al.*, 2015). There are differences in the way and manner with which male and female placentas respond to adverse intrauterine environment, this is as a result of some sex specific differences in the placenta (Brett *et al.,* 2014), hence the placenta is considered a central regulator of foetal programming (Tarrade *et al.,* 2015). Placental lipase ensures delivery of free fatty acid to the foetus (Gauster *et al.,* 2007), Contrary to the result obtained in the male, placental lipase concentration increased in the female, which is in support of previous studies that shows some sex specific differences in the placenta (Brett *et al.,* 2014). Similarly, to the results obtained in the male, placental lactogen was

reduced in all the groups except oleic acid group thereby impacting on glucose delivery to the foetus because glucose delivery to the foetus is associated with placental lactogen (Kalhan and Parimi, 2000).

Also, placental GLUT-1 and lactogen are required for optimal glucose transfer to the foetus (Stuat *et al.,* 2012), of which reduction in the concentration could alter glucose delivery to the foetus.Fatty acids are essential for development of brain in the foetus and accretion of fat especially at the third trimester (Larque *et al.,* 2013) and transplacental transfer and uptake of free fatty acids to the foetus is ensured by placental FATP-1 (Duttaroy, 2009), although the concentration of triglycerides as well as FATP-1 increased in all the groups except stearic acid group in the study, the female pups still ended up with poor intrauterine growth, this may be as a result of overall reduced placental efficiency observed in the study. In addition, amino acids being an essential nutrient for development of foetal tissue/organ (Gude *et al.,* 2004), the transplacental amino acids transport to the foetus is linked to small neutral amino acid transporter such as SNAAT-1 (Jansson, 2001). The reduction in concentration observed may compromise the optimal foetal growth and development, which is evident by the alteration in birth morphometry and placental efficiency observed in the female offspring. The reduction in the placental lipid profile obtained from the study may be as a consequence of reduced level in maternal circulation, since lipids are essential in pregnancy and placental functions have been reported to be affected by adverse maternal conditions (hypolipidemia), thus resulting in modifications of placental efficiency (Tarrade *et al.,* 2015), which could lead to long term consequences in the female offspring. The result of the oxidative stress response in female suggests increase in placental oxidation of lipids in *Citrus limon* juice (CLJ) group, which may consequently lead to cell/tissue damage, thereby impacting negatively on the placental efficiency. However, the level of antioxidant enzymes increased thereby moping off the effects of lipid peroxidation in the tissue, thus the results corroborate the findings of Olukanni *et al.,* (2013) which shows *Citrus limon* juice to possess antioxidant property.

Placental insulin receptor is involved in the processes of insulin delivery to the placenta and foetus, although its expression varies with the stage of the pregnancy, it is abundantly expressed in the first trimester however low expression at term is associated with aleration in insulin delivery, consequently affecting glucose and fatty acid transport to the foetus (Desforges and Sibley, 2010). This is evident by the alteration in birth morphometry observed in the foetus, as the amount of insulin transfer to the foetus has been associated with altered birth morphometry (Ruiz-Palacios *et al*, 2017). Leptin is associated with amino acid transport regulation in the placenta (Jansson, 2001), by interacting with amino acid transporter such as small neutral amino acid transporter. There was reduction in placental amino acid transporter SNAAT-1 assessed, this may be due to the reduction in the placental transfer of leptin, thereby affecting the growth of the foetus and consequently resulting into intrauterine growth restriction. Peroxisome proliferating activating receptor gamma is associated with placental fatty acid transport, fatty acid being one of the macronutrients needed for optimal growth of the foetus, thus reduction in its supply has adverse effect on foetal development and consequently programming the offspring for metabolic diseases in later life (Desforges and Sibley, 2010). For instance birth weight, which is a pointer of intrauterine foetal growth and nutrient supply was observed to be reduced in the study and has been associated with glucose intolerance and risk of metabolic diseases in aldulthood (Barker, 2004).

Placenta plays an important role as a central regulator of foetal programming (Tarrade *et al.*, 2015), Foeto-placental ratio (FPR) is a marker of placental efficiency (Hayward *et al.*, 2016), and it refers to gram of foetus per gram of placenta. Low FPR is linked to neonatal and maternal morbidity while high FPR is associated with uterine and umbilical pulsatility index (Sirpurkar *et al.*, 2015). Thus, FPR is an indicative marker of immediate or long-term health risk for an individual. The reduction in foetal weight, crown-rump length, foeto-placental ratio in the female foetus as well as increase in placental thickness and lipid peroxidation are due to reduced transfer of nutrient substrates from placental to the foetal circulation, these effects results into less efficient placenta.

Previous study has linked reduced efficiency of placenta to upsurge in the occurence of intrauterine growth restriction (Vaughan *et al.,* 2008), consequently leading to short term outcome such as pre-eclampsia, c-section delivery and preterm delivery and also long-term outcome like metabolic, reproductive and cardiovascular diseases (Wallace *et al.,*2012).

CHAPTER SIX

SUMMARY AND CONCLUSION

6.1. Research Findings

This study was carried out to assess the effects of maternal administration of *Citrus limon* juice (lemon) and its major components (oleic acid, palmitic acid and stearic acid) on placental efficiency and foetal morphometry in Wistar rats. The specific objectives were to determine its effects on serum levels of pregnancy hormones (rat chorionic gonadotropin, placenta lactogen, progesterone and oestrogen), placental and foetal morphometry and morphology, expression of key proteins in the transfer of lipids, glucose and amino acids in the placenta as well as maternal and placental lipid profile and lipid peroxidation. A preliminary study was done to determine the effects of the juice on reproductive hormones, redox status and lipid profile in non-pregnant Wistar rats. The results of the study show that the juice has hypolipidemic and anti-oxidants effects as well as mild disruption of reproductive hormones and oestrus cycle in the rats.

Subsequently, on the basis of the hypolipidemic property of Citrus *limon* juice and its use by pregnant women to prevent miscarriage and alleviate morning sickness, the second phase was designed to assess the effects of the juice and its major components on maternal hormone, lipid profile and redox status as well as placental nutrient transporters (GLUT-1, SNAAT-1 and FATP-1), foetal and placental morphometry. The juice and its major constituents were administered orally for 20 days. On gestation day 20 (GD-20), anaesthesia was administered to the animals, thereafter, non-survival caesarean section was performed in order to expatiate placenta and foetuses. Administration of *Citrus limon* juice and its major components negatively affected placental efficiency and foetal morphometry via alterations in the concentration of major placental nutrient transporters and key regulators of lipid and energy metabolism.

6.2. Conclusion

Maternal administration of *Citrus limon* juice and its major components reduced placental hormone secretions, lipid profile, oxidative stress markers, receptor expression and nutrient transporters in male and female placenta. Although, sex differences were obtained in the results i.e sexual dimorphism, the effects were more pronounced in male than female foetuses. Placental inefficiency and foetal morphometric outcomes observed in the study is linked to increased lipid peroxidation, reduction in the maternal lipid profile, nutrient transporters and reduction in the hormones that regulate lipid and energy metabolism, thereby impeding on the normal foetal growth and development. This is linked to the oleic acid and stearic acid constituents of the *Citrus limon* juice.

6.3. Contributions to knowledge

Maternal administration of *Citrus limon* juice (CLJ) and its major components (oleic, palmitic and stearic acids) resulted in decrease in maternal feed consumption in CLJ and SA groups, similarly maternal lipid profile and oxidative stress markers were decreased across the groups.

Maternal administration of *Citrus limon* juice increased placental lipid peroxidation, reduced placental lipid profile, placental nutrients transporters and receptor expressions.

Maternal administration of *Citrus limon* juice and its major components resulted in the reduction of the efficiency of placenta as well as pup morphometry.

Oleic acid and Stearic acid has been shown to be linked to the activity of CLJ obtained in the study.

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APPENDICES

1. Effects of *Citrus limon* juice (CLJ) and its components on lipid profile in pregnant Wistar rat dams.

GROUP	TC (mg/dL)	LDL-C (mg/dL)	HDL-C (mg/dL)	TG (mg/dL)
CONTROL	145.9±3.7	121.9±1.7	40.7±6.8	61.3±3.7
CLJ	172.8±4.5*	102.9±6.8*	25.1±1.1*	42.1±1.7*
OA	164.4±3.5*	101.6±6.5*	19.2±3.5*	58.7±3.7*
PA	156.7±1.4*	101.8±3.3*	27.3±4.6*	50.1±3.7*
SA	163.5±4.2*	106.2±1.6*	15.6±0.6*	50.9±1.5*

Data are presented as mean± SEM, n=5, *p<0.05 compared with control. TC= Total Cholesterol, LDL-C= Low Density Lipoprotein- Cholesterol, HDL-C= High Density Lipoprotein- Cholesterol, TG=Triglycerides. CLJ=*Citrus limon* juice; OA=oleic acid; PA=palmitic acid; SA=stearic acid.

2. Effects of *Citrus limon* juice (CLJ) and its components on serum level of rat chorionic gonadotropin and insulin concentration in pregnant Wistar rat dams.

GROUP	**Chorionic gonadotropin (mIU/mL)**	**Insulin (mIU/mL)**
CONTROL	3.6±0.4	1.1±0.1
CLJ	4.1±1.1	1.1±0.1
OA	2.8±0.9	1.1±0.1
PA	2.9±1.1	1.1±0.1
SA	3.5±1.0	1.0±0.1

Data are presented as mean± SEM, n=5. CLJ=*Citrus limon* juice; OA=Oleic Acid; PA=Palmitic Acid; SA=Stearic Acid.

3. Effects of maternal *Citrus limon* juice (CLJ) and its major components on male placental transporters.

GROUP	Placental Lactogen (ng/mL)	GLUT-1 (ng/mL)	FATP-1 (ng/mL)	SNAAT-1 (ng/mL)
CONTROL	1.1±0.5	15.9±4.8	20.5±2.1	16.2±4.7
CLJ	0.5±0.1*	5.8±1.5*	14.1±3.8*	6.4±1.2*
OA	0.5±0.1*	7.0±0.9*	34.2±0.8*	7.6±0.5*
PA	0.7±0.1*	17.5±0.5*	39.3±2.0*	19.2±0.4*
SA	0.3±0.1*	8.9±1.4*	57.0±1.9*	10.6±0.5*

Data are presented as mean± SEM, n=5, *p<0.05 compared with control. CLJ=*Citrus limon* juice; OA=Oleic Acid; PA=Palmitic Acid; SA=Stearic Acid.

4. Effects of maternal *Citrus limon* juice (CLJ) and its major components on female placental transporters.

GROUP	Placental Lactogen (ng/mL)	GLUT-1 (ng/mL)	FATP-1 (ng/mL)	SNAAT-1 (ng/mL)
CONTROL	0.5±0.1	16.0±2.2	16.9±1.0	18.1±2.1
CLJ	0.3±0.1*	10.5±2.1*	21.6±2.6*	10.2±1.7*
OA	0.6±0.1	5.7±1.5*	38.9±3.6*	6.1±1.0*
PA	0.4±0.1	16.5±3.7*	22.5±3.2*	13.7±3.6*
SA	0.5±0.1	12.3±1.2*	13.7±2.1*	11.1±1.0*

Data are presented as mean± SEM, n=5, *p<0.05 compared with control. CLJ=*Citrus limon* juice; OA=Oleic Acid; PA=Palmitic Acid; SA=Stearic Acid.

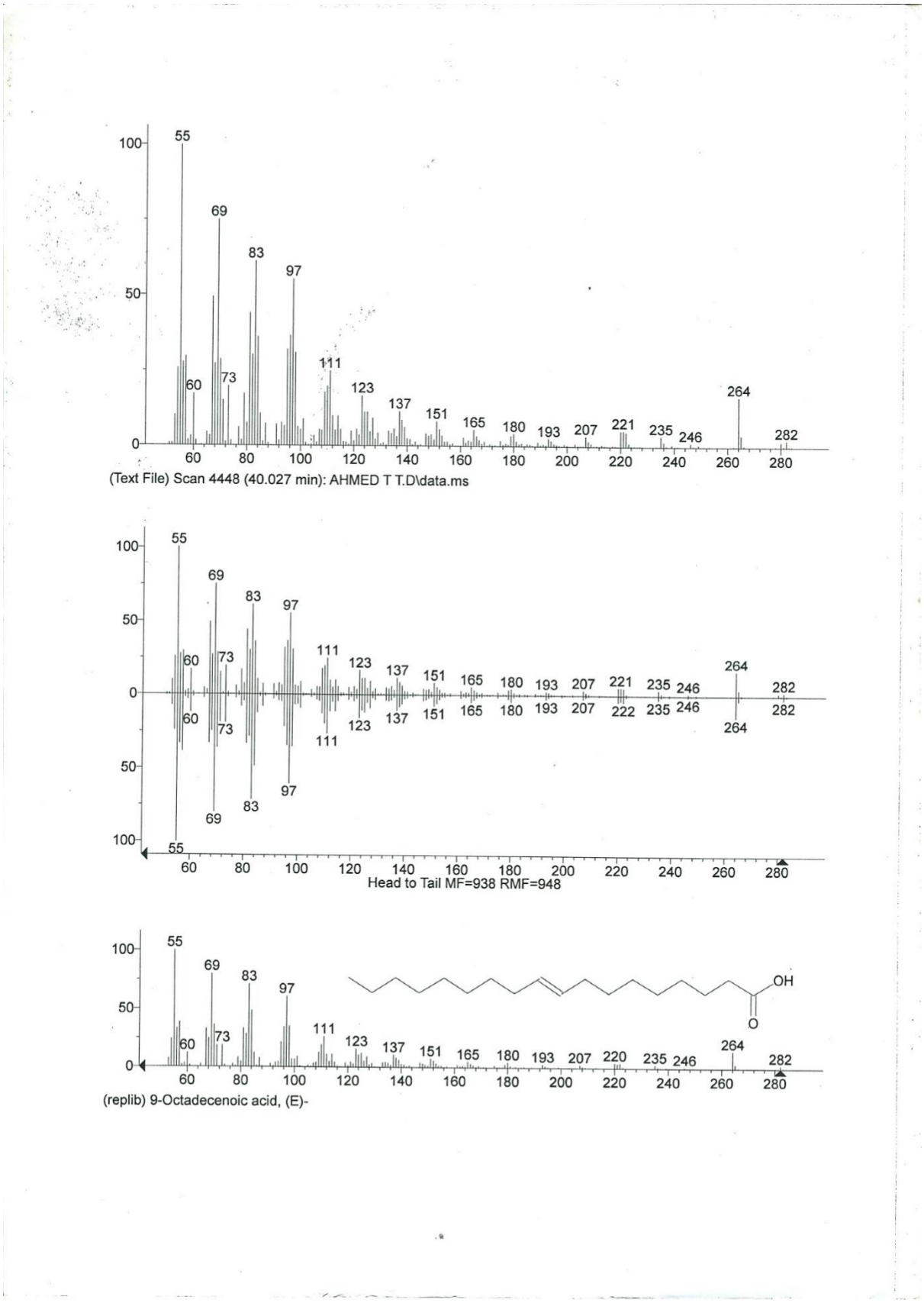

Figure 1 Fragmentation pattern and structure of 9-Octadecenoic (oleic acid)

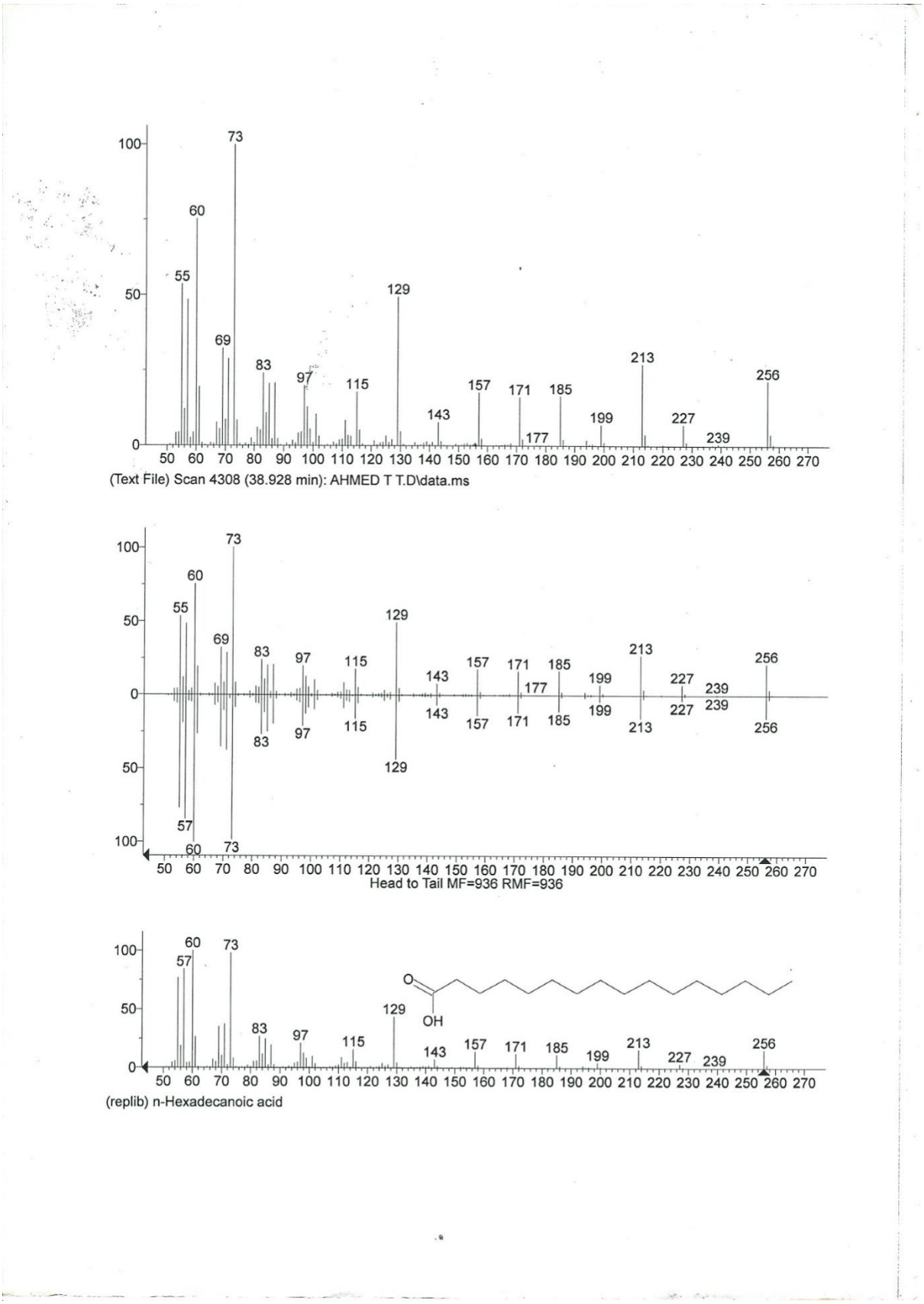

Figure 2 Fragmentation pattern and structure of n-Hexadecanoic (palmitic acid)

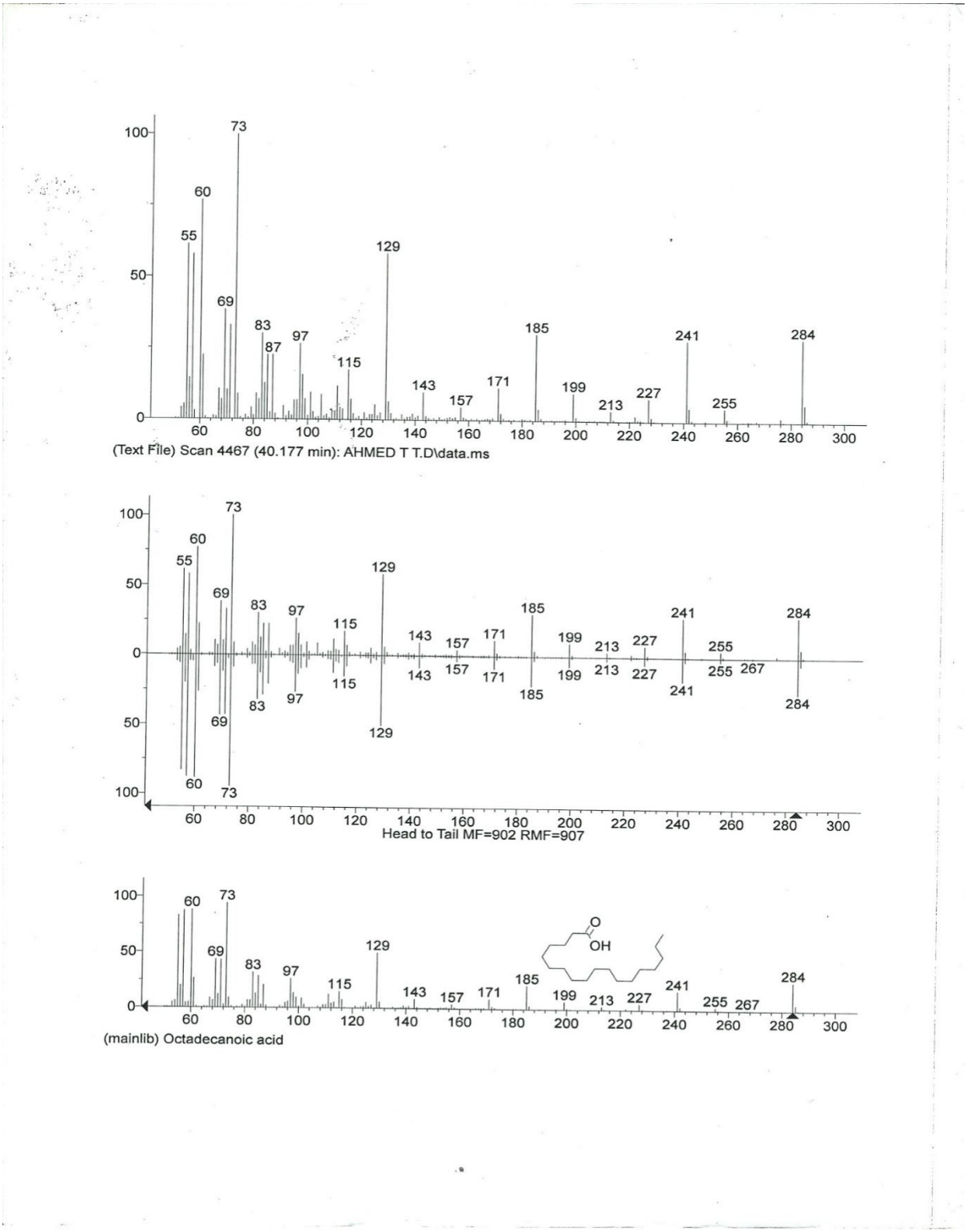

Figure 3 Fragmentation pattern and structure of Octadecanoic (stearic acid)

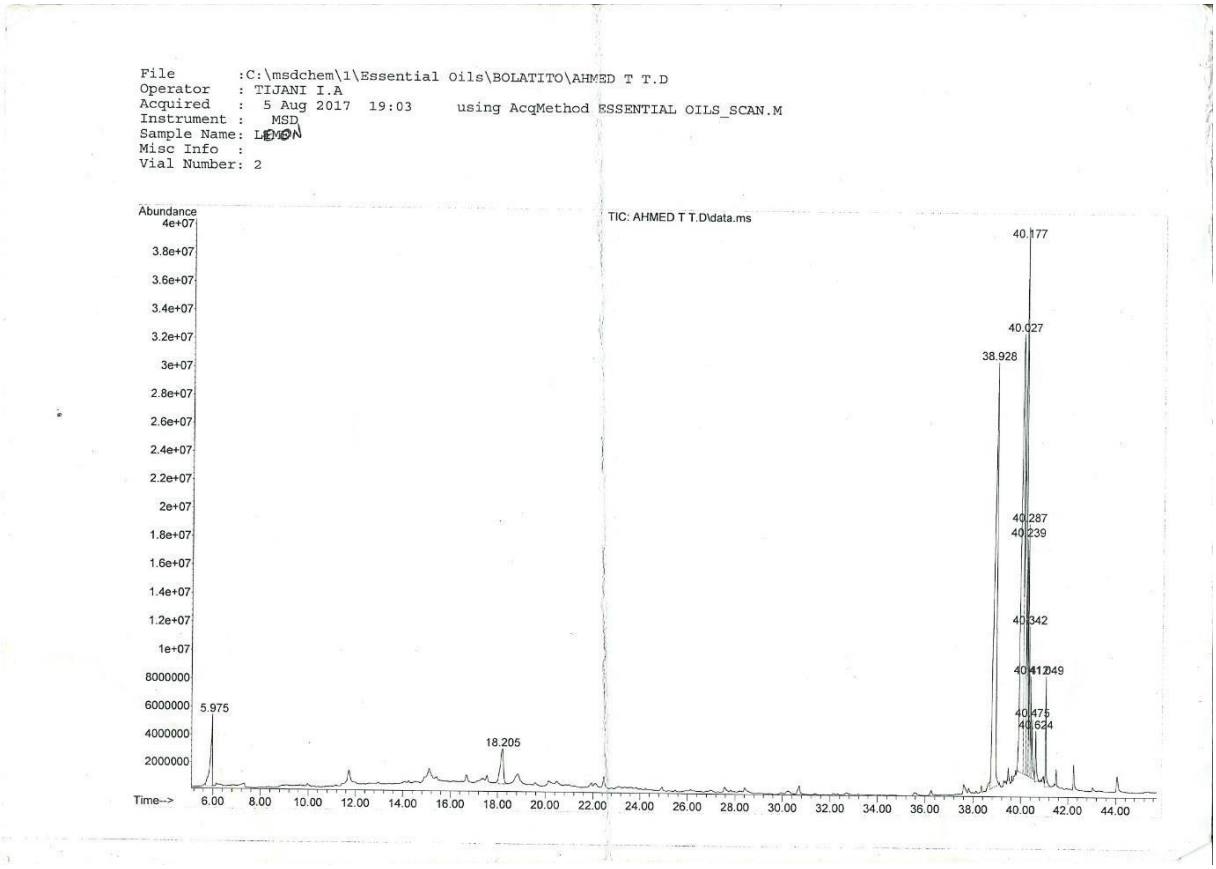

Figure 4 Gas chromatogram of the chemical constituents in *Citrus limon* juice