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ETHYL ACETATE EXTRACT OF *LANNAE EGREGIA* LEAVES ATTENUATES HAEMATOLOGICAL DISORDER AND DYSLIPIDAEMIA ASSOCIATED WITH METHOTREXATE TOXICITY IN RATS

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ABSTRACT

Methotrexate is a known chemotherapeutic agent with high degree of potency accumulating in tissues inducing varying degrees of toxicities. This study investigated the haematological disorder and dyslipidaemia associated with methotrexate toxicities and the effects of ethyl acetate extract of *Lannae egregia (EELE)* leaves, a local anticancer medicinal plant in rats. Forty-eight male Wistar rats averagely weighing 150 g were randomly selected into six groups **:** group A (distilled water only), group B (2.5 mg/kg.bw of methotrexate only for 21days at 7days interval), group C (pre-treated with 100 mg/kg.bw of EELE daily and 2.5mg/kg.bw of methotrexate at 7days interval), group D (pre-treated with 100 mg/kg.bw of ascorbic acid daily and 2.5mg/kg.bw of methotrexate at 7days interval), while group E and F were administered 100 mg/kg.bw of EELE and 100 mg/kg.bw of ascorbic acid daily respectively. Administration was done orally in 0.1 ml solution for 35 days. Plasma total protein levels, haematological parameters and lipid profile were determinates via quality methods. Results showed that group B, elicit significant ($p<0.05$) decreases in total protein levels, red blood cell count, haemoglobin concentrations, packed cell volume and high-density lipoprotein-cholesterol levels with corresponding significant ($p<0.05$) increases in white blood cell count, lymphocyte, platelets, triglyceride and cholesterol levels in the plasma. However, combined treatment with methotrexate and EELE (group C) as well as rats treated with methotrexate and ascorbic acid (group D) showed ameliorative effect following metabolic alterations associated with methotrexate administration as these parameters were reversed significantly with greater effects observed in rats treated with extract only (group E) and ascorbic acid only (group F). Results are indication of haematological disorder and alterations of lipid metabolism by methotrexate, while EELE attenuates these effects via possible anti oxidative effect that compared favourably with ascorbic acid. It also showed synergistic effect with ascorbic acid in attenuating methotrexate toxicity.

Keywords: *Lannae egregia*, haematological disorder, attenuates, ethyl acetate extract, dyslipidaemia, Anti-oxidative.

INTRODUCTION

The occurrence and management of cancer globally have led to ever increasing modalities employed for its treatment (Ochwang *et al*., 2014). Various methods used in the treatment of cancer includes immunotherapy, hormonal therapy, surgery, radiotherapy and the use of chemically derived drugs (chemotherapy) known to be common, effective and potent for treating different types and stages of cancer while its usage has also been shown to prevent recurrence following surgery (Poornima *et al*., 2014).

Methotrexate, chemically called 4-amino-10-methylpteroylglutamic acid is an antineoplastic, antimetabolites and cytotoxic agent widely employed in the treatment of broad range of cancers (Benedek, 2010). Methotrexate is a folate antagonist (substrate analoque) and an inhibitor of dihydrofolate reductase (Deepak *et al*., 2017). It also blocks a number of enzymes involved in purine and pyrimidine metabolism (Chen *et al*., 2011). The pharmacological efficacy reported for methotrexate against malignancies (Gossec *et al*., 2012) is accompanied with speculative bone marrow, haematological, hepatological, and pulmonary toxicities that are linked to its free radical producing potential and the accompanied oxidative stress (Gaies *et al*., 2012; Xiang *et al*., 2013).

It has been reported that methotrexate toxicity has adverse side-effects on the hematopoietic system without considering other toxicities, as methotrexate has been known to induced significant alteration in haemoglobin, RBC, WBC, packed cell volume, platelet, neutrophil and lymphocytes in rats (Patel *et al*., 2014; Deepak *et al*., 2017). Furthermore, increasing evidence revealed dyslipidaemia as hallmark of cancer during chemotherapy (Chen *et al*., 2011) as studies in patients with different

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types of cancers have demonstrated that levels of plasma lipids and lipoprotein fractions, particularly high-density lipoprotein cholesterol (HDL-c) and triglycerides (TG) are associated with cancer risk probably mediated via an excessive intake of dietary fats, saturated fats or trans fats. Thus, during chemotherapy altered blood lipids is associated with changes in regulatory factors, enzymes and transporters involved in lipid synthesis, lipid transport and lipid degradation which are known to be a major factor linked to dyslipidaemia (Chen *et al*., 2011). The etiology of lipid changes associated with chemotherapy is multifactorial and the relationship of lipid changes in cancer patient is still enigmatic.

The use of adjunvant drugs such as vitamin A, C, E and other agents with suitable antioxidant properties have been reported to reduce the toxic effect of chemotherapeutic drugs (Al-Motabagani, 2006). Evidences have shown that medicinal plants are important sources of antioxidant with potential to alleviate oxidative stress-induced complications, toxicities and inflammations (Adedosu *et al*., 2014). In as much as many of these plants are known to contain certain chemical constituents which may have been employed as herbal supplements for treatment of diseases locally (Tag *et al*., 2012). *Lannea egregia* belonged to the family of Anacardiaceae (Soladoye *et al*., 2010), geographically distributed in both tropical and sub-tropical African countries (Jasen, 2005). It is usually identified as false marula and locally known as ekudan in Nigeria (Yoruba) (Soladoye *et al*., 2010). Several species of *Lannae* have been used for a variety of indigenous medicine locally (Abdullahi *et al*., 2014) as their part (including the roots) had been employed in folk medicines in African countries to treat various ailments in humans (Paulsen and Malterud, 2014). The stem bark decoction is administered as a stomachic to improve the haemoglobin level and as part of vermifuge medicine (Arbonnier, 2004). The leaves of *Lannea egregia* are also used traditionally as an anticancer herb with other concoctions (Soladoye, 2010).

Hence, this study evaluated the potentials of ethyl acetate extract of *Lannae egregia* leaves to attenuate haematological and dyslipidemic alterations induced by methotrexate administration in rats, so as to validate its medicinal claims and as a possible template for drug discovery and also as a possible adjuvant source in the treatment and management of cancer in some poor African countries who still used orthodox drugs with traditional herbs.

METERIALS AND METHODS

Materials

In the present work we used dissecting sets, syringes and needles, electronic weighing balance, measuring cylinders, triple weighing balance, pH meter, centrifuge, plasma bottles, micropipette, separating funnels, spectrophotometer, thermometer, refrigerator, stopwatch and other relevant materials.

Reagents

We used good quality reagent obtained mostly from Sigma USA and Chemelex Barcelona Spain. These are Tris buffer, distilled water. Laboratory kits for quantitative determination of total protein (TP), total cholesterol (TC), total triglycerides (TG) and high density lipoprotein (HDL). Methotrexate, a product of Ebewe Pharmaceutical company, Austria, was obtained at Boluke pharmaceuticals Agege, Lagos state, Nigeria.

Plant collection and preparation of Ethyl acetate Extract

Lannae egregia leaves was collected at Igbeti area of Oyo State, Nigeria and identified at the Botany Unit of the Department of Pure and Applied Biology, Ladoke Akintola University of Technology, Ogbomoso with herbarium voucher number LHO 520 deposited. The plant was air dried in the laboratory at room temperature and powdered after dryness while 1500 g of the powdered leaves were soaked in 5000 ml ethyl acetate for 72 hours in a dark cupboard and filtered using filter paper. The filtrate was concentrated to dryness at room temperature to obtain dried crude ethyl acetate extract (Wu *et al*., 2009).

Designing of Experimental Animals Groupings

For this study, 48 male Wistar rats averagely weighing 150 g were obtained from the animal house of College of Health Sciences, Ladoke Akintola University of Technology, Ogbomoso, Oyo State. We strictly followed guidelines on ethics and conducts for handling experimental animals for research institutions which conforms with the international standards and also the ARRIVE Guidelines (Animal Research: Reporting of In Vivo Experiments), 2010 (https://www.nc3rs.org.uk/sites/default/files/documents/Guidelines/NC3Rs%20ARRIVE%20Guidelines%202013.pdf) and PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines, 2017 (https://norecopa.no/prepare).

Experimental animals were housed in cages and acclimatized for two weeks under standard laboratory conditions. For the experiments, test animals were randomly divided into six groups with eight animals each, namely A, B, C, D, E and F. The various groups and treatment received are shown below:

Group A: Animals received distilled water only.
Group B: Animals treated with methotrexate only.
Group C: Animals treated with extract and methotrexate
Group D: Animals treated with ascorbic acid and methotrexate
Group E: Animals administered extract only
Group F: Animals treated with ascorbic acid only
Group G: Animal treated with extract and ascorbic acid

Experimental Protocols

Using corn oil as vehicles, 100 mg/kg body weight of extract were made into 0.1 ml of corn oil, while 100 mg/kg body weight of ascorbic acid were made into 0.1 ml of distilled water respectively and given to the rats once daily for 14days (pre-treatment) by intubation. Methotrexate (2.5 mg/kg body weight made into 0.1 ml of distilled water) was administered orally for 21days at 7 days interval to group B, C and D. The animals were fasted and sacrifice 24hrs after last administration through cervical dislocation.

Preparation of plasma

The study animals were euthanized using mild anaesthetic chloroform and blood was obtained through cardiac puncture from the jugular vein using a 5 ml syringe and needle. The obtained blood was transferred into a centrifuge bottle and centrifuged (model 3538) at 4000 rpm for 10 minutes. The plasma (supernatant) was extracted into plasma bottle, covered and stored at 4^{o}C inside refrigerator (Jin and Manabe, 2009).

Biochemical indices studied and their determination

The total protein concentrations of the plasma were determined by Biuret (colorimetric) method as described by Gomail (1949). While haematological parameters were determined by Flow cytometry which involved the passage of cells in single files in front of a laser where they are detected, counted and sorted as blood cell components are fluorescently labelled and then excited by the laser to emit light at varying wavelengths after heamolysis. Also, levels of total plasma cholesterol were determined spectrophotometrically by the enzyme hydrolysis of cholesteryl esters (Naito, 1984), while triglycerides concentration was determined by enzymatic colorimetric method using standard diagnostic triglycerides kits as described by Buccolo *et al*. (1973). The quantitative determination of high density lipoprotein (HDL-C) cholesterol was based on HDL-cholesterol (HDL-C) precipitating method of Naito (1984).

STATISTICAL ANALYSIS

Data were expressed as mean ± SD, analysed by one way ANOVA and considered significant at $P<0.05$ using Statistical Package for Social Sciences (SPSS) 21.0 and graph pad prism.

RESULTS AND DISCUSSION

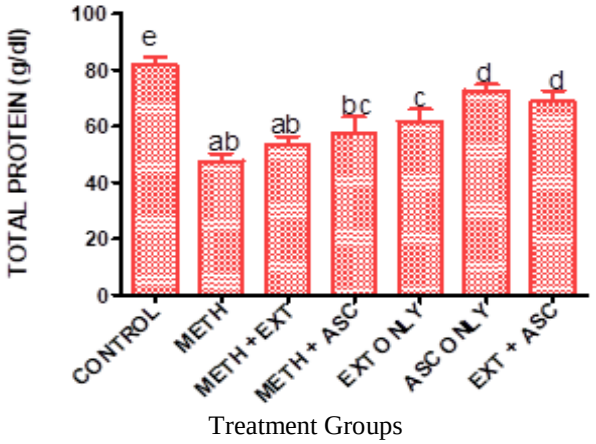
Complications arising from chemotherapy are major concern for cancer patient undergoing treatment (Fatoki *et al*., 2018). Evidences have shown that chemotherapeutic agents perturb normal haematological and lipid homeostasis as various studies have implicated haematopoietic disorder, lipid and lipoprotein abnormalities as common risk factors associated with hemoglobinemia, erythrocytopenia, leucopenia, thrombocytopenia and atherosclerotic pathogenesis (Deepak *et al*., 2018). Similar observations in blood parameters following methotrexate administration has been reported earlier (Rofe *et al*., 1994).

Proteins such as albumin and globulin are constituents of muscles, enzymes, hormone and many other key functional and structural entities in the body. These proteins are carriers of many ions, transporters of materials and are involved in maintenance of normal water distribution between blood and tissue, while their determination and metabolism has been of good index for etiology of diseases.

The observed reduction in plasma total protein level (Fig. 1), is possibly attributed to low circulating albumin due to alteration in plasma protein binding to tissue suggestive of protein degradation and an indication of methotrexate behaviour in malignant diseases (Leeb *et al*., 1995; Rajnics *et al*., 2017). Interestingly, the ability of both extract (group C) and ascorbic acid (group D) when used singly with METH or combined to reverse this trends may be suggestive of their ability to increase protein synthesis probably by eliciting decreases in reactive oxygen species (ROS) production, an indication of their possible potential in ameliorating or alleviating methotrexate-induced toxicity via oxidative stress (Heaney *et al*., 2008; Yiang *et al*., 2014; Wu *et al*., 2017). In addition, the ability of the combined treatment (extract and ascorbic acid) to increase total protein level is an indication of their ability to synergistically boost and protect the antioxidant status of the body against intoxication and degradation of body proteins.

One of the major roles of the blood is the maintenance of homeostasis as a special circulatory tissue. It contains haematological components which are employed as valuable tools in monitoring dietary toxicity that affect blood as well as the health status of animals and individuals. The observed significant ($p<0.05$) increases in levels of the defensive cells of the blood; the white blood cells, lymphocytes and platelets (Figs. 2,3 and 4), due to methotrexate administration (group B) possibly indicate the ability of these cells to fight against toxins or abrupt change in cellular environment or presence of antigens which may elicit leukocytosis, lymphocytosis, and thrombocythemia, respectively presenting the toxic effect of methotrexate on blood erythropoietic system. However, the decreases observed in the levels of these blood indices in combined treatment of methotrexate with extract (group C) and ascorbic acid (group D) respectively, counteracts methotrexate-induced toxicity, and an hint to the plant extract and ascorbic acid potentials to reversed this effect may be attributed to their antioxidant potential and their protective effect on membrane where they successfully protect these

components from peroxidative damage due to methotrexate-induced oxidative stress Antunes *et al.* (1996) and Cesquini *et al.* (2003). Additionally, the observed decreases in the levels of the nutritive cells of the blood; red blood cell, haemoglobin count and packed cell volume due to methotrexate administration (Figs. 5, 6 and 7) in this study revealed red blood cell and haemoglobin loss suggestive of anaemia which could be attributed to cell degradation, haemolysis and failure of the bone marrow to produce more red blood cells due to intoxification. Also, the reduction in the values of these blood parameters have been linked to mild or moderate methotrexate-induced suppression of bone marrow where profuse bleeding due to intestinal injury and free radical-induced red cell damage have contributed to lowered red blood cell counts and haemoglobin concentration (Pradhan *et al.*, 2016). However, co-administration of extract and methotrexate (group C), elicits increases ($p<0.05$) in red blood cell count, haemoglobin levels and packed cell volume comparably with ascorbic acid.



KEYS for Figs. 1 to 10 : **A** [Control], **B** [Methotrexate], **C** [Methotrexate + Extract] **D** [Methotrexate + Ascorbic acid, **E** [Extract only], **F** [Ascorbic acid] **G** [Extract and Ascorbic acid] METH = Methtrexate, Ext = Extract, ASC = Ascorbic acid

Fig. 1. Plasma total protein concentrations in various treatment groups. Values are given as mean and standard deviation of eight determinations. Bars with different superscripts are significantly different from each other ($p<0.05$).

Interestingly, the marked increases in white blood cell, lymphocytes and platelet counts accompanying corresponding decreases in red blood cell, haemoglobin counts and packed cell volume have been linked to activation of the immune system to form defensive cells (Ohbayashi *et al.*, 2010). Nevertheless, the behaviour of ethyl acetate extract of *Lannae egregia* leaves and ascorbic acid when administered alone or in combination with methotrexate from this study showed the extract potential to ameliorate the effects of methotrexate toxicities to blood components probably due to its bioactive constituents.

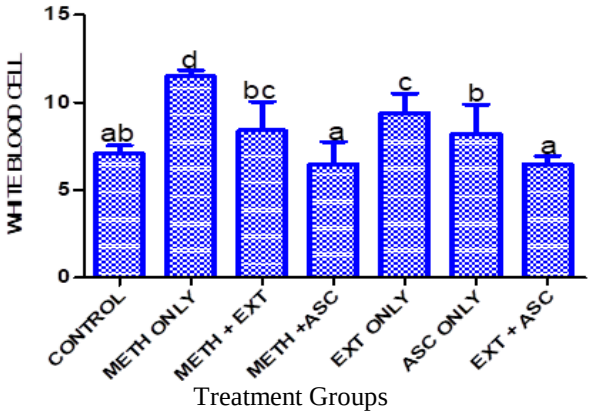


Fig. 2. White blood cell counts in the various treatment groups. Values are given as mean and standard deviation of eight determinations. Bars with different superscripts are significantly different from each other ($p<0.05$).

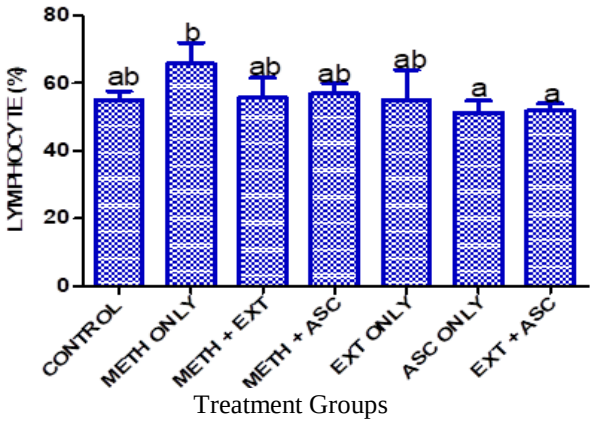


Fig. 3. Total Lymphocyte counts in the various treatment groups. Values are given as mean and standard deviation of eight determinations. Bars with different superscripts are significantly different from each other ($p<0.05$).

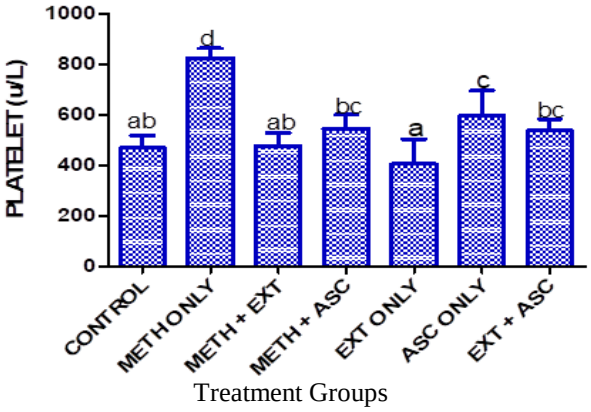


Fig. 4. Platelet counts in the various treatment groups. Values are given as mean and standard deviation of eight determinations. Bars with different superscripts are significantly different from each other ($p<0.05$).

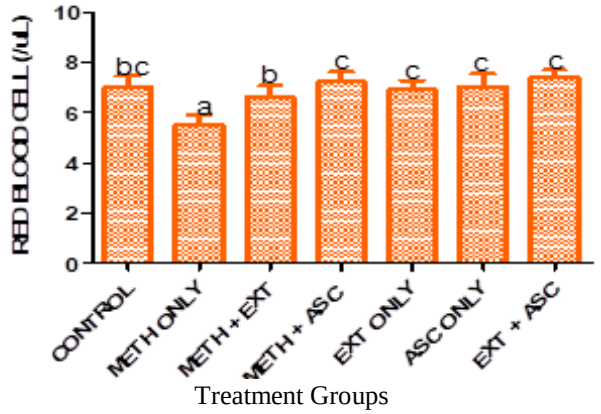


Fig. 5. Red blood cell concentration in the various treatment groups. Values are given as mean and standard deviation of eight determinations. Bars with different superscripts are significantly different from each other ($p<0.05$).

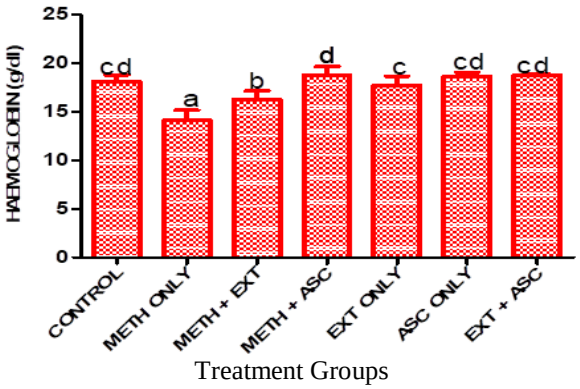


Fig. 6. Haemoglobin concentrations in the blood samples of various treatment groups. Values are given as mean and standard deviation of eight determinations. Bars with different superscripts are significantly different from each other ($p<0.05$).

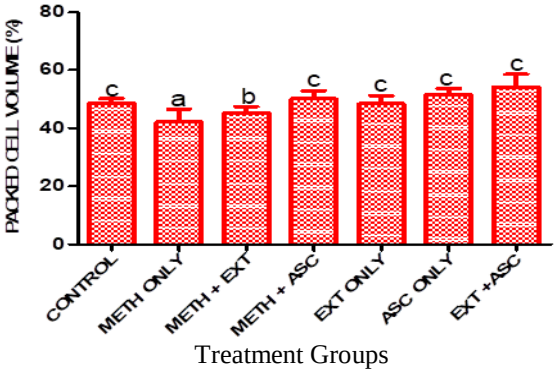


Fig. 7. Packed cell volume of the various treatment groups. Values are given as mean and standard deviation of eight determinations. Bars with different superscripts are significantly different from each other ($p<0.05$).

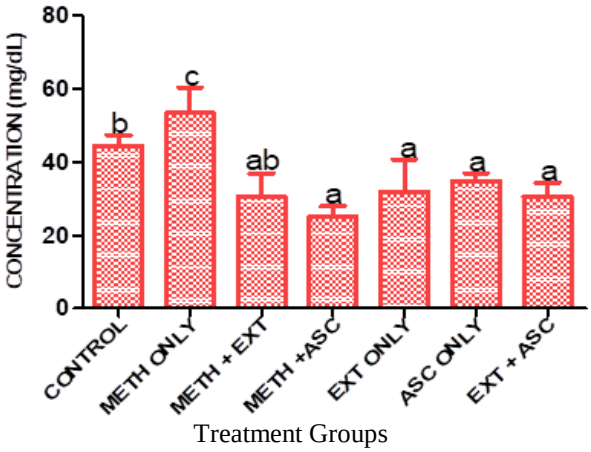


Fig. 8. Plasma total triglyceride levels in various treatment groups. Values are given as mean and standard deviation of eight determinations. Bars with different superscripts are significantly different from each other ($p<0.05$).

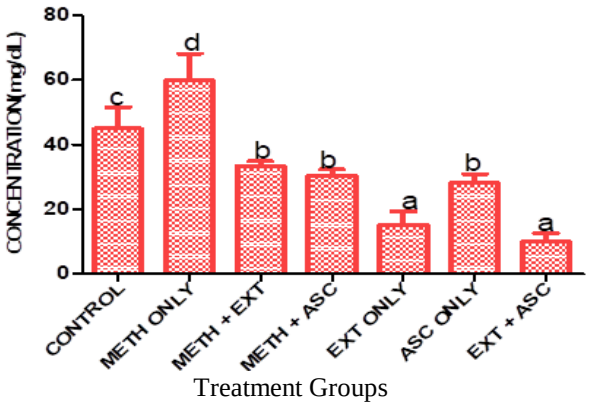


Fig. 9. Plasma total cholesterol levels in various treatment groups. Values are given as mean and standard deviation of eight determinations. Bars with different superscripts are significantly different from each other ($p<0.05$).

Determination of the plasma lipid profile is deployed as a marker of dyslipidemia which correlates with many disorders of lipid metabolism especially in pathological states. The results of the lipid profile (Fig. 8 and 9), respectively showed elevated levels of total triglycerides and total cholesterol in methotrexate treated animals only (group B) compared with control and other treatment groups. The observed increase in total triglyceride and cholesterol levels were associated with decreased level of high density lipoprotein cholesterol (HDL-c) as seen in Figure 10, suggestive of methotrexate-induced dyslipidaemic alterations, and an indication of increasing hepatic fatty acid synthesis accompanying a provoked rise in key enzyme activities Girotti (1998) and Hockenberry *et al*. (2014). However, the significant ($p<0.05$) decrease observed in the levels of total

triglycerides and total cholesterol as well as the increase in HDL-c levels with the treatment of extract and ascorbic acid either singly or in combination with methotrexate is connected with possible anti oxidative potential of the extract reducing oxidative stress and limiting membrane lipid peroxidation due to the action of methotrexate following concomitant supplementation.

The lipid-lowering effect of extract and ascorbic acid either when used singly or combination with methotrexate may also probably due to inhibition of hepatic fatty acid synthesis by lowering key enzyme activities supplying the substrates (Feingold and Grunfeld, 2018). Behaviour exhibited by the plant extract in this study have been favourably linked to their phytochemical constituents in which flavonoids have been majorly identified which may have justify their medicinal use in folk medicines (Okoth *et al*., 2013; Kumar and Jain, 2015), while the presence of flavonoids in many plants have indicated their significance in pharmaceutical industries for the production of antimicrobial, antioxidant ,anticancer and other anti-inflammatory drugs (Havsteen, 2010).

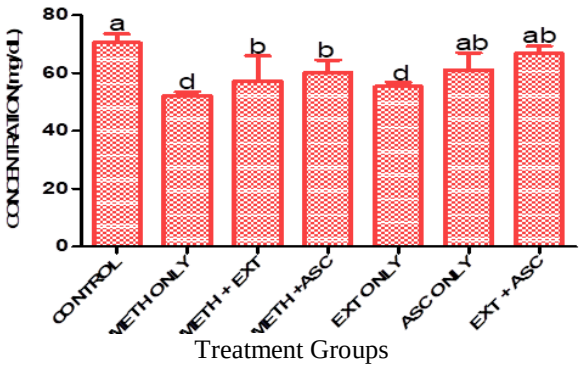


Fig. 10. Plasma high density cholesterol concentrations in various treatment groups. Values are given as mean and standard deviation of eight determinations. Bars with different superscripts are significantly different from each other ($p<0.05$).

CONCLUSION

Studies have shown that anticancer activities can be detected from herbal medicine via screening of these plants in varieties while exploitation of these resources may have breakthrough via clinical trials and identification of drug template in these plants. Hence this study validated methotrexate toxicity shown by haematological disorder and dyslipidaemia in rats via possible induction of oxidative stress as its mode of action. However, the ethyl acetate extract of *Lannae egregia* leaves demonstrated potent anti-oxidative, tissue protective and ameliorative effects compared with ascorbic acid against methotrexate toxicity, justifying its usage in folk medicine in the treatment and management of cancer and other related diseases connected to oxidative stress while its further study as possible drug template is recommended.

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