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Immunological Biomarkers Determined In Female Rats Administered With Pro-Fertility Extract Of *Anthocleista Vogelii*

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ABSTRACT
OBJECTIVE:*Anthocleista vogelii* Planch, is a plant that has been widely used by Traditional medicine practitioners either singly or in combination with other plant materials to treat several diseases or ailments in humans, including infertility problems both in male and female.
DESIGN: Ethanolic extract of *Anthocleista vogelii* were administered orally for 14 days to rats placed in different groups. Temporary infertility in female rats was induced with Micronor (norethisterone) or N-acetylcysteine (NAC) which was given orally for seven (7) days prior to other treatment. The rats were sacrificed after the completion of extract administration. Blood samples from experimental animal groups were collected through cardiac puncture and transferred into potassium ethylene diamine tetra acetate (K_3EDTA) tubes and plain tubes. The absolute counts of clusters of differentiation CD4+ and CD8+ was performed using the Becton Dickinson's (BD) FACS Count Automated technique. The haematological parameters were performed using the Sysmex® Automated Hematology Analyzer KX-21N. Oestradiol analysis was performed on sera obtained from the experimental animals using commercial standard enzyme-linked immunosorbent assay kit.
RESULT / OUTCOME: The extract was found to possess Anthraquinones, Terpenoids, Flavonoids, Saponins, Alkaloids, Phenols and Phytosterols The obtained results of the ethanolic extract of *Anthocleista vogelii* administered group compared with control showed a statistically significant decrease ($P<0.05$) in CD4+ and CD8+ counts, from (851.33±96.34) to (451.00±21.02) and from (1058.67±93.31) to (636.00±28.93) for CD4+ and CD8+ cells respectively. The haematological parameters showed a significant increase ($P<0.05$) in absolute middle cells (basophils, eosinophils and monocytes) count. The obtained results showed a significant increase of estradiol concentration in the female rats, from (184.65±30.06 pg/ml) in the control group compared to (288.29±30.06pg/ml) in the extract treated group.
These findings suggest that *Anthocleista vogelii* may have a role in creating the environment required for successful pregnancy by decreasing the levels of CD4+ and CD8+ cytokines production and increasing monocytes and granulocytes activation. The plant can be used to induce oestrogen production therefore, supports the claims on the traditional use of *Anthocleista vogelii* to enhance fertility in female.
KEY: Ethanolic extract, *Anthocleista vogelii*, WBC, RBC, female fertility, CD4+ and CD8+, `Oestradiol.

1.1 INTRODUCTION

Infertility is a common problem, affecting perhaps one couple in six; among those that seek medical care (Glazener *et al.,* 1987). Although diagnostic problems make it difficult to establish the extent of the female's contribution with certainty, a number of studies suggest that male problems represent the commonest single defined cause of infertility (Van den Eede, 1995). The use of plant extracts as fertility enhancer in human is now in the increase because of the shifting of attention from synthetic drugs to natural plant products (Dada and Ajilore, 2009).

In developing countries, 80% of the population continues to use medicinal plants and plant products in handling primary medical problems due to their accessibility, availability and affordability (Pierre *et al.,* 2009). In the African countries, a variety of plants are claimed to have fertility regulating properties and a few have been tested for such effect (Baker *et al.,* 1999; Telefo *et al.,* 2002; Ganguly *et al.,* 2007; Cherdshewasart *et al.,* 2007).

Aside from the possession of fertility enhancing activities, now it is well known that some herbal medicines act as immunomodulator through their dynamic regulation of information molecules such as cytokines (Anjana *et al.,* 2010). This may offer an explanation for the effect of some of these herbs on the immune system (Spellman *et al.,* 2006). Plants that were once considered of no value are now being investigated, evaluated and developed into drugs, with little or no side effects (Adedeji *et al.,* 2009). Since differences in immune responses between sexes and reproductive phases are accompanied by variations in sex hormones, the variations in immune responses are usually suggested to be due to these hormonal variations (Bouman *et al.,* 2005).

Anthocleista vogelii Planch, belongs to the family Loganiaceae; and also classified by the Angiosperm Phylogeny Group (APG) to the family Gentianaceae (de Ruijter, 2007). Traditional medicine of West African region indicates that the root, stem bark and leaf extracts of *Anthocleista vogelii* Planch, are used to improve fertility in both male and female human. Several *Anthocleista* species, including *A. vogelii*, has been used in

traditional medicine to treat various ailments. However, there is little or no scientific data to justify most of these traditional claims, which include the fertility enhancement claims of *Anthocleista vogelii*. This research work therefore aims to provide a scientific validation for the claims of the traditional medicine practitioners of the usage of *Anthocleista vogelii* Planch, as fertility enhancer in females and also to study the immune-modulating properties of the plant using female albino rats as model.

1.2. RESEARCH METHODS:

1.2.1 REAGENTS AND EQUIPMENT

Some chemicals/reagents/equipment made used of in this study include: N-Acetyl cysteine (NAC), Micronor (Norethisterone), K_3EDTA, Cannula, Vacuum Tubes, BD FACS Count™ Instrument System with FACS Count™ Control Kit and BD FACS Count™ Reagent Kit, Sysmex® Automated Haematology Analyser KX-21N, commercial standard estradiol ELISA kit, Microplate Reader.

1.2.2 PLANT MATERIAL

Leaves of *Anthocleista vogelii* were collected in large quantities from the forests of Imoshe, Ogun State, Nigeria in mid – June, 2013. These was taken for authentication at the Taxonomy Unit, Department of Botany, University of Lagos and obtained the voucher specimen (No: LUH/5652) The leaves was deposited in the herbarium of the Institute and also deposited at the Department of Biochemistry, Lagos State University for future reference.

1.2.3 PREPARATION OF THE ETHANOLIC EXTRACT

The leaves of the plant were washed well with water, dried under shade for 14days and powdered to fine grade using electric blender. A batch of 200g of powdered material was subjected to cold maceration extraction in 50% (v/v) of 1 L of Ethanol with intermittent shaking at room temperature for 3 days (72 hours). The extract was then filtered, that yielded 620ml of crude which was lyophilized to get 15g of the ethanolic extract corresponding to an extraction yield of 7.5%, stored in the freezer. This extract was re–dissolved in distilled water when ready for administration.

1.2.4 PHYTOCHEMICAL STUDIES

Phytochemical screening of the extract was carried out using standard procedures to identify the constituents (Trease and Evans, 1989) in modified methods of Somkuwar and Kamble (2013).

1.2.5 Toxicity Test

Acute toxicity test was performed according to Guessom, *et al.,* (2013). Nine (9) rats were randomly selected (three per group) and starved overnight. Extract doses of 150, 250 and 400 mg/kilogram body weight were administered to each animal group respectively. The animals were kept under the same natural condition and observed for toxicity signs and mortality for 72 hours. All dosage administered were found to be non-lethal as earlier determine as the safe doses reported by Guessom, *et al.,* (2013).

1.2.6 ANIMAL STUDY:

Sixty (60) healthy female Wister albino rats of average weight of 100g were procured from an inbred stock at University of Ibadan, Oyo State, Nigeria. The animals were acclimatized with the laboratory environment for 3 weeks following the Animal Care Ethics of the Biochemistry Department, Lagos State University.

Animal Treatment

Forty-two (42) female Wister albino rats with an average weight of 120g were randomly selected and divided into seven (7) groups with six (6) animals per group. The infertile group was obtained using N-acetylcysteine (NAC) or Micronor (Norethisterone, a proven female contraceptive) used to induce reversible infertility in this rat groups. The treatments for each group were as follows:

Group I: Rats were administered with 1ml of distilled water once a day for 21days.
Group II: Rats were administered with micronor (norethisterone) at dose of 20µg/kg b.w. once a day in a volume of 1ml for 7 days.
Group III: Rats received NAC (N-Acetylcysteine) at a dose of 1000mg/kg b.w. once a day in a volume of 0.74ml for 7 days.
Group IV: *Anthocleistavogelii* extract was administered to rats at a dose of 100mg/kg b.w. once a day in a volume of 0.25ml for 21 days.

Group V: Rats were administered with micronor (norethisterone) at a dose of 20µg/kg b.w. once a day in a volume of 1ml for 7 days and thereafter administered with *Anthocleistavogelii* extract at a dose of 100mg/kg b.w. in a volume of 0.25ml for 14 days.
Group VI: Rats were administered with micronor (norethisterone) at a dose of 20µg/kg b.w. once a day in a volume of 1ml for 7 days and thereafter administered with *Anthocleistavogelii* extract at a dose of 200mg/kg b.w. in a volume of 0.5ml for 14 days.
Group VII: Rats received NAC (N-Acetylcysteine) at a dose of 1000mg/kg b.w. once a day in a volume of 0.74ml for 7 days and thereafter administered with *Anthocleistavogelii* extract at a dose of 100mg/kg b.w. in a volume of 0.25ml for 14 days.

All administration was performed orally with the aid of cannula. On completion of administration, the rats in the different groups were anaesthesia with diethyl ether via inhalation. Blood samples were collected from the animals through cardiac puncture and collected into properly labelled K_3EDTA vacutainer tubes and plain tubes for analysis.

1.2.7 IMMUNOLOGICAL BIOMARKERS ANALYSIS
$CD4^+$ and $CD8^+$ analysis was performed using the BD FACS Count Automated $CD4^+$/$CD8^+$absolute count technique. A successful control run was necessary before running the test samples to ensure reliable results.

1.2.8 HAEMATOLOGICAL PARAMETERS ANALYSIS
WBC (white blood cell) count, absolute count of small cells (lymphocytes), absolute count of middle cells (basophils, eosinophils and monocytes), absolute count of large cells (neutrophils), RBC (red blood cell) count and Haemoglobin (HGB) volume analyses were carried out automatically using the Sysmex® Automated Haematology Analyser KX-21N.

1.2.9 HORMONAL ASSAY
Estradiol analysis was performed on the serum samples obtained from the animals using commercial standard Enzyme – Linked Immunosorbent Assay (ELISA) kit.

STATISTICAL ANALYSIS
Data were analysed using One Way Analysis of Variance (ANOVA, SPSS Version 20) and expressed as mean ± Standard Error Mean (SEM). Differences between groups were regarded significant at $P<0.05$ and post-hoc tests were then performed using the Tukey's test.

1.3 RESULTS:
1.3.1 PHYTOCHEMICAL CONSTITUENTS
The phytochemical studies revealed the presence of anthraquinones, saponins, flavonoids, alkaloids, terpenoids, phenols and phytosterols (Table 1).

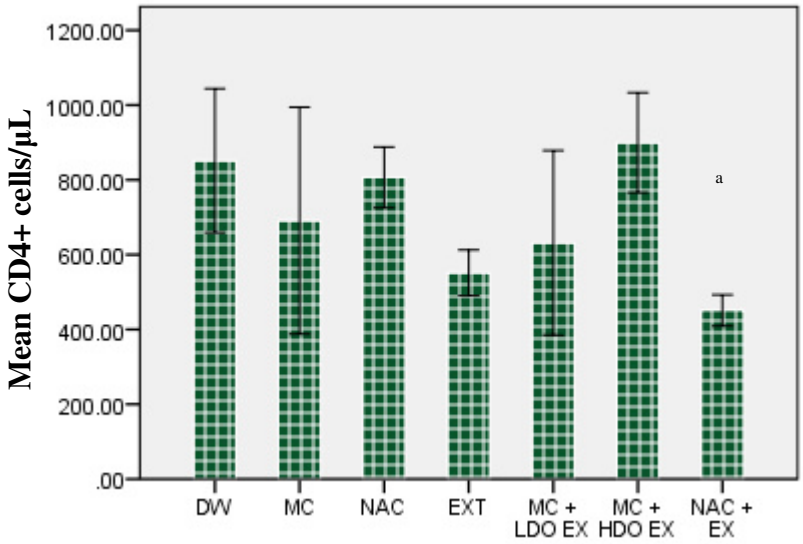
Table 1: Phytochemical investigation of the leaf of *Anthocleistavogelii*.

Test	Inference
Reducing sugar	-ve
Anthraquinones	+ve
Terpenoids	+ve
Flavonoids	+ve
Saponins	+ve
Tannins	-ve
Alkaloids	+ve
Cardiac glycosides	-ve
Protein/Amino acids	-ve
Phenols	+ve
Phytosterols	+ve

+ve: present; -ve: absent

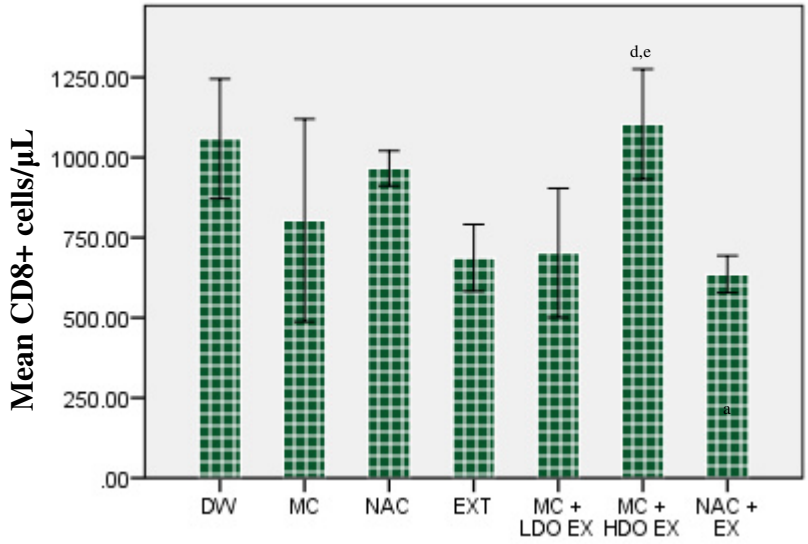
1.3.2 EFFECTS ON IMMUNOLOGICAL BIOMARKERS

The CD4 and CD8 cells concentration as counted from all the animal groups are presented in Figure 1 and Figure 2 below for CD4 and CD8 cells respectively:



KEY:
DW – Distilled Water
MC – Micronor
NAC – N-acetyl cysteine
EXT – Extract
MC+LDO EX – Micronor + Extract (Low Dose)
MC+HDO EX – Micronor + Extract (High Dose)
NAC+EX – N-acetyl cysteine + Extract

Figure 1: Graphical representation of CD4+ cells count.
[a]represents significant value compared to control group (i.e. Distilled Water) ($P < 0.05$, ANOVA post hoc Tukey HSD test).



KEY:
DW – Distilled Water
MC – Micronor
NAC – N-acetyl cysteine
EXT – Extract
MC+LDO EX – Micronor + Extract (Low Dose)
MC+HDO EX – Micronor + Extract (High Dose)
NAC+EX – N-acetyl cysteine + Extract

Figure 2: Graphical representation of CD8+ cells count.
[a]represents significant value compared to control group (i.e. Distilled Water), [d] represents significant value compared to Extract group, [e] represents significant value compared to MC+LDO EX group ($P < 0.05$, ANOVA post hoc Tukey HSD test).

1.3.3 EFFECTS ON LEUKOCYTES

The leukocytes levels of the blood obtained from each animal group were assessed and the values obtained are presented in Table 2. corresponding to total WBC count, absolute lymphocytes count, absolute middle cells count and absolute neutrophils count respectively:

Table 2: Total leukocytes count

Group	Haematological Parameters			
	WBC $x10^9/L$	Lymphocytes $x10^9/L$	Middle Cells $x10^9/L$	Neutrophils $x10^9/L$
Distilled Water	10.17 ± 2.10	7.08 ± 1.23	1.38 ± 0.38	1.70 ± 0.58
Micronor	10.20 ± 1.53	6.70 ± 0.79	1.27 ± 0.21	2.23 ± 0.73
N-acetyl cysteine	11.33 ± 1.55	6.50 ± 0.73	1.77 ± 0.22	3.07 ± 0.60
Extract	12.72 ± 1.83	5.07 ± 0.80	3.50 ± 0.70[a,b,c]	4.15 ± 0.83
Micronor + Extract (Low Dose)	8.00 ± 0.80	3.40 ± 0.27	1.92 ± 0.23	2.68 ± 0.57
Micronor + Extract (High Dose)	11.17 ± 2.06	5.70 ± 1.60	2.32 ± 0.30	3.15 ± 0.25
N-acetyl cysteine + Extract	8.15 ± 0.56	2.40 ± 0.18[a, c]	1.85 ± 0.38	3.90 ± 0.00

All values are expressed as Mean ± SEM of six observations (n = 6).
[a]represents significant value compared to control group (i.e. Distilled Water), [b] represents significant value compared to Micronor group and [c] represents significant value compared to N-acetyl cysteine group ($P < 0.05$, ANOVA post hoc Tukey HSD test).

1.3.4 EFFECTS ON OTHER HAEMATOLOGICAL PARAMETERS

The haemoglobin and red blood cell (RBC) levels of the blood obtained from each animal group were assessed and the values obtained are presented in Table 4 below.

The red blood cells (RBC) level in the control group is (5.39±0.51). There was no significant difference ($P<0.05$) observed in RBC level in all of the treated groups compared to the control. Extract administration in the NAC treated groups has no significant effect ($P<0.05$) on the haemoglobin level. Also, extract administered into micronor treated groups has no significant difference ($P<0.05$) in haemoglobin level.

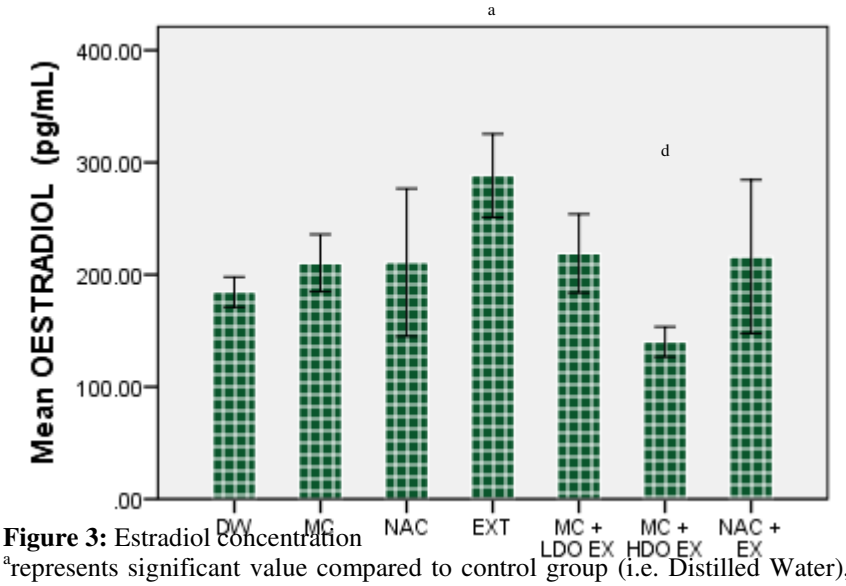
Table 3: Haematological Parameters

Group	Haematological Parameters	
	Haemoglobin g/dL	RBC $x10^{12}/L$
Distilled Water	11.02 ± 1.18	5.39 ± 0.51
Micronor	11.40 ± 0.48	5.87 ± 0.33
N-acetyl cysteine	11.97 ± 0.49	5.95 ± 0.29
Extract	11.72 ± 0.83	5.47 ± 0.44
Micronor + Extract (Low Dose)	12.27 ± 0.82	6.09 ± 0.41
Micronor + Extract (High Dose)	14.82 ± 0.28[a,b]	7.24 ± 0.18
N-acetyl cysteine + Extract	11.80 ± 0.54	5.10 ± 0.71

[a]represent significant value ($P < 0.05$, ANOVA post hoc Tukey test) compared to control (i.e. Distilled Water).
[b]represent significant value ($P < 0.05$, ANOVA post hoc Tukey test) compared to Micronor group.

1.3.5 EFFECTS ON FEMALE SEX HORMONE

The estradiol concentration of the blood obtained from each animal group was analysed and the values obtained are presented in Table 5 below. The values are also represented graphically (see Figure 3) below:



KEY:
DW – Distilled Water
MC – Micronor
NAC – N-acetyl cysteine
EXT – Extract
MC+LDO EX – Micronor + Extract (Low Dose)
MC+HDO EX – Micronor + Extract (High Dose)
NAC+EX – N-acetyl cysteine + Extract

Figure 3: Estradiol concentration
[a]represents significant value compared to control group (i.e. Distilled Water), and [d]represents significant value compared to extract group ($P < 0.05$, ANOVA post hoc Tukey HSD test).

1.4 DISCUSSION

The results obtained from this study for the effect of ethanolic extract of *Anthocleista vogelii* on CD4+ and CD8+ cells, showed that the extract caused reduction in the CD4+ cells and CD8+ cells count, although not to a significant level ($P<0.05$). In the groups administered with NAC followed by extract treatment, a significant reduction in CD4+ and CD8+ cells counts occurred. This may suggest that the effect of the ethanolic extract of *Anthocleista vogelii* on CD4+ and CD8+ cells was being enhanced after the treatment with NAC. On the other hand, significant increase ($P<0.05$) in CD8+ cells count was observed in the group treated with micronor followed by high dose extract administration (MC+HDO EX) group when compared to the group treated with micronor followed by low dose extract administration (MC+LDO EX) group.

The ethanolic extract of *Anthocleista vogelii* caused a significant increase ($P<0.05$) in the absolute middle cells (basophils, eosinophils and monocytes) count. This significant increase in the absolute middle cells count is observed in the extract treated group compared with the control ($P<0.05$). Although the ethanolic extract of *Anthocleista vogelii* as shown by this study increased the total white blood cells (WBC) count and absolute neutrophils count but these increases are not statistically significant ($P<0.05$). The effect of the extract on absolute neutrophils count of the extract only treated animals was found to be similar to its effect on animals with prior NAC treatment. This was also seen, in the micronor treated animals, the extract caused a dose-dependent increase in absolute neutrophils count, which however, is not significant ($P<0.05$).

Immunology have been shown to play pertinent role in pregnancy and at different reproductive processes, including ovulation, menstruation, which are influenced by the immune system, (Bouman *et al.,* 2005; Menzies and Henriquez, 2009). The study by Nieuwenhoven *et al.,* (2003), showed that the ratio of type 1 and type 2 cytokine productions of lymphocytes is decreased during pregnancy, while monocytes and granulocytes are activated. Moreover the cytokine environment has been shown to be important for a successful pregnancy, with such studies showing that Th1 environment is associated with abortion and a Th2 environment allow the successful continuation of pregnancy (Menzies and Henriquez, 2009). Therefore, the findings of this study suggested that *Anthocleista vogelii* may have a role in fertility by creating the environment required for successful pregnancy. This is shown by the reduction of CD4 cells level (Th1 to Th2 ratio), which in-turn causes slight reduction in CD8 cells count because the CD4 molecule have a direct role in CD8+ T cell function by modulating expression of IFN-γ and Fas ligand, two important CD8+ T cell effector molecules (Kitchen *et al.,* 2004). Also, this effect of the extract may suggest that *Anthocleista vogelii* increases the plasma concentration of ovarian steroid hormones (oestrogen) which is in support of the findings of Weinberg *et al.,* (2011) that the increase in steroid hormones levels during the menstrual cycle was associated with lower cell mediated immunity.

The ethanolic extract of *Anthocleista vogelii* has no significant effect (P<0.05) on the absolute lymphocytes count. While the animals treated with NAC prior to extract administration showed a significant reduction (P<0.05) in absolute lymphocytes count. Other investigations are in support of these findings on absolute lymphocytes count, as most literatures suggest no changes in total circulating numbers of lymphocytes and no variation in percentage of lymphocyte subtypes during the menstrual cycle (Mathur *et al.,* 1979, in Bouman *et al.,* 2005; Bouman *et al.,* 2001). Other mechanisms involved in controlling lymphocyte counts in women cannot be excluded. One of these factors may be related with ageing (Miller, 1996; Chakravarti and Abraham, 1999). So, from these reports, it may also be suggested that *Anthocleista vogelii* have some roles in the treatment of menstrual cycle disorder. However, these findings may be supported by previous literatures which reported that synthetic hormones in Oral Contraceptives (OCC) preparations do not affect absolute numbers or percentages of lymphocytes, T cells and subsets of T cells (Baker *et al.,* 1985; Yovel *et al.,* 2001) and also that the effects of synthetic hormones are not clear, since depending on the type of OCC used, OCC may or may not increase granulocyte numbers (Klinger *et al.,* 2000; Yovel *et al.,* 2001).

Ethanolic extract of *Anthocleista vogelii* showed an increase in haemoglobin level, however, the increase is not significant in the extract only treated group (P<0.05) when compared with the control group. However, a dose-dependent increase in haemoglobin level was observed in the groups treated with extract prior to micronor administration and the increase was significant (P<0.05) only in high dose extract (MC+HDO EX) group. Though, the extract caused an increase in haemoglobin level also in the animals with prior treatment with NAC; the increase is not significant (P<0.05) and similar to that observed in the animal group treated with extract only. This study also showed that the ethanolic extract of *Anthocleista vogelli* has no significant effect on Red Blood Cell (RBC) level (P<0.05). Red blood cells level was found to be increased more in the in the groups administered with micronor followed by high dose extract treatment (MC+HDO EX) but this increase was found to be not significant (P<0.05). Therefore, the results obtained for both haemoglobin and RBC level may suggest that the ethanolic extract of *Anthocleista vogelii* has no significant effect on haematological parameters; the total red cell volume is unaltered by the extract itself.

This research showed that *Anthocleista vogelii* leaves extract causes increase in the level of estradiol. The increase in the level of estradiol was found to be statistically significant at P<0.05. The increase in estradiol, and probably increase in progesterone, may be responsible for the effect of this plant as fertility agent as, claimed by traditional medicine practitioners. This is so because optimum levels of estradiol and progesterone is required for stimulation of follicular growth and maturation, induction of female to begin displaying oestrous behaviour to facilitate mating, preparation of the external genitalia for copulation, creating favourable conditions for the development of fertilised egg cells and maintenance of pregnancy if it occurs. Estrogens also contribute to the growth and development of mammary tissue and prepare the uterus for parturition (Beagley and Gockel, 2003; Wira *et al.,* 2008) hence promotes female fertility.

The phytochemicals found present in the leaves of *Anthocleista vogelii* include: anthraquinones, saponins, flavonoids, alkaloids, terpenoids, phenols and phytosterols. Various phytosteroids have been shown to promote fertility (Ruiz-Luna *et al.,* 2005). However, the type of steroid present in *Anthocleista vogelii* has not been evaluated but this may have a contributory effect on its profertility properties. Also, flavonoids present in this plant have been shown to possess many pharmacological properties such as: anti-oxidant activities, anti-inflammatory activities, anti-cancer activities and anti-microbial effects hence, flavonoids also may have a contributory effect on its pro-fertility properties and other pharmacological effects that the plant possesses (Uche and Obianime, 2008; Uche, *et al.,* 2008; Okwu and Josiah, 2006).

The findings of this study revealed that N-acetyl cysteine (NAC) showed a cooperative activity with the ethanolic extract of *Anthocleista vogelii* in most of the parameters examined. Therefore, this may deemed NAC non-suitable as an Oral Contraceptive. Also, it is unclear from the findings of this study if *Anthocleista vogelii* extract can be used to reverse immediately, the infertility induced by an oral contraceptive. The effects shown by *Anthocleista vogelii* on the immune system were found to be similar to that of the ovarian sex hormones (estrogen and progesterone) on the immune response during the reproductive processes of pregnancy. Some of the effects of the extract have also been found to be similar to those of these female sex hormones on the immune system during menstruation conditions.

1.5 CONCLUSION:

These findings reveals the richness of ethanolic extract of *Anthocleista vogelii* in Anthraquinones, terpenoids, flavonoids saponins, alkaloids phenols and phytosterols which suggest its potentials to elicit the plethora of effects observed by African traditional medicine practitioners. The other results also suggest that the extract may

have a role in creating the environment required for successful pregnancy by decreasing ratio of CD4+ and CD8+ linked Th1 and Th2 cytokines production and increasing monocytes and granulocytes activation; and that the plant may also have a role in treatment of menstrual cycle disorder. The plant may be administered to induce oestrogen production. Therefore, this study provides support for the claims of the traditional use of *Anthocleista vogelii* to improve reproductive function in female fertility. However, further study needs to be done to isolate, identify and characterize the active principle responsible for this effect. Such scientific validation is important since it will facilitate the medicinal efficacy of Nigerian herbal medicinal system.

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