Original article

The effects of methanol extract of cleistopholis patens on the reproductive system of female Wistar rats

Amadi CN[1], Odigie JO[2], Siminialayi IM[2]

[1]*Department of Clinical Pharmacy, Faculty of Pharmaceutical Sciences*, [2]*Department of Pharmacology, College of Health Sciences, University of Port Harcourt, PMB* 5323, *Port Harcourt*

Abstract

Objective: In Port Harcourt and its environs, extracts of *Cleistopholis patens* are used by traditional medicine healers for the treatment of menstrual irregularities and other gynaecological disorders. The objective of this study was therefore to determine the effects of orally administered methanol extract of the stem bark of *Cleistopholis patens* on the reproductive organs of non-pregnant albino (Wistar) rats. **Methods**: 3g/kg (low dose) and 6g/kg (high dose) of the extract were administered orally, daily to two different groups of animals, respectively, over a period of 28 days. A third (control) group of animals received distilled water only, orally over the same period. Five animals from each of the groups were sacrificed on day 8, 15 and 29. Venous blood samples and reproductive organs respectively were taken from each group of sacrificed animals for hormonal and histopathological analysis. **Results**: Results of the hormonal assay revealed a general increase in the levels of Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH), progesterone, and estrogen. The highest levels were noticed in the animals sacrificed on the 29th day (LH = 5.48 ± 0.04 IU/L; FSH = 3.80 ± 0.00 IU/L; Progesterone = 7.14 ± 0.15 nmol/L; Estrogen = 0.168 ± 0.002 nmol/L). These increases were statistically significant compared to those of the control animals (LH = 2.90 ± 0.00 IU /L; FSH = 1.28 ± 0.02 IU/L; Progesterone = 3.80 ± 0.00 nmol/L; Estrogen = 0.130 ± 0.002 nmol/L; $P < 0.05$), and were also dose dependent. Results of the histopathological studies showed presence of chronic inflammatory cells in the tissues of the fallopian tubes and uterus on the 29th day. However, no changes were observed in the ovaries. **Conclusion**: The administration of the extract produced a dose and time-dependent increase in FSH, LH, progesterone and estrogen levels. We postulate that these observed effects may have been induced by the phytoestrogens (known to have 1/1 000 th of the efficacy of natural oestrogens) in the extract. The hormonal and histopathological changes may explain the effects described by patients following ingestion of extracts of this plant in traditional medical practice. However, it remains to be determined if these effects are harmful or beneficial in disease conditions.

Keywords: Methanol extract; *Cleistopholis Patens*; Histopathological; Hormonal; Effects; Female; Rats

INTRODUCTION

Over the years, man has relied on natural sources (plants and animals) for his vital medical needs. Many African plants are used in traditional medicine as antimicrobial agents but only a small proportion of such uses are documented[1]. Fewer still of these uses have been subjected to any scientific investigations[2-5].

Cleistopholis patens is one of three or four species of the family Annonaceae[6] that is native to the tropical forests of West and Central Africa[7]. The

Correspondence to: Siminialayi IM, Department of Pharmacology, College of Health Sciences, University of Port Harcourt, PMB 5323, Port Harcourt.
E-mail: imsiminialayi@aol.com
Tel: +2348035310680

red juice that the tree exudes when cut resembles palm oil and tastes salty, hence the Ghanaians dubbed it (as translated) "the salt and oil tree." It is a widely occurring ever-green tree, mostly found in the rain forests of southern Nigeria and grows to a height of about 30-70cm.

An extract of *Cleistopholis Patens* is a herbal medicine in Yoruba (Western Nigeria) Odu incantations for curing cough, hence the Yoruba name "*apako*" meaning killing cough[8]. The leaves are chewed with kola, and the liquid squeezed out from the leaves is taken in draught for fever in Sierra-Leone and Gabon[9]. The stem bark is used to treat stomach pain and bronchial diseases and to induce emesis[10, 11]. In Gambia nursing mothers drink a medicine made from the seeds to ensure a good milk supply[12]. There is anecdotal evidence that the aqueous extract of *Cleistopholis patens* is effective in treating uterine fibroids as well as menstrual irregularities, in Port Harcourt, Rivers State, and its environs. This finely powdered medicine, which is taken as porridge, is prepared from the stem bark of the plant[13]. A pilot study with the aqueous extract of the stem bark of *Cleistopholis patens* had demonstrated histological and hormonal changes following 28-day oral administration to non-pregnant Wistar albino rats. Phytochemical studies have showed that the Methanol extract of *Cleistopholis patens* contains lipids, steroids and the flavonoids (quercetin and dihydroquercetin)[13-17]. The objectives of this study were to determine the effects of the methanol extract of *Cleistopholis patens* on the reproductive organs and sex hormones of nonpregnant Wistar rats.

MATERIALS AND METHODS

Plant Collection and Identification

The stem bark of the plant was collected from the University of Port Harcourt botanical forest. The plant was identified and authenticated by H. B Onyeachusim of the Department of Botany, University of Port Harcourt.

Extraction of Plant Material

The collected and duly identified stem-bark was cut into small pieces and dried in an oven at 35℃ for 24hours. The dried stem bark was then reduced to smaller sizes and ground to a fine powder. The dry powder was cold extracted with 70% methanol for 24 hours. The extract was then filtered using No 1 Whatman filter paper and reduced to dryness using a rotary evaporator[18]. The extract was kept in the refrigerator at 4℃ until when required for administration to the experimental animals.

Animals

Female non-pregnant Wister albino rats weighing between 125g and 200g were used for the experiment. They were kept in the animal house of the Department of Pharmacology and Toxicology, University of Port Harcourt in multiple cages at room temperature and maintained on a standard animal feed and water. They were allowed two weeks for acclimatization.

Acute Toxicity Test/LD_{50} Determination

A pilot study was conducted to determine the maximum dose of the extract that did not produce death and the minimum dose that caused 100% death[19]. The animals tolerated as much as 15g/kg via oral route, without lethality.

Animal Experiment

The animals were divided into three groups of fifteen animals each. Group one received a low dose of the extract (3g/kg) daily. Group two received a high dose (6g/kg) daily and group three (which was the control) received corresponding volumes of distilled water via the oral route daily. All drugs were administered with the aid of an inflexible oral cannula. Five animals from each group were sacrificed on day 9, 15, and 29 respectively. Blood samples were collected from the animals and the uterus, ovaries, and fallopian tubes harvested for hormonal analysis and histopathological studies respectively. The animals were allowed free access to food and water throughout the experiment.

Hormonal Assay

Collected blood samples were assayed for the following hormones: Luteinizing Hormone (LH), Follicle Stimulating Hormone (FSH), progesterone and estrogen. The assay was carried out according to the Direct Human Serum Enzyme Immuno-Assay (EIA) manual, (Immunometrics, UK, 2004) for progesterone and estrogen, ELISA manual, (Diagnostic Automation Inc. UK, 2004) for LH and FSH respectively.

Histopathological Analysis

The animal tissues (ovaries, uterus, and fallopian tubes) were fixed in 10% formalin solution for at least 12 hours, and then dehydrated through graded percentage of alcohol. The tissues were then cleared with pure xylene solution for 2 hours before infiltration took place, and transferred into molten paraffin wax (M. P 54-58℃) for 2hours on hot plate. The embedded tissue blocks were sectioned with a Shaddon AS 325 rotary microtone and slides were prepared with the sections . The tissues were stained with Ehrlich's haematoxylin and eosin blue using Lillie's method[20]. Each of the slides was placed in the photomicroscope and photographed under magnifications of ×100; the photographic transparencies were obtained and later developed into photo micrographic pictures.

Statistical Analysis

All data are expressed as mean ± S. E. M. Analysis was by the One- way ANOVA using Epi Info version 6.04D (CDC, Atlanta, Georgia, USA).

RESULTS

Hormonal Analysis Results

The results are as shown in table 1. Administration of *Cleistopholis patens* produced both a time-dependent and a dose-dependent effect on the rats. Over a period of 28 days, there was a significant increase ($P < 0.05$) in the levels of luteinizing Hormone (LH), follicle stimulating hormonal (FSH), progesterone, and estrogen compared to controls.

For luteinizing Hormone, there was a dose dependent decrease on day 8 and day 15 as compared to the controls, and subsequently, an increase on day 29 as compared to the controls and this increase was statistically significant.

Similarly, for follicle stimulating hormone (FSH), there was a decrease ($P < 0.05$) in level on day 8 and 15 and subsequently an increase ($P < 0.05$) on day 29 as compared to the controls of the different days. For progesterone, there was a dose-dependent increase on day 8, a decrease on day 15 which was not statistically significant, and a statistically significant increase on day 29 as compared to the controls of the different days.

Lastly, for estrogen, there was a decrease on day 8, 15 and 29 as compared to the controls of the different days. However this finding was not significant.

Table 1 Hormonal analysis results.

Day	Dosage(g/kg)	n	LH (IU/L)	FSH (IU/L)	Prog(nmol/L)	Oestr (nmol/L)
0	0 (baseline)	5	2.00 ± 0.00	1.28 ± 0.02	3.80 ± 0.00	0.130 ± 0.002
8	0 (control)	5	3.90 ± 0.02	2.02 ± 0.02	3.48 ± 0.02	0.158 ± 0.002
8	3	5	2.70 ± 0.02	1.42 ± 0.02	3.70 ± 0.00	0.136 ± 0.002
8	6	5	2.88 ± 0.02	1.50 ± 0.00	3.60 ± 0.00	0.144 ± 0.004
15	0 (control)	5	4.74 ± 0.04	2.50 ± 0.00	8.02 ± 0.04	0.222 ± 0.002
15	3	5	2.74 ± 0.04 *	1.98 ± 0.06	6.62 ± 0.06	0.286 ± 0.002 *
15	6	5	3.28 ± 0.16	2.30 ± 0.00	7.66 ± 0.07	0.272 ± 0.002
29	0 (control)	5	3.38 ± 0.02	2.22 ± 0.02	4.50 ± 0.03	0.147 ± 0.004
29	3	5	5.04 ± 0.07	3.42 ± 0.12 *	6.26 ± 0.15	0.168 ± 0.002
29	6	5	5.48 ± 0.04 *	3.80 ± 0.00 *	7.14 ± 0.15	0.170 ± 0.003

* $P < 0.05$

Histopathology results

The administration of distilled water to the control rats for 7, 14 and 28 days did not induce any changes (Fig 1, 2 and 3) in the ovaries, fallopian tubes, or uterus. There were also no histological changes observed in the ovaries for both the low

(3g/kg) and high (6g/kg) dose of the extract in the animals sacrificed on days 8, 15 and 29.

The fallopian tubes of the animals sacrificed on day 8 were found be cystically dilated and containing secretory materials in the lumen (Fig4 and 5). This effect occurred with both the low and high doses of the extract. The tissues obtained on day 15, show the fallopian tubes to have numerous glandular spaces in the mucosa (Fig 6 and 7), following treatment with both the low and high doses of the extract. The fallopian tubes of the animals sacrificed on day 29 were infiltrated with chronic inflammatory cells in response to both the low and high doses of the extract (Fig 8 and 9).

The tissues of the uterus in animals sacrificed on day 29, showed the presence of chronic inflammatory cells in the endothelial lining and the presence of cystically dilated glands (Fig 10 and 11). These changes occurred with both the low and high doses of the extract administered.

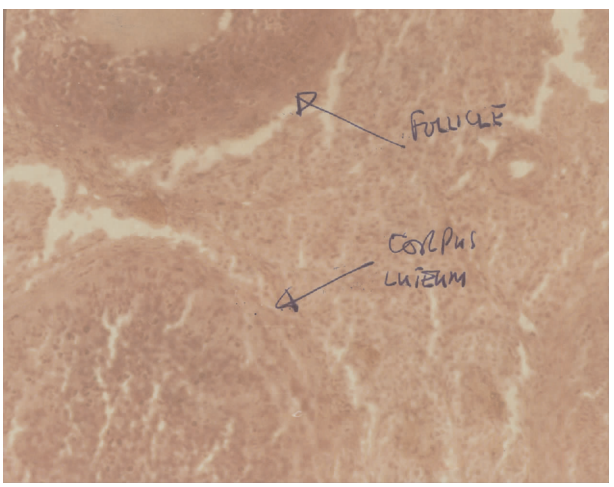

Figure 1 Histology of the ovary (control) ×100.

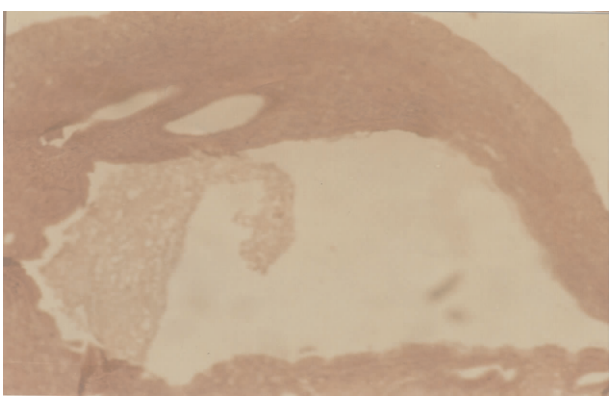

Figure 2 Histology of the fallopian tube (control) ×100.

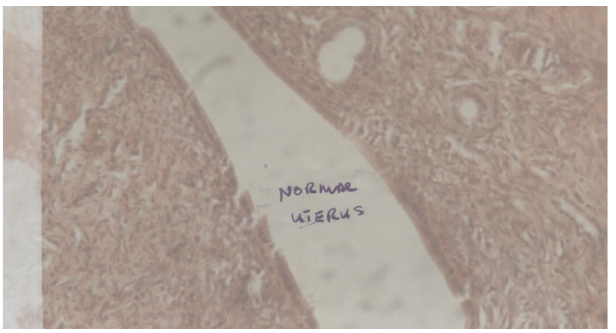

Figure 3 Uterus (control) ×100.

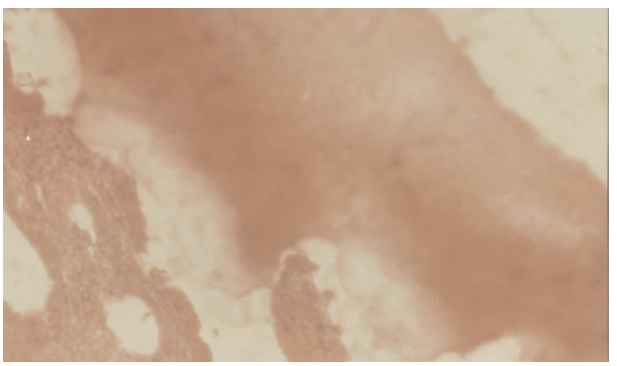

Figure 4 Fallopian tube day 8 (3g/kg) ×100, showing cystically dilated tubular tissue containing secretory material in the lumen with glandular spaces.

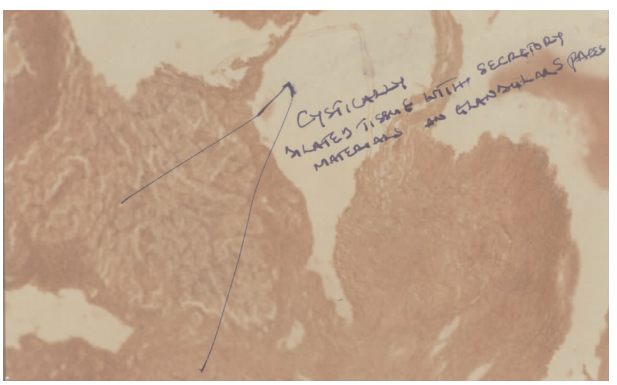

Figure 5 Fallopian tube day 8 (6g/kg) ×100, showing cystically dilated tubular tissue containing secretory material in the lumen with glandular spaces.

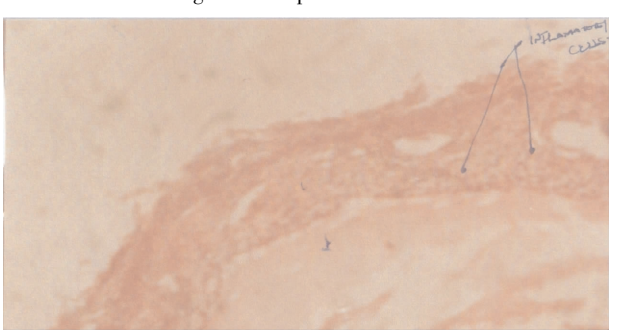

Figure 6 Fallopian tube day 15 (3g/kg) ×100. Muscular are fluntered with glandular spaces in the submucosa.

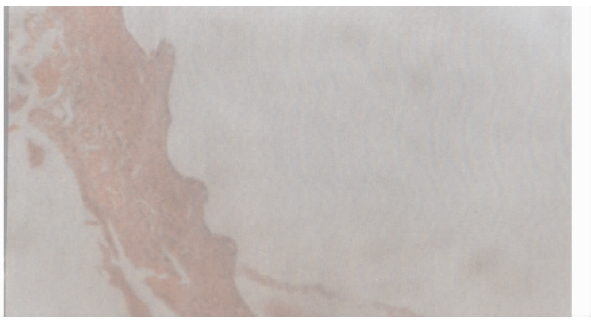

Figure 7 Fallopian tube day 15 (6g/kg) ×100, showing cystically ditated tube with blunting of the mucosal cell.

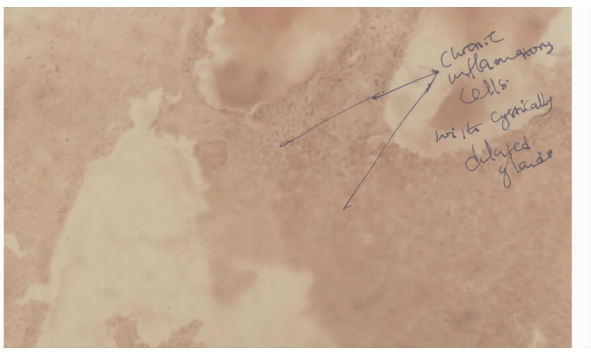

Figure 8 Fallopian tube day 29 (3g/kg) ×100, showing chronic inflammatory cells.

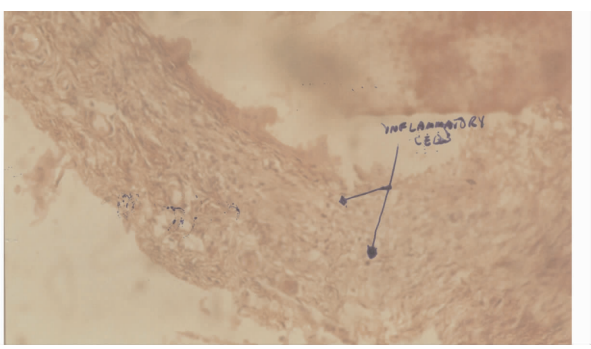

Figure 9 Fallopian tube day 29 (6g/kg) ×100, showing chronic inflammatory cells.

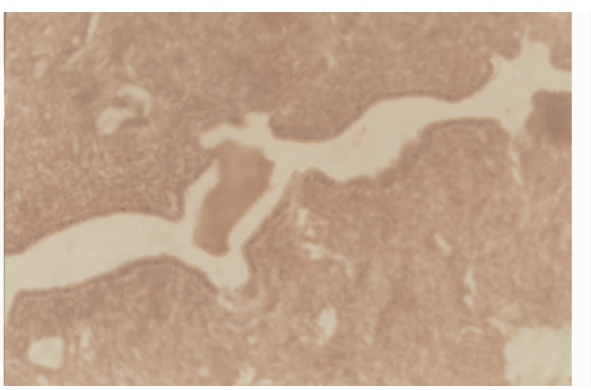

Figure 10 Uterus day 29 (3g/kg) ×100. The endometrial lining is infiltrated by chronic inflammatory cells with cystically dilated glands.

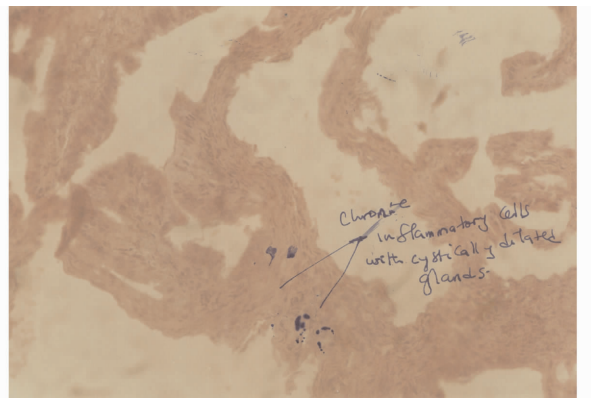

Figure 11 Uterus day 29 (6g/kg) ×100. The endometrial lining is infiltrated by chronic inflammatory cells with cystically dilated glands.

DISCUSSION

In this study, we investigated the possible effects of the methanol extract of *Cleistopholis patens* on the reproductive system of non-pregnant female albino rats. Results of administering the extract orally, daily, for 28 days revealed a general increase in the levels of follicle stimulating hormone (FSH), luteinizing hormone (LH), progesterone, and estrogens.

Phytochemical studies[13] indicate that the methanol extract of *Cleistopholis patens* contains steroids and flavonoids. Chemicals in plants that contain a steroidal nucleus are considered to be phytosterols[21]. These phytosterols (phytoestrogens) occurring in plants are very similar to the steroidal hormones found in humans and other animals but they are less efficacious; about 1/1000 times the efficacy of the estrogens[21, 22]. Many plants now found to be high in steroidal molecules (very similar to those observed in animals) have been used for centuries, if not millennia for hormonal, menstrual, and obstetrical conditions[21]. In addition, Phytoestrogens are believed to occupy estrogen receptors and thus promote increased metabolism of the displaced and free estrogen[23,24]. The displacement from binding sites results in high amount of estrogen in circulation, reflected in the high concentrations measured. The flavonoids in *Cleistopholis patens* (quercetin and dihydroquercetin) have been documented to be phytoestrogens[25]. These flavinoids might have also contributed to this observed increment in estrogen

levels.

High concentrations of endogenous estrogen are known to inhibit the secretion of FSH and LH by a negative feedback effect on the anterior pituitary[26]. It has been reported[27] that phytoestrogens interfere with estrogen negative feedback by binding to estrogen receptors in the anterior pituitary or hypothalamus thus preventing the endogenous oestrogens from binding to those receptors. With the observed increase in estrogen level in the present study, a decrease in FSH and LH was expected. However, an increase in both FSH and LH was observed. This could be an indication that the phytoestrogens in the extract competed with the endogenous estrogen for the receptors in the pituitary and hypothalamus, thereby reducing the feedback effects of endogenous estrogen[22].

Estrogen given cyclically with a progestogen induces an artificial cycle in adults with primary amenorrhoea[26] while in hypogonadism the cycle will not occur normally, and there is irregular menses. Prolonged cycles are frequently associated with failure of ovulation, presumably because of insufficient secretion of LH, which is necessary for ovulation[13]. The observed increase in the level of estrogen, progesterone, FSH and LH following chronic administration of the extract as compared to the control animals, suggests a possible explanation for the use of the plant traditionally in the correction of menstrual irregularities. From literature, FSH and LH act on the ovaries to promote development of follicles, each containing an ovum and progesterone stabilizes the endometrium and induces regular sloughing, while estrogen prepares the follicle for the release of an egg[28].

The initial decrease in the levels of LH and FSH as compared to the controls after 7 and 14 days of administration of the extract, preceding the increase noticed after 28 days, suggests that the effect of the extract on these hormones is time-dependent. The increase in the levels of these hormones was also higher with the higher dose of the extract, suggesting a dose-dependent effect.

No histopathological changes were observed in the ovaries in this study. Inflammatory cells were observed in the fallopian tubes and the uterus following administration of the extract for 28 days. This however, was not dose-dependent. In the animals sacrificed on day 8, the tissues of the fallopian tubes were found to be cystically dilated and containing secretory materials in the lumen. This was also not dose-dependent i. e. similar results were obtained for both high (6g/kg) and low (3g/kg) doses of the extract. In the animals sacrificed on day 15, the fallopian tubes were observed to be fluttered with glandular spaces in the mucosa. These suggest that the extract may have some sort of direct cytotoxic effect on the fallopian tubes and uterus. The inflammatory cells observed in the fallopian tube and the uterus on the 28th day were time-dependent effects.

The hormonal effects of phytoestrogens (flavinoids)[21] and other botanical medicines[21] on the female genital tract has been previously documented, as well as evidence of the presence and effects of the flavinoids contained in the genera *Cleistopholis* on the female reproductive organs[12,14-17]. This study quantitatively demonstrates the hormonal effects and further elucidates the histopathological effects of *Cleistopholis patens*.

CONCLUSION

The results of this study suggest that the administration of the methanol extract of the stem bark of *Cleistopholis patens* produced time- and dose-dependent increases in FSH, LH, progesterone and estrogen levels. These findings may explain the effects observed by traditional medical practitioners and indicate that the extract may correct irregular menstrual cycles. The cytotoxic effects produced in the fallopian tube and the uterus may constitute serious adverse effects. The effects of this extract in a pathological setting (such as uterine leiomyomas) needs tobe determined. The active component(s) of the extract responsible for the observed effects also need(s) to be determined.

REFERENCES

1 **Lewis WH**, Elvin-Lewis MPF. Medical botany: Plants affecting man's health. New York: John Wiley; 1977:120-134.

2 **Meckes M**, Torres J, Calzada F, Revera J, Camorlinga

M, Lemus H, et al. Anti-bacterial properties of *Hellanthemum glomeratum*, a plant used in Maya traditional medicine to treat diarrhoea. *Phytother Res.* 1997; 11:128-131.

3 **Okunade AL**, Hufford CD, Richardson MD. Peterson JR, Clark AM. Antimicrobial properties of Alkaloids from *Kanthorhiza simplicissima* . *J Pharm Sci.* 1994; 83:404-406.

4 **Ebi GC**, Ofoefule SJ. Investigation into the folkloric antimicrobial activities of Landolphia owrrience. *Phytother Res.* 1997; 11: 149-157.

5 **Okunade AL**, Hufford CD, Clark AM, Lentz D. Antimicrobial Properties of *piper aduncum*. *Phytother.* 1997; 11: 142 -144.

6 **Hutchinson J**. The Genera of Flowering Plants. Oxford: Clarendon Press; 1964:71-109.

7 **Le Thomas A**. Annonacées, in: A. Aubreville, editors. Flore du Gabon. paris: Muséum national d´Histoire naturelle. 1969:87-91.

8 **Verger PF**. A won ewe Osanyi. Yoruba medicinal leaves. Ist edition. Ife, Nigeria: University of Ife press; 1967. 5-30.

9 **Walker AR**, Sillans R. Les plantes utiles du Gabon. Paris: pau le chevalier; 1961.

10 Raponda-Walker A, Sillans R. Les Plantes Utiles du Gabon. In: Sépia Ed. Fondation Raponda-Walker, Centre Culturel Francais de Libreville, Libreville: Gabon; 1995: 65.

11 **Neuwinger HD**. African Ethnobotany, Poisons and Drugs. London: Chapman and Hall; 1996:3-42.

12 **Seidel V**, Bailleul F, Waterman PG. Novel Oligorhamnosides from Stem Bark of Cleistopholis glauca. *J Nat Prod.* 2000; 63 (1):6-11.

13 **Ebi GC**, Kamalu TN. Phytochemical and Antimicrobial properties of Constituents of Ogwu Odenigbo´a popular Nigerian Herbal Medicine for Typhoid Fever. *Phytother Res.* 2001; 15:73-75.

14 **Ngiefu CK**, Paquot C, Vieux A. Oil plants of zaire. ll. Botanical famities producing oils of medium nonsaturation Dleagineut. 1976; 31:545-547.

15 **Tané P**, Ayafor JF, Sondengam BL, Lavaud C, Massiot G, Connolly JD, et al. A substituted cinnamoyl ester from cleistopholis staudtii *Tetrahedron Lett.* 1988; 29; 1837-1840.

16 **Woods N**. Synthetic and Structural Studies in the Natural Product Field. [dissertation]. Scotland, University of Glasgow; 1989.

17 **Etse JT**, Gray AI, Waterman PG, Bates CD, Watson DG, Thomas DW. Chemistry in the Annonaceae. XXV. Sesquiterpenes from the stem bark of Cleistopholis glauca. *J Nat Prod.* 1988; 51: 314-318.

18 **Evans WC**. Trease and Evans Pharmacognosy. 15th edition. London: WB Saunders Company Limited; 2002:138.

19 **Aliyu YO**, Nwude N. Determination of the median lethal dose (LD_{50}) in Veterinary Pharmacology and toxicology experiments. Zaria: Baraka Press. 1982; 104-122.

20 **Lillie RD**, Fulllmer HM. Histopathologic thechnique and practical histochemistry. 4th edition. NY: MacGrawhill; 1976:35-60.

21 **Stansbury J**. Botanical medicines acting on the female reproductive system. Greenbush Home index. 2007. http://www. greenbush. net//morthanyouev. html.

22 **Ward-Dean MD**. Hormone Replacement Alternatives for women. Vitamin Research products. 2008. http://www. E:/phytoestrogen. htm.

23 **Anderson JJ**, Garner SC. Phytoestrogens and human function. *Nutr Today*. 1997; 32(6):232-39.

24 **Adlercreutz H**, Mazur W. Phyto-oestrogens and Western diseases. *Ann Med.* 1997; 29:95-120.

25 **Burton JL**, Wells M. The effect of phytoestrogens on the female genital tract. *Journal of Clinical Pathology*. 2002; 55(6):401-407.

26 **Rang HP**, Dale MM, Ritter JM, Moore PK. Pharmacology. 5th edition. Edinburgh: Churchill Livingstone; 2003: 431-434.

27 **Adams NR**. Detection of the effects of phytoestrogens on the sheep and cattle. *Journal of Animal Science*. 1995;73 (5):1109- 1515.

28 **Loose DS**, Stancel GM. Estrogens And Progestins. In: Bruton L, Lazo JS, Parker KL. Goodman and Gilman's the pharmacological basis of therapeutics. 11th edition. New York: Wiliams and Wilkins McGraw Hill; 2006; 1541-1567.