Expression of Oestrogen and Progesterone Receptors in the Ovary Following Administration of Combined Extracts of *Lophira lanceolata* and *Alchornea cordifolia* on Menopausal Albino Wistar Rats

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ABSTRACT

Many women still believe herbal medicines will delay the onset of menopause, regularise oestrous cycles in the perimenopausal period and improve the chances of conceiving. At menopause oestrogen and progesterone are no longer produced by the ovaries. This study examined the expression of oestrogen and progesterone receptors in the ovary of menopausal rat models. Twenty-five healthy female albino wistar rats were randomly divided into 5 groups of five animals each. Groups A, C, D and E consisted of menopausal rats. Group B consisted of young rats. Groups A was negative control (irregularly cycling menopausal rats). Group B served as a positive control (young regularly cycling rats). Groups C, D, and E were the experimental groups made up of irregularly cycling menopausal rats. Extract administration of *Lophira lanceolata* and *Alchornea cordifolia* to the treated groups was for 21 days. Animals were sacrificed on the 22nd day, on the pro-oestrus phase and the ovaries excised for further analysis. Ovaries from positive control and the treatment groups showed normal histological features consistent with the pro-oestrus phase. Group C animals treated with combined extract had multiple Graafian follicles. The results of this study showed that progesterone receptors were poorly expressed in the negative control as well as in all the treatment groups. The positive control and the combined extract-treated groups showed strong staining intensity while the other groups showed moderate staining intensity. The results demonstrate likely oestrogen expression in ovaries of rats that received extracts particularly in combination and possible delayed onset of menopause.

INTRODUCTION

Menopause, defined as twelve months of amenorrhoea without any obvious pathologic cause, is usually preceded by a period of marked menstrual cycle irregularities known as the perimenopausal period. This signals the beginning of the end of the reproductive life of a woman. Perimenopause and menopause can be accompanied by a series of unpleasant symptoms (e.g. abnormal uterine bleeding, hot flushes, skin and hair changes, fatigue, mood disorders, myalgia, atrophic vaginitis, sexual dysfunction, and osteoporosis).
All of these symptoms are due to the changing serum levels of oestrogen (Decherny et al., 2013). During the oestrous cycle, the hormones secreted by the ovaries are the oestrogens (mainly oestradiol) and progesterone. These are steroid hormones produced principally by the cells of the theca interna (oestrogen) and the corpus luteum (progesterone). The preovulatory period of the oestrous cycle is characterised by the growth of ovarian follicles and a concomitant enhanced secretion of oestrogen. The rate of secretion of estradiol into ovarian venous plasma is low on oestrus, begins to rise significantly by late metoestrus through the morning of dioestrus and reaches peak concentration by the afternoon of pro-oestrus (Hamid & Zakaria, 2013). Peripheral plasma levels of oestradiol are basal through oestrus in the 4-day-cycling rat. Plasma levels begin to rise late on metoestrus through early dioestrus and continue through dioestrus and early pro-oestrus, to reach peak values and plateau by mid-pro-oestrus. During the early evening, shortly before the dark interval in the colony, oestradiol levels fall rapidly, reaching basal values by the early morning of oestrus. The dominant progestin secreted into ovarian vein blood during the oestrous cycle in adult rats in 20-alpha-hydroxyprogesterone, a metabolite of progesterone. During the cycle, there are two peaks of 20-alpha-hydroxyprogesterone and progesterone secretion. The first peak occurs during the afternoon of metoestrus and both steroids arise from the newly formed corpora luteal (Hamid & Zakaria, 2013). The second peak occurs during the late afternoon of pro-oestrus (Hamid & Zakaria, 2013). At this time, the 20 alpha-hydroxyprogesterone comes from the corpora lutea, whereas progesterone arises from granulosa cells of the preovulatory follicle. The secretion of 20 alpha-hydroxyprogesterone in 4-day-cycling rats appear quite variable; during the morning of dioestrus, the lowest levels are observed but peripheral levels rise during the evening of dioestrus and morning of pro-oestrus to reach peak levels on pro-oestrus. Progesterone secretion is more regular, a large increase originating in the follicles occur during the afternoon and evening of pro-oestrus. This increase occurs nearly simultaneously with the major ovulation-inducing increase of luteinizing hormone (LH) peak secretion and reaches peak level around the time of LH peak in the early evening and returns to basal levels by the morning of oestrus. A second major peak of luteal origin begins about midday metestrous and falls to levels on dioestrus.

The transition to menopause also called perimenopause, is marked by menstrual irregularities, and associated with distressing symptoms such as hot flushes, weight gain, night sweats, mood disorders, sexual dysfunction, headaches, and depression, to mention just a few (Grady, 2006). The perimenopause can last for four to eight years, it usually begins in the mid-late 40s, but can also begin in the late 30s. There is a sharp decline in fertility at this stage due to hormonal imbalance (Grady, 2006; Gold, 2011). In the early peri-menopause period, levels of oestrogen remain normal or slightly raised, follicle-stimulating hormone (FSH) rises but remains within the upper border of normal. Later on, in the perimenopausal period, the levels of oestrogen drop significantly, while FSH rises. When menopause is reached, oestrogen and progesterone are no longer produced by the ovaries, but the production of testosterone continues (Decherny et al., 2013).
Globally, the average age at childbearing has been rising sharply. In the United Kingdom (UK), a rise from eight percent in the 1990s to eighteen percent in 2009 of all pregnant women being 35 years or above was reported Ngowa et al., (2013), in South Australia, an increase of 4.6% in 1981 to 21.1% in 2009 was also reported. In USA, older women comprise the only age group whose birth rate is on the rise, increasing by 6% for those in the 40-44 years between 2007 and 2009 (Orazuilike et al., 2015). Various reasons suggested for this trend include delay in marriage, more focus on education and careers, effective birth control, fertility problems and advances in assisted reproductive technologies (ART). In addition to the above reasons, in the developing countries, other reasons could include lack of or ineffective family planning methods, favourable cultural disposition towards large family size and poverty (Orazuilike et al., 2015).

With ART, successful pregnancies are becoming common, even in post-menopausal women. Most of these successes are with donor eggs, and this is unacceptable for some women, mostly on religious grounds. Many women still believe that herbal medicines will delay the onset of menopause, regularise their cycles in the perimenopausal period, and therefore improve their chances of conceiving with their ovum or egg. Although the advent of assisted reproductive techniques has brought succour to a lot of couples, they are very expensive. Hence, a lot of infertile couples, both educated and uneducated, seek herbal solutions to their problem (Akinloye & Truter, 2011). In view of the above, any plant that holds the potential to be effective in the treatment of perimenopausal symptoms, or delay in onset of menopause, menstrual cycle disorder, and infertility is worth researching. Regularisation of the menstrual cycle, either in the perimenopausal period or earlier in the reproductive life, would improve fertility. Alleviation of the symptoms associated with perimenopause and menstrual irregularities will improve women’s quality of life. A possible delay in the onset of menopause would imply a reduced risk for all the diseases associated with menopause.

*Lophira lanceolata* is used in the treatment of both male and female infertility. It is used particularly in the treatment of female infertility, alleviation of symptoms of menopause and menstrual cycle disorders. Depending on the symptoms presented to the herbal practitioner, it can be used either alone or in combination with other herbs in the management of the case. Similarly, *Alchornea cordifolia* is used in the treatment of menstrual cycle irregularities and infertility. Most often, these two herbs (*L. Lanceolata* and *A. cordifolia*) are used in combination with the treatments of these conditions. The efficacy of plant infertility treatment is highly acclaimed. It is therefore in the light of this that this research seeks to evaluate the scientific basis, if any, for the use of these herbs.

This study is aimed at assessing the histological and immunohistochemical effects of the oral administration of leaf extracts of *L. lanceolata* and *A. cordifolia*, on the expression of oestrogen and progesterone receptors in the ovary and microscopic structure of the ovary of menopausal albino Wistar rats.

**MATERIAL AND METHODS**

**Animal Care and Experimental Design:**

All experimental procedures were carried out in accordance with the ethics of animal handling as approved by the Animal Research Ethics Committee of the Faculty of Basic Medical Sciences, University of Calabar, (FAREC/PA/016A30217). Twenty-five healthy female albino wistar rats were weighed and randomly
divided into 5 groups (A, B, C, D, and 
E) of five (5) animals each. Groups A, C, D, and E consisted of aged (menopausal) rats; 24-30 months old. Group B consisted of 5 young rats (between 4-6 months old). All the rats were kept in the animal house of the College of Medicine, University of Calabar, under standard laboratory conditions (12hrs light/dark cycle, temperature, humidity). Water and food (rat chow) were allowed ad 
libitum. The rats were allowed a period of acclimatization of two weeks following which the experiment commenced.

**Determination of Oestrous Cycle Pattern (cycling of animals):**

To determine the oestrous cycle of the rats, the vaginal smear pattern method was used. The vaginal smear of the rats was taken daily between 8 am to 9 am. The method used is that described by Marcondes *et al.*, (2002). The animals were smeared daily for 3 weeks. The cycle was designated as a period extending from the first day of leukocytes (L) which corresponds to di-oestrous and which was preceded by a cornified (C) vaginal smear until the end of the next sequence of C smears. A normal cycle is expected to follow the sequence pro-oestrus, oestrus, met-oestrus and di-oestrus. Any cycle in which a particular phase was maintained for 4-5 days was considered irregular. If the phases do not follow the sequence outlined above, the cycle was also termed irregular. Only rats confirmed to have regular and irregular cycles were used for the research work. Groups A was the negative control group (5 irregularly cycling menopausal rats). Group B served as a positive control group (5 young regularly cycling rats). Groups C, D, and E were the treatment or experimental groups and were made up of 5 irregularly cycling menopausal rats. Extract administration was completed on the 21st day from the commencement of administration. Sacrifice was done serially from the 22nd day, on pro-oestrus phase of the oestrous cycle of each animal, using the chloroform inhalation method. The ovaries were dissected out, cleaned in saline, and fixed in 10% buffered formalin for tissue processing using the H&E method and Avidin-Biotin complex method for microscopic viewing.

**Preparation and Administration of Extract:**

Fresh leaves of *Lophira lanceolata* and *Alchornea cordifolia* were authenticated by a botanist in the Department of Botany, University of Calabar, Calabar, and voucher numbers were deposited for *Alchornea cordifolia* (BOT/UC/HERB/010) and *Lophira lanceolata* (BOT/UC/HERB/013). The ethanolic extracts were administered as daily oral doses for a duration of three weeks as shown below.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dosage/ Extract of administration</th>
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<tbody>
<tr>
<td>A</td>
<td>Normal saline</td>
</tr>
<tr>
<td>B</td>
<td>Normal saline</td>
</tr>
</tbody>
</table>
| C      | 500mg/kg body weight each of *L.*
|        | *lanceolata* and *A. cordifolia* |
| D      | 500mg/kg bodyweight of *L. lanceolata* |
| E      | 500mg/kg bodyweight of *A. cordifolia* |

Daily vaginal smear observation was continued throughout the duration of this experiment.
RESULTS
Histological section of the ovary of Group A rats (negative control), using H and E staining method showed normal ovarian features of menopausal rats. There were corpora lutea of various sizes, a prominent primary follicle, with multiple layers of follicular cells and an oocyte within. No primordial or Graafian follicle is seen in this section (Fig. 1). The ovary of Group B animals (positive control) showed normal ovarian features. A matured (Graafian) follicle is seen, with characteristic features of an antral cavity, oocyte surrounded by cumulus oophorus and corona radiata, granulosa cells, theca interna and theca externa. Other structures shown include corpus albicans, corpus luteum, atretic follicles, and primordial follicles (Fig 2). The ovary of the group treated with 500mg/kg body weight of the combined extract showed normal structure, with two Graafian follicles prominently shown. Primary follicles, primordial follicles, corpus luteum, and blood vessels can be seen in the medulla (Fig 3). For Group D (L. lanceolata treated), a section of the ovary showed a Graafian follicle, with an oocyte within, corpus albicans, atretic follicles, and corpus luteum. The ovarian surface epithelium can also be seen, elevated by corpus luteum at some points (Fig. 4). Section of an ovary of Group E rats treated with 500mg/kg bodyweight of leaf extract of A. cordifolia showed normal ovarian histology, with a prominent Graafian follicle, corpus luteum and primary follicle (Fig. 5).

Fig. 1: Photomicrograph of the ovary of Group A animals (negative control) given normal saline stained with H & E (x100). Ovary showing large corpora lutea (CL), a multilayered primary follicle (MPF) with an oocyte (OC), and blood vessels.
Fig. 2: Photomicrograph of the ovary of Group B (positive control) animals given normal saline stained with H&E (X100). Ovary showing a secondary follicle (SF) with antral cavity (AC), oocyte (OC), theca interna (TI), theca externa (TE), primordial follicle (PF), corpus luteum (CL) with vacuolization, corpus albicans (CA), degenerating (atretic) follicle (AF) and are blood vessels (BV).

Fig. 3: Photomicrograph of the ovary of Group C animals treated with combined extracts of *Lophira lanceolata* and *Alchornea cordifolia* (500mg/kg body weight each), stained with H&E (x100). Section showing normal ovary with prominent Graafian (matured) follicles (GF), multilayered primary follicle (MPF), unilaminar primary follicle (PF), corpus luteum (CL), surface epithelium (SE) elevated by follicles at some points, and blood vessels (BV) in the medulla (MD).
Fig. 4: Photomicrograph of the ovary of Group D animals treated with, 500mg/kg body weight *Lophira lanceolata* stained with H&E (X100). Ovary section showing Graafian follicles (AF), corpora lutea (CL), a primary follicle and blood vessels in the medulla (MD).

Fig. 5: Photomicrograph of the ovary of group E animals treated with 500mg/kg body weight of *Alchornea cordifolia* stained with H&E (x100). Section showing ovary with Graafian follicle (GF) containing oocyte (OC), follicular antrum (FA), corpus luteum (CL), primary follicle (PF) and surface epithelium (SE).
Demonstration of oestrogen and progesterone receptors in the ovary

Section of the ovary of Group A animals (negative control) that received normal saline and stained with Avidin Biotin Complex (ABC) method showed negligible staining intensity for progesterone receptors (indicated by the brown areas) in the corpora lutea and stromal cells. However, there was a weak staining intensity in the secondary follicle (Fig. 6). Section of the ovary of Group B animals given normal saline showed the moderate intensity of staining for progesterone receptors in the stromal as well as follicular and corpus luteum cells (Fig. 7). Section of the ovary of Group C animals treated with 500mg/kg of combined extracts of *L. lanceolata* and *A. cordifolia* showed weak staining intensity for progesterone receptors (Fig. 8). Section of the ovary of Group D animals treated with *L. lanceolata* showed the weak intensity of staining for progesterone receptors in the stroma, follicular and corpus luteal cells (Fig. 9). The ovaries of Group E animals treated with 500mg/kg of *A. cordifolia extract* showed a weak intensity of staining for progesterone receptors (Fig. 10).

Section of the ovary of Group A (negative control) animal given normal saline showed weak staining intensity for oestrogen receptors (Fig. 11). Sections of the ovary of Group B animal (positive control) given normal saline showing strong staining intensity for oestrogen receptors (Fig. 12). Group C animals treated with 500mg/kg of the combined extract showed the moderate intensity of staining for oestrogen receptors (Fig. 13). Sections of ovaries of groups D and E treated with 500mg/kg of *L. lanceolata* and *A. cordifolia* respectively showed moderate staining intensity for oestrogen (Figs. 14 &15).

![Photomicrograph of the ovary of negative control animal rat, stained for progesterone receptors, Avidin Biotin method (x400).](image)

The ovary showed almost no staining in the corpus luteum and stromal cells but showed very weak staining intensity in the follicle (arrow).
Lophira lanceolata and Alchornea cordifolia on oestrogen and progesterone receptors in the ovary of menopausal rats

Fig. 7: Photomicrograph of the ovary of Group B (positive control) animal given normal saline stained for progesterone receptors, Avidin Biotin Complex method (x400).
Section of ovary showing moderate intensity of staining for progesterone receptors in both stromal and follicular, and corpus luteum cells indicated by the brown colours within cells (arrows).

Fig. 8: Photomicrograph of immunohistochemistry study of the ovary of animals treated with 500mg/kg each of combined extracts of L. lanceolata and A. cordifolia, stained for progesterone receptors, Avidin Biotin method (x400).
Section of ovary showing weak intensity of staining for progesterone receptors (arrows).
Fig. 9: Photomicrograph of the ovary of Group D animal treated with 500mg/kg of *L. lanceolata*, stained for progesterone receptors. Avidin Biotin Complex method (x400). Section of ovary showing weak intensity of stain for progesterone receptors in the stroma cells but strong intensity in the follicular and corpus luteal cells (arrows).

Fig. 10: Photomicrograph of Group E of the ovary of Group D animal treated with 500mg/kg of *A. cordifolia* stained for progesterone receptors. Avidin Biotin Complex method (x400). Section of ovary showing weak intensity of stain for progesterone receptors (arrows).
Lophira lanceolata and Alchornea cordifolia on oestrogen and progesterone receptors in the ovary of menopausal rats

Fig. 11. Photomicrograph of the ovary of negative control rat given normal saline stained for oestrogen receptors, Avidin Biotin method (X40). The ovary showing with weak intensity for oestrogen receptors.

Fig. 12: Photomicrograph of the ovary of Group B (positive control) animals given normal saline stained for oestrogen receptors, Avidin Biotin method (x400). The ovary showing strong staining intensity for oestrogen receptors.
Fig. 13: Photomicrograph of the ovary of Group C rat treated with 500mg/kg each of combined leaf extracts of *A. cordifolia* and *L. lanceolata*, stained for oestrogen receptors, Avidin Biotin method (X400). Section of the ovary showing weak staining intensity for oestrogen receptors.

Fig. 14: Photomicrograph of the ovary of Group D rat treated with leaf extracts of *L. lanceolata*, stained for oestrogen receptors, Avidin Biotin method (x400). The ovary showing weak staining intensity for oestrogen receptors.
DISCUSSION

Phytomedicines are widely used for the treatment of a variety of diseases with proven efficacies (Ebong, 2015). Acute toxicity test carried out on L. lanceolata and A. cordifolia showed that administrations of up to 5000mg/kg bodyweight of ethanolic leaf extract of either plant showed no adverse effects. This is in line with previous studies that reported similar findings (Etuk & Mohammed, 2010; Kouakou et al., 2013; Onyeto et al., 2014; Igboeli et al., 2015; Fischer & Fischer, 2017a) for L. lanceolata, and for A. cordifolia (Ansah et al., 2011; Ajibade & Olayemi, 2015; Fischer & Fischer, 2017b). These plants are relatively safe, and they have been used over the years in phytomedicine for the treatment of diverse health conditions such as infertility and menstrual cycle disorders.

In the present study, the ovaries from Group B (positive control) and all the treatment groups (Groups C-D) showed normal histological features consistent with the pro-oestrus phase of the oestrous cycle. These include pre-ovulatory (Graafian) follicles, corpora lutea at varying stages of degeneration from previous cycles. Other structures present were primordial follicles, primary follicles, secondary follicles, atretic follicles, and corpus Albicans, to varying degrees, which are normal findings of ovarian tissue. Group C animals treated with combined extract had multiple Graafian follicles. The menopausal Group A animals (negative control) however, did not show any Graafian follicle, and primordial follicles were not seen in the sections taken. There were, however, corpora lutea, and a primary follicle. The presence of these structures can be explained by the fact that in rats, follicles are not completely depleted even in aged rats, and also prolactin is luteotropic, therefore it can maintain the corpus luteum and create a pseudopregnant state. Prolactin secretion can be activated by irritation or mechanical stimulation of the

Fig. 15: Photomicrograph of the ovary of Group E animals treated with 500mg/kg of A. cordifolia stained for oestrogen receptors, Avidin Biotin Complex method (x400). The ovary is stained with weak intensity for oestrogen receptors.
vagina, as might occur during the collection of vaginal smears. No histopathological feature was observed in any of the groups. The features observed in the negative control is normal for aging or aged rats. Other medicinal plants have been reported to have no effect on ovarian histology too. Amah et al. (2011) reported that Momordica charantia had no effect on ovarian and uterine histology despite distorting the oestrous cycle. In contrast to this, Onyegeme-Okerenta and Essien (2015) reported that Millettia aboensis leaf extract caused the destruction of ovarian cells in a dose-dependent manner, Aspilla africanus disrupts was also reported to cause ovarian stromal cell degeneration and disruption of the endometrium of the uterus (Oyesola et al., 2010).

Oestrogen and progesterone receptors are distributed widely in the ovary, from the granulosa cells, thecal cells, stromal cells, to surface epithelial cells, where they mediate the actions of oestrogen and progesterone (Woodruff & Mayo, 2005). The expression of these receptors varies with the phase of the oestrous cycle, thus at pro-oestrus, oestrogen receptors will be found in high intensity within granulosa cells, while progesterone will be poorly expressed prior to the LH surge (Woodruff & Mayo, 2005). The results of this study showed that progesterone receptors were poorly expressed in the negative control as well as in all the treatment groups, as represented by the weak staining intensity of the cells. The positive control group showed moderate staining intensity while the other groups showed strong staining intensity. This is expected, because of the ongoing folliculogenesis, and the presence of corpus luteum. The ovary is both a site of production and target of oestrogen which is involved in early folliculogenesis (Teresa et al., 2005). In fact, oestrogen receptor knockout mice are infertile and have attenuated folliculogenesis and anovulation (Woodruff & Mayo, 2005).

**Conclusion**

Our findings demonstrated that no abnormal feature was noted in the histology of the ovary, rather the features were consistent with normal expectations in the pro-oestrus phase of the cycle. The receptor expression had a similar intensity of stains in the treated groups and the positive control group, but weak in the negative control. The combined extract was the most potent, gave the best results relative to the individual herbs. A. cordifolia and L. lanceolata may be useful, especially when used in combination, in the management of sub-fertility in the perimenopause period, alleviation of perimenopause and menopause symptoms (because of their oestrogenic properties), menstrual cycle irregularities and probably delay the onset of menopause.

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