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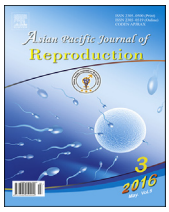

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Alteration in oestrus cycle and implantation in *Mus musculus* administered aqueous wood ash extract of *Azadirachta indica* (neem)

T. Auta[1*], A.T. Hassan[2]

[1]*Department of Biological Sciences, Federal University Dutsin-Ma, Katsina State, Nigeria*

[2]*Department of Zoology, University of Ibadan, Ibadan, Nigeria*

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ABSTRACT

Objective: The study was designed to investigate the alterations in oestrus cycle and implantation in *Mus musculus* administered aqueous wood ash extract of *Azadirachta indica*.

Methods: Mice were randomly grouped into four groups (5/group) where 0, 5, 50 and 100 mg/kg doses of the extract were orally administered for 20 d and microscopy of vaginal smear carried out daily to determine anti-ovulatory activity. Oestrus cycle, including metestrus, diestrus and oestrus phases and histopathology of uterus were examined. To assess abortifacient activity, pregnant mice were also administered extracts for 7 d and laparotomised on the 10th day of pregnancy to observe implantation in the horns of the uterus.

Results: Duration of metestrus and diestrus phases varied significantly, while the frequency at which the oestrus phase occurred was lowest at 100 mg/kg, (2.8 ± 0.2) and (1.2 ± 0.4) d, with degeneration of epithelial cells of glands in the uterus. Failure of implantation (0% implantation) was recorded in mice exposed to 50 and 100 mg/kg of the extract.

Conclusions: Aqueous wood ash extract of *A. indica* altered the duration of metestrus, diestrus and oestrus phases of the oestrus cycle and compromised implantation in female mice.

1. Introduction

The use of wood-ash extracts, including that of *Azadirachta indica* as food additives and for medicinal purposes by some ethnic groups in the Middle-Belt Region of Nigeria has been an age long practice [1]. The physical and chemical composition of ash contents are variable among tree species and also depend on soil type and climate. They vary significantly depending upon the method and manner of combustion, efficiency of the boiler, and other supplementary fuel used with wood [2,3]. Auta *et al.* wood ash extract of *A. indica* has been reported to contain high concentrations of heavy metals and Polycyclic Aromatic Hydrocarbons (PAHS), with presence of some phytochemicals [1].

A. indica has been well known in the traditional system of medicine for more than 2000 years as one of the most versatile medicinal plants, with a wide range of biological activity [4]. Several beneficial products such as antimalarials, spermicidals, antituberculosis agents, antipyrrhetics, antiviral drugs, antiseborrheics, antiallergic medicines, antienzymic, and antifungal agents, have been extracted from the *A. indica* [5]. It has been reported to have antiseptic, anti-helminth, anti-fungal, antibacterial, antipyretic, antimalarial, anti-diabetic and anti-fertility properties [6].

Several plants have been confirmed as antifertility, abortive, uterine stimulant, estrogenic or cytotoxic agents in animals and humans [7]. The aqueous wood ash extract of *A. indica* has been reported to have deleterious effects on sperm motility, count, morphology and pathological alterations of testes in mice, hence compromising male fertility [8]. There is dearth of information in the open scientific literature that has reported on the reproductive toxicity of aqueous wood ash extract of *A. indica* in female mice. Thus, this study has shown alteration in the oestrus cycle and implantation in female albino mice treated with varied dose levels of aqueous wood ash extract of *A. indica*.

*Corresponding author: T. Auta, Department of Biological Sciences, Federal University Dutsin-Ma, Katsina State, Nigeria.
E-mail: autatimz@gmail.com

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2. Materials and methods

2.1. Collection of woods and preparation of ash and extract

Fresh *A. indica* (neem) wood was collected from Katsina State, North-Western Nigeria. Stalk of the plant, carrying leaves and flowers, were collected and authenticated at the herbarium of Department of Biological Sciences, Ahmadu Bello University, Zaria, with a voucher number of 90051. Collection and processing of wood ash was as described by Auta *et al.* [1].

2.2. Selection of dose levels

The doses of 5, 50 and 100 mg/kg, as used in this work were selected based on a range finding test carried out.

2.3. Anti-ovulatory activity

This study was carried out using method described by [9]; Female mice (12–14 weeks of age) with normal oestrus cycle (average 4–6 d of proestrus, oestrus, metoestrus and dioestrus) were divided into 4 groups (n = 5/group), totalling 20 mice. Mice in group 1 received 0 mg/kg (distilled water) while groups 2, 3 and 4 received 5, 50 and 100 mg/(kg·bw) per day doses of neem wood-ash extract for 20 consecutive days. Vaginal smears were examined daily during this period to check the stage of oestrus they are in. Persistent dioestrus, non –cyclic and lengthy oestrus cycles were considered as toxic signs. Different stages of the oestrus cycle were determined based on the presence or absence of leukocytes, cornified epithelial, and nucleated epithelial cells according to [10]. Diestrus index was calculated using the formula:

$$\text{Diestrus } index = \frac{\text{Numbers of days with clear diestrus smear}}{\text{Total duration of treatment (Days)}} \times 100$$

2.3.1. Histopathology of uterus

Samples from the uterus of the four groups were processed histologically for paraffin sections. 5–7 µm sections was prepared and stained by haematoxylin and eosin stain.

2.4. Abortifacient activity

Regularly cyclic female virgin mice (12–14 weeks of age) were mated. Presence of copulation plugs or sperms in the vaginal smear on the following morning were regarded as confirmation of mating. The day copulation plugs or sperms appeared was assumed the day one of pregnancy. Pregnant female mice were randomly divided into 7 groups (n = 5/group). Mice in group 1 received 0 mg/kg (distilled water) bw/day while groups 2, 3 and 4 received 5, 50 and 100 mg/kg bw/day doses of neem wood ash extract and groups 5, 6 and 7 received 5, 50 and 100 mg/kg bw/day locust bean wood ash extract from day 1–7 of pregnancy. During pregnancy, the mice were evaluated for survival, altered appearance and any clinical signs of toxicity such as changes in food and water intake, piloerection, diarrhoea, changes of locomotor activity and vaginal bleeding. Autopsies were performed on the 10th day and the number of corpora lutea, implantation sites and the number of live/dead foetuses were recorded. Implantation index was calculated by the formula:

$$\text{Implantation } index = \frac{\text{Total number of implantation sites}}{\text{Number of corpora lutea}} \times 100$$

Abortion (%) was calculated by the formula:

$$\text{Abortion } (\%) = \frac{\text{Number of dead foetuses}}{\text{Number of live + Number of dead}} \times 100$$

2.5. Statistical analysis

The values are expressed as mean ± standard error (SE). An analysis of variance (one way ANOVA) was used to determine the significance between different doses of exposure and was followed by Duncan Multiple Range (DMR) test. The significant difference between the groups was considered significant at $P < 0.05$.

3. Results

The control mice, administered distilled water showed regular oestrous cycle and normal duration of each phases of oestrous cycle. Mice administered aqueous extract of *A. indica* at 5, 50 and 100 mg/kg showed significant dose dependent decrease in number of proestrus and oestrus phases, while metestrus and diestrus phases showed significant increase in number compared to the control group; diestrus index decreased with increase in dose concentration (Table 1).

Histopathology results of the uterus tissues of mice used for the experiment, as presented in Figure 1 showed moderate dilation of lumen; glands and endometrium lined by high columnar epithelium; moderate degeneration of epithelial cells of glands in the control group. Mice exposed to 5 mg/kg of aqueous extract of *A. indica* showed mild/slight hyperplasia of endometrial lining which is composed of simple to low stratified cuboidal epithelial cells. Glands are simple columnar. Mice in group administered 50 mg/kg showed undulating endometrial lining composed of stratified columnar epithelium with a few degenerate epithelial cells; glands are stratified epithelium. The 100 mg/kg group showed moderate dilatation of lumen; glands

Table 1
Effect of aqueous wood ash extract of *A. indica* wood ash on oestrous cycle in albino mice.

Group	Number of mice	Number of cycles	Duration in days (Mean ± SEM)				Diestrus index
			Proestrus	Oestrus	Metestrus	Diestrus	
Dw	5	4.80 ± 0.20^{a}	6.20 ± 0.37^{a}	5.80 ± 0.37^{a}	4.60 ± 0.40^{a}	3.40 ± 0.24^{a}	19.00
5AI	5	4.60 ± 0.24^{a}	4.80 ± 0.37^{b}	4.40 ± 0.40^{b}	5.80 ± 0.49^{ab}	5.00 ± 0.55^{b}	25.00
50AI	5	4.40 ± 0.24^{a}	4.60 ± 0.40^{b}	3.20 ± 0.20^{c}	6.00 ± 0.55^{ab}	6.20 ± 0.37^{bc}	31.00
100AI	5	3.40 ± 0.24^{b}	3.80 ± 0.20^{b}	2.80 ± 0.20^{c}	6.40 ± 0.40^{b}	7.00 ± 0.45^{c}	35.00

Values are expressed as Means ± SEM (n = 5 per group). Means in same column with different superscript letters are significantly different; $P < 0.05$.

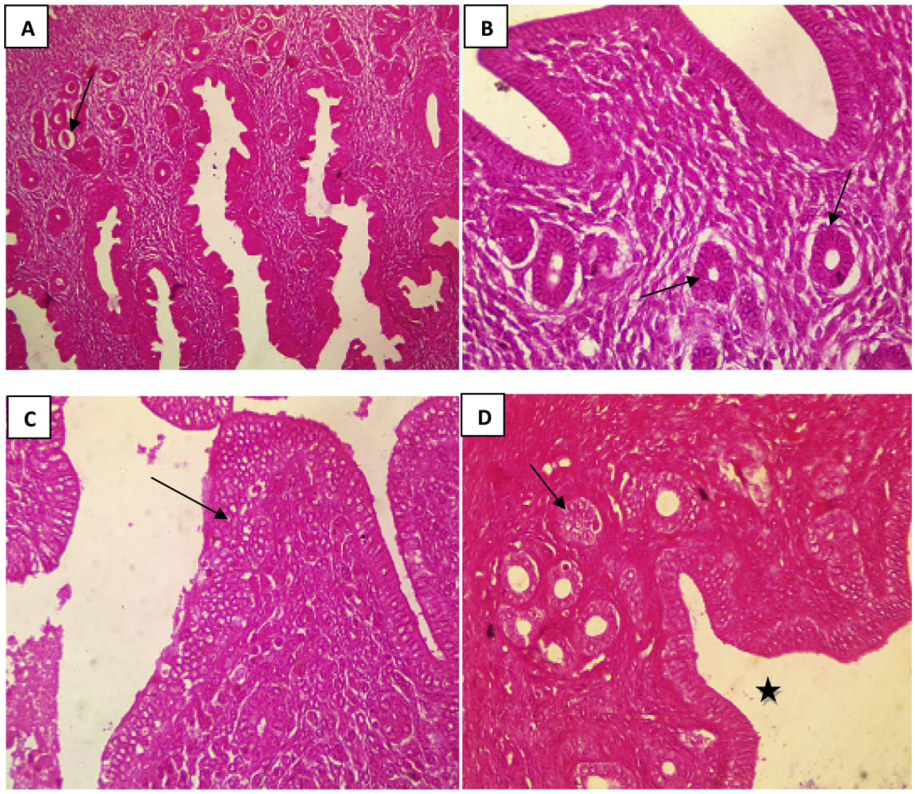

Figure 1. Photomicrograph of mice uterus exposed to aqueous extract of *Azadirachta indica* wood ash.
A (control), there is moderate dilation of lumen; glands and endometrium lined by high columnar epithelium and the glands show mild degeneration (arrow). B (5 mg/kg), there is mild hyperplasia of endometrial lining which is composed of simple to low stratified cuboidal epithelial cells. Glands (arrows) are simple columnar. C (50 mg/kg), the endometrial lining (arrow) is undulating and composed of stratified columnar epithelium with a few degenerate epithelial cells; glands are stratified epithelium. D (100 mg/kg), there is moderate dilatation of lumen (star). The glands and endometrium lined by high columnar epithelium and there is moderate degeneration (arrow) of epithelial cells of glands. H&E. X100(A). X400 (B–D).

Table 2
Abortifacient effect of aqueous extract of *A. indica* wood ash.

Group	Number of mice	Number of corpora lutea	Number of implantation	Implantation index	Number of live foetuses	Number of dead foetuses	Abortion (%)
Control	5	12.80 ± 1.02	9.40 ± 0.51	74.53 ± 4.16	9.00 ± 0.32	0.40 ± 0.24	3.82 ± 2.34
5AI	5	5.40 ± 0.75	2.80 ± 0.86	45.71 ± 12.49	0.80 ± 0.37	1.60 ± 0.68	40.33 ± 16.64
50AI	5	2.00 ± 0.32	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
100AI	5	1.20 ± 0.37	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Values are expressed as Means ± SEM (n = 5 per group). Means are significantly different at $P < 0.05$.

and endometrium lined by high columnar epithelium; moderate degeneration of epithelial cells of glands.

Results (Table 2) obtained from the study of effects of aqueous extract of *A. indica* wood ash in mice, sacrificed on the tenth day of pregnancy, after exposure for 7 d revealed sharp decrease in the number of corpora lutea and number of implantation. The implantation index decrease significantly with increase in the concentration of dose, with 0 (zero) implantation index among mice administered 50 and 100 mg/kg of the extract. The number of live foetuses equally decreased tremendously, with number of dead foetuses greatly increased. Abortion index significantly increased among the groups. Those administered 50 and 100 mg/kg (group 50AI and 100AI respectively) did not conceive at all.

4. Discussion

In this study, the variations in the phases of oestrus cycle in the experimental mice groups showed a dose dependent pattern. The regular oestrus cycle of 4–5 d observed among control mice, those orally administered distilled water indicated proper steroid production, which is in turn controlled by the gonadotropic hormones [11]. An oestrus cycle is a rhythmic reproductive cycle in sexually matured female mammals and is influenced by the release of gonadotropin releasing hormone from the pituitary gland and sex hormones from the gonads. While female cyclicity characterised by vaginal changes as observed in oestrus cycle is an index of good functioning of the neuroendocrine-reproductive system and ovarian activity, the distortion of the normal oestrus cycle in this study indicates the disruption of ovarian progesterone and oestrogen balance [7,12,13].

This alteration in the oestrous cycle observed: prolonging of the duration of the metestrus and diestrus phases and subsequently lowering of the frequency at which the oestrus phase occurs; increase of diestrus index with increase in dose in all the groups following the administration of the extracts implies reduction in frequency of ovulation. Hence, fertility may therefore be impaired [14–19].

Cyclic changes of the vaginal smear observed in oestrous cycle gives a reasonable index of the ovarian activity and its

hormonal synthesis of oestrogen and progesterone. The levels of these hormones are controlled by hypothalamus releasing gonadal hormones and pituitary gonadotropins [20]. Oestrogen chemicals are known to cause infertility by shortening the time of transport of egg, disrupting oestrus cycle, lowering the plasma progesterone and decreasing pregnanediol which finally stops development of endometrium [21,22]. It has also been reported in literatures that anti-fertility plants with estrogenic effect can directly influence the pituitary action through peripheral modulation of luteinizing (LH) and follicle-stimulating hormones (FSH) by decreasing the secretion of these hormones and blocking ovulation [23]. In addition, the plant may also intercept the synchronized development of the ovum and endometrium while others may have abortifacient or antiprogestational effects [24]. Oestrogen and progesterone are the hormones responsible for histology and functional modifications of female genital tract [25]. These extract might have interfered with the hormone synthesis which might have brought about changes in the mechanism of oestrus cycle and cell cytology during oestrus phase [26].

The presence of tannin, flavonoids, saponins and steroids in the aqueous wood ash extract of *A. indica* as reported by [1] might be a contributing factor in disruption of oestrus cycle. They have been reported to disrupt oestrus cycle [27]. The endometrial changes in the uterus, the disarrangement of endometrium, endometrial stroma and reduction in endometrial height may be due to imbalance in hormonal level caused by the presence of phytoestrogens in the aqueous wood ash extract of *A. indica* [1], which are reported to have an antifertility effect [28].

Failure of implantation observed in this study could be attributed to alteration or interference in the production of hormones (oestrogen and progesterone). Thus, the lining of the uterus does not grow enough to support or nourish fertilized eggs, hence preventing implantation [29–33]. It may also be due to the effect of the extracts on blastocysts or the final stage of implantation, probably due to alteration in the endometrial environment or a combination of both. Implantation can only be successful when the activated state of the blastocyst coincides with the receptive state of the uterus [34–37].

Abortifacient activity of aqueous extract of *A. indica* wood ash in this study may also be due to the presence of flavonoids and other phytochemicals, which are known to exhibit anti-fertility activity [38–42]. It could also be due to uncontrolled uterine contractions, leading to abortion depending on the oestrogen levels in the tissues that could be due to uterotonic effects of combination of enzymes [43].

In conclusion, aqueous wood ash extract of *A. indica* altered the duration of metestrus, diestrus and oestrus phases of the oestrus cycle and compromised implantation in female mice. It could lead to infertility in females.

Conflict of interest statement

The authors declare that no conflict of interests.

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