Original Research

Lactogenic study of the ethyl-acetate fraction of *Hibiscus sabdariffa* linn seed on pituitary prolactin level of lactating albino rats

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Summary. The effect of ethyl-acetate fraction of *Hibiscus sabdariffa* l. seed on pituitary prolactin and milk production was evaluated in albino rats. Twenty four lactating rats were randomly grouped at parturition into control, metoclopramide-treated and Ethyl-acetate-treated group consist of six rats in each group (n=6). The lactating rats were administered control (normal saline), metoclopramide (5mg/kg) and ethyl-acetate fraction (100 and 200mg/kg) respectively from day 3-17 of lactation. Milk yield for rats were estimated by pup weight and weight gain. The animals were then euthanized on the day 18 and pituitary prolactin was analyzed using prolactin kit. The prolactin level of ethyl-acetate fraction of *Hibiscus sabdariffa* showed a significant increase (P<0.01) when compared to control group. Pup weight gain was also significantly higher (P<0.05) than the control group. This can be inferred that ethyl-acetate fraction of *Hibiscus sabdariffa* l. seed has lactogenic activity because it increases pituitary prolactin level and milk production in lactating female albino rats. The LD50 of Ethyl-acetate fraction of *Hibiscus sabdariffa* was found to be above 5000mg/kg.

Industrial relevance. The outstanding advantage of this galactagogue option is that, it is safer, affordable and tolerable, and it is taken as an alternative in preference to anti-psychotic drugs that have side effect of drowsiness and depression. The plant calyces, leaves and seeds are eaten as foods because it contain substantial amount of essential fatty, Tocopherol (Vitamin E), ascorbic acid (Vitamin C), mineral salts calcium, magnesium, sodium, potassium and phosphorus.

Keywords. *Hibiscus sabdariffa*; prolactin; lactation; milk; pituitary

INTRODUCTION

Prolactin is a polypeptide hormone that is synthesized in and secreted from specialized cells of the anterior pituitary gland, the lactotrophs (Freeman et al., 2000). Prolactin has a multiple biological activities, however its principal role is in reproduction during lactation, and the synthesis and secretion is from anterior pituitary gland (Bern and Nicoll, 1968; Freeman et al., 2000). The best-known physiological stimulus affecting prolactin secretion is the sucking stimulus applied by the nursing young. This has been characterized as a classical neuro-endocrine reflex, just as muscle contraction evoked by an electrochemical stimulus is described as a stimulus-contraction reflex (Freeman et al., 2000). In mammals, the control exerted by the hypothalamus over pituitary prolactin secretion is largely inhibitory (Ben-Jonathan, 1985). On the other hand, the hypothalamus is involved in the acute stimulatory control of prolactin secretion by removal of the inhibition (disinhibition) and/or superimposition of brief stimulatory input (Ben-Jonathan and Liu, 1992). In addition, prolactin secretion is also influenced by numerous factors released by the lactotrophs themselves (autocrine regulation) or by other cells within the pituitary gland (paracrine regulation) (Ben-Jonathan and Liu, 1992; Taffetani et al., 2007). Prolactin secretion is affected by a large variety of stimuli provided by the environment and internal milieu like the effect of the chemistry of lactation. There have been reports that some of the African women with lactation insufficiency particularly those living in villages depend largely on herbal supplements for milk yield increase or milk induction despite the modern alternative bottle feeding (Okasha et al., 2008). With a serious constraints to modern alternative breast feeding, rural women use herbal decoction to boost (Sholapukar, 1986; Morton, 1987) lactation so that they can breastfed their newborn babies very well. *Hibiscus sabdariffa* l. is an erect, glabrous, annual sub-shrub, 0.5-3m high, with a strong tap-root (Morton, 1987) figure 1. It is cultivated for leaf, fleshy calyx, seed or fibre, according to the respective properties of several varieties, some of which are red coloured (in calyx, foliage, stem and sap), others are green or green tending.

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...to a red tinge (Morton, 1987). *Hibiscus sabdariffa* l. is also used in folk medicine against many complaints that include high blood pressure, liver diseases and fever (Dalziel, 1973; Morag et al., 1975; Wang et al., 2000; Chen et al., 2003). *Hibiscus sabdariffa* has been reported to be antiseptic, aphrodisiac, astringent, chologogue, demulcent, digestive, diuretic, emollient, purgative, refrigerant, sedative, lactogenic, stomachic and tonic (Ben-Jonathan and Liu, 1992; Olaleye, 2007; Okasha et al., 2008). In light of this, the study is designed to evaluate the pituitary prolactin and lactogenic effect of the ethyl acetate fraction of *Hibiscus sabdariffa* seed.

Figure 1: Plant of *Hibiscus sabdariffa* l. (Morton, 1987).

**MATERIALS AND METHODS**

**Chemicals and drugs.** Ethyl acetate puriss Reg No 27227 Sigma-Aldrich®, 1-Butanol puriss Reg. No 33065 Sigma-Aldrich®, Prolactin ELISA 96 test kits (Fortress® Diagnostics Limited, BT41 IQS, UK), Metoclopramide (NAFDAC Reg. no. 04-5946) and Chloroform Poole, BH15 1TD England.

**Preparation of the plant extract.** The samples of *Hibiscus sabdariffa* l. seed were collected in Gaya Hong Local Government in Adamawa state of Nigeria in November 2010. The plant was identified in the Department of Biological Sciences, Ahmadu Bello University, Zaria by a taxonomist authenticated with a voucher number 1056 and deposited in the Herbarium section of the Department of Biological Sciences, Ahmadu Bello University Zaria, Nigeria. Extraction and Fractionation was conducted in Department of Pharmacognosy and drug development, Ahmadu Bello University Zaria. Extraction was done using maceration method while the crude aqueous extracts were fractionated by using Ethyl acetate and n-Butanol reagents.

**Acute toxicity studies.** The lethal dose (LD₅₀) of the plant fraction was determined by the method of Lorke (1983) using 13 rats. In the first phase rats were divided into 3 groups of 3 rats each and treated with the ethyl acetate fraction of *Hibiscus sabdariffa* l. seed at doses of 10, 100 and 1000 mg/kg body weight intraperitoneal. They were observed for 24 h for signs of toxicity. In the second phase 4 rats were divided into 4 groups of 1 rat each and also treated with ethyl acetate fraction of *Hibiscus sabdariffa* l. seed at doses of 1000, 1600, 2900 and 5000 mg/kg bodyweight (i.p). The median lethal dose (LD₅₀) was calculated using the second phase.

**Phytochemical Analysis.** The ethyl acetate fraction of *Hibiscus sabdariffa* l. seed was subjected to preliminary phytochemical screening to identify the chemical constituents. The methods of analysis employed were those described by Brain and Turner (1975).

**Experimental Protocol.** The study was compiled with the ethical committee guidelines of Ahmadu Bello University Veterinary Teaching Hospital, Zaria and the procedures followed were in accord with the ethical standards of Ahmadu Bello University, Zaria, Nigeria with registration number ABUGVTH/PGO/COMM/0048. Twenty Four female albino rats weighing 180-240g were obtained from the Animal house of Department of Human Physiology Ahmadu Bello University, Zaria. The animals were housed and mated with the male rats in a stainless steel metal cage under standard laboratory condition with 12h dark/light cycle. They were fed with commercial feeds and tap water *ad libitum*. Following birth, the litters' weights were recorded and culled to 6 litters per dam. The twenty four lactating rats were randomly divided at parturition into four groups (control, metoclopramide-treated (Alexandre et al., 2002), Ethyl acetate-treated) groups consist of six rats each (n=6). All groups received control (normal saline), metoclopramide-treated (5mg/Kg metoclopramide) and Ethyl acetate-treated groups (100mg/Kg and 200mg/Kg of ethyl acetate fraction) orally for fourteen days starting from day 3 to day 17 of lactation (Vogel and Vogel, 1997). The animals were then euthanized on day 18 and pituitary gland was removed, weighed and homogenized with phosphate buffer pH 8 and then finally analyzed using prolactin kit (Dombrwics et al., 1992).
Diagnostics® Limited, 29 -AH-R011 US in microplate was designed for the quantitative evaluation of rat prolactin. The prolactin species specific enzyme-linked immunosorbent assay (ELISA) kit ALPCO was centrifuged at 1000 × g for 20 min and supernatant was analyzed immediately to evaluate the prolactin level of the gland with sufficient amount of phosphate buffer to obtain 20mg of pituitary tissue per one millilitre of buffer. The tissue was weighed (w5) (Sampson and Jansen, 1984; Ouedraogo et al., 2004). They were subsequently left with their dams during the night. Milk yield 23 h after gavage was estimated as w5 – w4 with a correction for weight loss due to metabolic processes in the pups as [(w2 – w1) + (w4 – w3)]/8.

Milk yield 18 hour after the gavage was estimated as w3 – w2 with a correction for weight loss due to metabolic processes in the pups as [(w2 – w1)/4. For Group IV that received 200mg/kg ethyl acetate fraction *Hibiscus sabdariffa* l. Milk production was estimated 18 h after gavage. The pups were weighed every day during the study period at 07: 00 h (w1) and then isolated from their dams for 4 h (Sampson and Jansen, 1984; Ann and Linzell, 2003). At 11:00 h, the pups were weighed (w2), returned to their dams and allowed to feed for 1 h. At 1200 h, they were weighed (w3). Milk yield 18 hour after the gavage was estimated as w3 – w2 with a correction for weight loss due to metabolic processes in the pups as [(w2 – w1)/4. For Group IV that received 200mg/kg ethyl acetate fraction *Hibiscus sabdariffa* l. Milk production was estimated at 18 and 23 h after gavage. For the measurement of milk yield 18 h after gavage, the same procedure as described above for Group III was followed. For measurement of milk yield 23 h after gavage, the pups were subsequently isolated between 12:00 and 16:00 h. After weighing at 1600 h (w4), they were reunited with their dams for 1 h of feeding and, finally, they were weighed (w5) (Sampson and Jansen, 1984; Ouedraogo *et al.*, 2004). They were subsequently left with their dams during the night. Milk yield 23 h after gavage was estimated as w5 – w4 with a correction for weight loss due to metabolic processes in the pups as [(w2 – w1) + (w4 – w3)]/8.

**Prolactin Analysis.** The pituitary gland was removed, weighed and then homogenized in phosphate buffer pH 8 and diluted with sufficient amount of phosphate buffer to obtain 20mg of pituitary tissue per one millilitre of buffer. The tissue was centrifuged at 1000 x g for 20 min and supernatant was analyzed immediately to evaluate the prolactin level of the gland (Dombrwics *et al.*, 1992). The analysis was conducted in the Department of Chemical Pathology Ahmadu Bello University Teaching Hospital, Shika-Zaria. The prolactin species specific enzyme-linked immunosorbent assay (ELISA) kit ALPCO Diagnostics® Limited, 29-AH-R011 US in microplate was designed for the quantitative evaluation of rat prolactin. The microplate is coated with a first monoclonal antibody specific for rat prolactin. Calibrators and samples are pipetted into the antibody coated microplate. During 2 hours incubation endogenous rat prolactin in the sample bind to the antibodies fixed on the inner surface of the wells. Non-reactive sample components are removed by a washing step. Afterwards, a second polyclonal horseradish peroxidase-labeled antibody, directed against another epitope of the prolactin molecule, was added. During 1 hour incubation, a sandwich complex consisting of the two antibodies and the rat prolactin is formed. An excess of enzyme conjugate is washed out. A chromogenic substrate, TMB (3,3',5,5'-Tetra-Methyl-Benzidine), was added to all the wells. During 30 minutes incubation, the substrate is converted to a colored end product (blue) by the fixed enzyme. Enzyme reaction is stopped by dispensing of hydrochloric acid as stop solution. The color intensity is direct proportional to the concentration of rat prolactin present in the sample. The optical density of the color solution is measured with a microplate reader at 450 nm. A standard curve was obtained by plotting the concentration of the standard versus the absorbance. The PRL concentration of the specimens and controls run concurrently with standards were calculated from standard curve. The lowest detectable level of prolactin with this test was 0.6 ng/mL and a range of 6.64 -23.26 ng/mL for male, 13.7-23.3 ng/mL for female (Dombrowicz *et al.*, 1992).

**Interaction of dopamine with Ethyl Acetate fraction of Hibiscus Sabdariffa L.** The ethyl acetate fraction of *Hibiscus sabdariffa* l. is the active fraction that has prolactin responses and lactogenic activity was interacted with dopamine. The highest dose of Ethyl acetate fraction of *Hibiscus sabdariffa* l. was interacted with dopamine to assess wether it can block the lactogenic response.

**Statistical analysis.** All data are expressed as mean ± standard of error mean (Mean ± S.E.M.). The data obtained were analyzed using t- test student-Newman Keul’s test (Betty and Jonathan, 2003) *post hoc* test for multiple comparisons. The (P<0.05) will be accepted as significant.

### RESULTS

**Toxicity studies.** The plant seed fractions are characterized by a very low degree of toxicity. The acute toxicity LD₅₀ of ethyl acetate fraction of *Hibiscus sabdariffa* l. seed in albino rats was found to be above 5000 mg/kg according to the method of Lorke.

**Phytochemical Analysis.** The preliminary phytochemical screening of ethyl acetate fraction of *Hibiscus sabdariffa* l. seed found the presence of saponins, steroidal rings, cardiac glycosides and flavonoids.
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Table 2. Phytochemical constituents of Ethyl acetate fraction of *Hibiscus sabdariffa* l.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Ethyl acetate</th>
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<tbody>
<tr>
<td>Alkaloids</td>
<td>-</td>
</tr>
<tr>
<td>Anthraquinones</td>
<td>-</td>
</tr>
<tr>
<td>Cardiac glycosides</td>
<td>++</td>
</tr>
<tr>
<td>Deoxy-sugar</td>
<td>-</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>++</td>
</tr>
<tr>
<td>Phlobatannins</td>
<td>-</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
</tr>
<tr>
<td>Steroidal ring</td>
<td>+++</td>
</tr>
<tr>
<td>Tannins</td>
<td>-</td>
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</table>

+++ = High concentration was recorded if a definite heavy precipitate or flocculation observed;
++ = Moderate concentration was recorded if a definite turbidity, but no flocculation observed.
+ = Low concentration was recorded if the reagent produced only a slight opaqueness.
- = Not detected.

*Milk production.* Milk production of both groups receiving 100 and 200 mg/kg of ethyl acetate fraction of *Hibiscus sabdariffa* l. seed was higher than that of the control group as shown figure 2 (a). Milk yield increased from 1.56±0.2, 1.92±0.3 and 2.08±0.2 g/pup/day to about 5.26±0.1, 5.92±0.3 and 6.78±0.2 g/pup per day for the control and ethyl acetate group respectively. The significant differences observed started from day 4 until the end of treatment day 17 (P<0.01). The mean milk yield was 3.02±0.4, 3.83±0.3 and 4.36±0.4 g/pup per day throughout the experimental period respectively (P<0.05) as shown in figure 2 (b). Milk production data at 18 and 23 hour after gavage showed that milk production was significantly in all groups receiving ethyl acetate fraction at both time points. The mean milk yield for the control group was 0.42±0.02 and 0.52±0.03 g/pup at 18 and 23 hour after gavage with normal saline respectively. For the group receiving the ethyl acetate fraction, the milk yield was 0.64±0.04 and 0.72±0.03 g/pup at 18 and 23 hour after treatment respectively.

**Figure 2a:** Effect of ethyl acetate fraction *Hibiscus sabdariffa* l. on milk production 18 h after administration. There was significant difference (P<0.01) throughout the period of administration for 200mg/ml, while significant differences was not recorded throughout the period of administration for 100mg/ml.
Body weight. All pups gained weight during the study period and the rate of weight gain for the ethyl acetate fraction groups was significantly higher than the control as shown in figure 3(a). Body weight increased from 6.8±0.13 to 30.79±1.23 g/pup per day for the control, from 7.82±0.21 to 32.29±1.41 g/pup per day for those receiving 100mg, and from 9.64±0.14 to 35.24±1.23 g/pup per day for receiving 200mg of ethyl acetate fraction of *Hibiscus sabdariffa* l. The daily average weight gain was 1.57±0.05, 1.96±0.06 and 2.25±0.08 g/pup respectively, as shown in figure 3(b). There was a significant difference between all ethyl acetate fraction groups and the control (P<0.05). While no significant effect on the body of the dams was seen.

Figure 2 (b) mean milk production per day was significant for 100mg/ml (P<0.05) and 200mg/ml (P<0.01)
Lactogenic effect of *Hibiscus sabdariffa* l.

Figure 3b: mean weight gain of pup was very significant (P<0.01) when control compared to treated group.

*Pituitary prolactin.* The results obtained in this experiment showed that the Ethyl acetate seed fractions of *Hibiscus sabdariffa* l. have increased pituitary prolactin level significantly (P<0.01) in lactating albino rats as shown in Table 3. Ethyl acetate fraction for 100 and 200mg increased the pituitary prolactin significantly to 31.45±2.39 ng/ml and 34.03±1.67 ng/ml respectively, while the control that received normal saline have the prolactin value of 20.68±0.35ng/ml. In regard to metoclopramide effect on serum prolactin level, it also produced an appreciable (26.40±0.94 ng/ml) increase in prolactin level when compared to the normal saline treated group. This means that both metoclopramide and the ethyl acetate fraction had similar effect on pituitary prolactin level of lactating female rats as shown in table 3 this implies that the potency of the ethyl acetate fraction is almost the same or higher than the standard drug metoclopramide.

Table 3: Pituitary prolactin levels in the control, metoclopramide-treated, and Ethyl acetate- treated groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Prolactin ng/ml</th>
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<tbody>
<tr>
<td>Control Norma saline</td>
<td>20.68±0.35</td>
</tr>
<tr>
<td>Metoclopramide 5mg/kg</td>
<td>26.40±0.94*</td>
</tr>
<tr>
<td>Ethyl acetate 100mg/kg</td>
<td>31.45±2.39**</td>
</tr>
<tr>
<td>200mg/kg</td>
<td>34.03±1.67**</td>
</tr>
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</table>

*P<0.05; **P<0.01*

*Effect of dopamine on Ethyl Acetate fraction of Hibiscus Sabdariffa L.* The ethyl acetate fraction of *Hibiscus sabdariffa* l. is the active fraction that has prolactin responses and lactogenic activity. Ethyl acetate fraction were interacted with dopamine, the highest dose 200mg/kg ethyl acetate-treated group (34.03±1.7ng/mL) increased pituitary prolactin significantly (P<0.01) when compared to the control group (20.68±0.4ng/mL). Nevertheless, the ethyl acetate-treated plus dopamine group (21.13±0.6ng/mL) increased pituitary prolactin but not significant when compared to the control group.
DISCUSSION
The accurate estimate of milk yield in the rat is an important component in lactation research. The measurement of milk production rates in rats is difficult, (Morag et al., 1975; Sampson and Jansen, 1984) however a more feasible direct method is to use the pups to remove the milk from the dam and to determine the amount of milk sucked by the litter (Morag et al., 1975). Milk yield estimations for rats by means of pup weight and weight gains have been used in several studies (Morag et al., 1975; Sampson and Jansen, 1984; Kamani et al., 1987; Kim et al., 1998). The purpose of this study was essentially to determine whether ethyl acetate fraction of Hibiscus sabdariffa seed is the lactogenic components responsible for milk production. Milk production was significantly higher in the ethyl acetate-treated group than in the controls. In addition, milk yield appears to be significantly stimulated about 24 h after administration of the extract and the pup growth rate was significantly improved. The ethyl acetate fraction of Hibiscus sabdariffa seed produced an appreciable increase in pituitary prolactin level when compared to the control with potency higher than the standard drug metoclopramide. The ethyl acetate fraction of seed of Hibiscus sabdariffa exhibited a lactogenic activity by increasing the pituitary prolactin in lactating rats. The effect of Ethyl acetate fraction may be responsible for the lactogenic effect displayed by the aqueous seed extract of Hibiscus sabdariffa (Okasha et al., 2008). Human breast milk is widely accepted to be the optimal source of nutrition for the newborn infant, containing all the proteins, lipids, carbohydrates, micronutrients and trace elements required for growth, development and immune protection (Ostrom, 1990). Herbs and seeds has been reported in other plants (Asparagus racemosus, fennel seed, Grape sap, Acacia nilotica, milk thistle and goat’s rue) (Joglekar et al., 1967; Narendranath et al., 1986; Sholapurkar, 1986; Oketch-Rabah, 1998; Goyal et al., 2003; Ouedraogo et al., 2004) to have lactogenic effect. The presence of cardiac glycosides, flavonoids and steroidal ring in higher concentration in seed extract of Hibiscus sabdariffa may be responsible for the lactogenic effect of ethyl acetate fraction. The mechanism through which Hibiscus sabdariffa exerted its effect might be by dopaminergic influence, as dopamine receptor antagonist, since dopamine blocked the largest dose. It can therefore be inferred that ethyl acetate fraction of Hibiscus sabdariffa seed decoction can improve milk production in lactating women may be valid.

REFERENCES

Figure 4. Pituitary prolactin level of control & ethyl Acetate-treated and control & ethyl Acetate-treated plus dopamine groups in lactating rats.

Ethyl A. = ethyl acetate fraction of Hibiscus sabdariffa l.
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