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Original Research Article

Mammogenic and Lactogenic Effects of Leaf Extract and Fractions of *Launaea taraxacifolia* and Resveratrol in Lactating Wistar Rats

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ABSTRACT

The mammary gland is a dynamic organ subjected to structural and physiological changes during the reproductive cycle of a female animal. The aim of this study was to carry out preliminary lactogenic studies of crude methanol extract (CME), hexane (HEXF) and ethyl acetate (EAF) fractions of *Launaea taraxacifolia*, phytochemical screening of the CME and the effect of HEXF on mammogenesis in lactating Wistar rats. For preliminary studies, 18 lactating rats were treated with 250, 333 and 500 mg/kg each of CME, EAF and HEXF of *L. taraxacifolia* daily at 7.00pm and weighed. Twenty-five mature nulliparous lactating female Wistar rats were divided into five groups of 5 dams each. The dams in groups I, II, III, IV and V were treated by gavage daily at 7:00 pm with distilled water (DW, 2 ml/kg), metochlopromide (MET, 15 mg/kg), resveratrol (RES, 5 mg/kg), HEXF (333 mg/kg) and the combination of RES and HEXF (CO, 5 + 333 mg/kg), respectively. The administration commenced on day 2 through to day 16 of lactation. The results showed HEXF treated group had significantly ($P < 0.05$) higher milk yield compared to CME and EAF. Using thin layer chromatography (TLC), CME was positive for phenols, alkaloids, anthraquinones, flavonoids, steroids and triterpenes. Haematoxylin and eosin staining of mammary gland tissue showed greater alveolar and ductal development in PLT group than in other treatment groups. Therefore, it is concluded that n-hexane fraction of *Launaea taraxacifolia* enhanced milk yield and mammogenesis than the other fractions when administered during lactation.

Keywords: Resveratrol, Milk yield, Lactiferous ducts, *Launaea taraxacifolia.*

Introduction

One of the major food problems in Nigeria is the gross deficiency in protein intake, both in quantity and quality. The low protein intake has been responsible for reduced human productivity with high incidence of infant mortality, severe malnutrition and general weakening of human body which pre-dispose people to diseases, low health status, and shorter lifespan.[1] For instance, the average consumption of animal protein per day is lower than the minimum 35 grams recommended (from animal sources) by the Food and Agriculture Organization (FAO) for daily maintenance of the health of the population.[2] The country-specific analysis of FAO data for 1988-1990 also found that Nigeria was among the countries whose fat-to-energy ratio (FER) fell below the minimum recommendation of 15% dietary energy supply from animal fat.[3]

Dairy production is one of the most important agricultural industries in Nigeria and total milk production in Nigeria is 243, 423 tons, while cow milk accounted for 98 percent of this production.[4] Even though milk production per cow has increased in the last ten years in Nigeria, milk production per cow is still rather low compared to that of European Union (EU) countries. The relatively low milk yield and the decrease in cattle stock coupled with an increase in per capital income and population has resulted in milk supply shortage in Nigeria.[5]

Milk production (lactogenesis), a neuro-endocrine event, is a complex neurophysiological process that involves interaction of a number of physical and emotional factors along with the action of multiple hormones, mainly prolactin.[6] Dopamine agonists and antagonists control milk production by regulating prolactin synthesis and secretion through interaction with the hypothalamus and anterior pituitary.[7]

Lactation is defined as the combined processes of milk synthesis, secretion and removal. Milk secretion refers to the release of milk by myoepithelial cells (MEC) and the passage of milk into the lumen of the alveoli of the mammary gland. Milk removal is the passive removal from the cisterns after the ejection of milk from the lumen of the alveoli to the milk duct.[8] Galactopoiesis is described as the enhancement and maintenance of an already established lactogenesis. Galactopoietic hormones, growth factors, and regular milk removal are essential for regulation and maintenance of lactation. Prolactin, somatotropin, glucocorticoids and thyroid hormones are typically required for full maintenance of lactation.[9]

Lactogenesis is divided into two stages: lactogenesis I and lactogensesis II. In the lactogenesis I, the myoepithelia cells differentiate into milk-producing lactocytes that are maintained by lactocyte renewal. This process is regulated by oestrogen, progesterone, prolactin, metabolic hormones and growth factors.[10] Lactogenesis II involves secretion of milk constituents into the lumens of the lobular alveoli. This process occurs as a feedback response to the withdrawal of progesterone with resultant increase in the circulating levels of prolactin, insulin and cortisol.[11] Low milk production and precarious activation of mammary gland involution to

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the non-lactating state have been reported in defective lactogenesis I and II. This could explain why over 50% of women stop breastfeeding prematurely.[12]

Galactogogues are foods, herbal medicines or pharmaceutical drugs used to induce or enhance milk secretion.[13] While, the term galactopoiesis is used independently to describe the mechanisms that enhance milk production in an animal already in lactation.[14] Galactogogues may be synthetic, plant-derived or endogenous products. They act through exerting an influence on adreno-hypothalamo-hypophyseal-gonadal axis by blocking hypothalamic dopaminergic receptors or by inhibiting dopamine producing neurons. Thus, increasing prolactin secretion by antagonizing dopamine receptors.[15] Galactogogue effect of various plants have been studied, and there is evidence that they increase milk synthesis and secretion and that most of them are safe in humans,[16] cows[17] and goats.[18]

Launaea taraxacifolia is a perennial herb belonging to the family *Asteraceae* (*Compositae*) and found mainly in the tropics. This plant is popularly called African lettuce in English, *Yanrin* in Yoruba and *Namijin dayii* in Hausa languages. The leaves are fed to lactating cattle in Northern Nigeria to increase milk yield, and to sheep and goat when mixed with ration for multiple birth.[19] The advantages of herbal medicines are due to the fact that the constituent chemicals develop as a result of co-evolution between flora and fauna through enzyme-driven synthesis leading to the development of optically pure chiral molecules with specific reactions in the mammalian body.[7] However, majority of these herbal preparations have however not been scientifically, systematically and thoroughly evaluated, but their traditional use suggests some safety and efficacy.[6]

The development of mammary gland structures is referred to as mammogenesis, which begins during early fetal life and proceeds beyond the initiation of lactation.[20] After birth, mammary ducts elongate through cell proliferation. At the onset of puberty, high concentrations of growth hormone, insulin-like growth factor in plasma stimulate mammary duct proliferation to form terminal end buds (TEBs) at the tips of the ducts. Under the influence of estrogen, TEBs actively proliferate to form ductal branches, which fill the mammary fat pad. After this stage, the TEBs regress. During pregnancy, progesterone and prolactin promote lobuloalveolar development to form alveolar buds. At the onset of lactation, mature alveoli capable of producing and secreting milk are formed. Suckling of the nipple by the neonate results in the contraction of the myoepithelial cells around the alveoli, causing the milk to be ejected through the ducts into the nipple.

Materials and Methods

Plant Materials

Plant collection and identification

Fresh leaves of *Launaea taraxacifolia* was obtained in Zaria in June, 2017. The leaves, flower and seeds of the plant was sent to the Herbarium, Department of Botany, A.B.U., Zaria, Nigeria, and voucher specimen number 648 was deposited by Mr. Namadi Sunusi. The leaves were dried in open air in the laboratory and the dried sample was kept in polythene bags until required for preparation of the extract.

Plant extraction

The dried leaves were ground to fine powder using mortar and pestle and 1.19 kg fine powder was obtained. The entire powder was extracted with methanol using cold extraction method. The first phase of the extraction was in the ratio of 1:3, where 300 g of powder was mixed with 900 mL of methanol in a separating funnel and allowed to stand for 48 hours before decanting. In the next phase, the same extracted powder was mixed with methanol in the ratio of 1:1 and left to stand for another 24 hours for complete extraction to take place before decanting. This procedure was done for all the powder. The whole extract was then pooled together and concentrated using a rotary evaporator. The crude methanol extract yielded 142.9 g after concentration.

Solvent partitioning of extract

For fractionation, ¾ of crude methanol extract was dissolved in distilled water (ratio of 1:10 w/v) to form an aqueous methanol extract (AME) and serially partitioned with n-hexane and ethyl acetate as solvents starting with the highly non polar solvent, using a separating funnel. Each partition process was repeated twice using equal volume of each solvent, and similar fractions were pooled together and concentrated at low temperature using rotary evaporator.

Determination of median lethal dose of the extract: limit test at 5000 mg/kg

The median lethal dose (LD_{50}) was determined according to Organization for Economic Corporation and Development (OECD) guideline.[21] Limit dose of 5,000 mg/kg was evaluated. Five healthy nulliparous adult female rats weighing between 120 and 150 g and aged 8-12 weeks were used. One rat was administered with 5,000 mg/kg by gavage and observed for a period of 48 hours. The rat survived after the period of observation, and 4 additional rats were dosed sequentially. Each rat was then observed for 14 days for signs of toxicity or death.

Phytochemical screening

Thin layer chromatographic plate (Merck, Germany) was used to develop chromatograms for the identification of phytoconstituents in the fraction. The plates were developed in hexane:ethyl acetate in a ratio of 9:1 and sprayed with ferric chloride (phenols), aluminium chloride (flavonoids), dragendoff (alkaloids), Liebermann-Burchard (steroids and triterpines) and Bontragers (anthraquinones) reagents. The chromatograms were visualized under day light, ultraviolet light and then heated at 100°C

Ethical Clearance

Ethical approval for the study was obtained from the Ahmadu Bello University Committee on Animal Use and Care (ABUCAUC) and approval number ABUCAUC/2018/053 was obtained.

Determination of most Efficacious Extract/Fraction

Twenty-five adult Wistar rats (nulliparous female n = 20; male n = 5) were used for this study. The rats were randomly divided into five groups of 5 rats each (4 females + 1 male) for natural breeding to occur. After the establishment of pregnancy, the pregnant rats were separated from the male rats and put in individual cages till time of parturition. After parturition, the dams were separated into three groups of 6 dams each. Each group was further subdivided into 3 with 2 dams each, and administered 250, 333 and 500 mg/kg of the extract or fractions respectively. Each dam was left with 5 pups for the period of the experiment.

The efficacy of crude methanol extract of *L. taraxacifolia* and its fractions on lactation were tested separately to determine the best extract or fraction to be used for the main experiment. The dams were dosed orally for 10 days starting from day 2 of lactation to day 12. The dosing was carried out daily at 7.00 pm. The efficacy of these trials on milk yield was carried out as described in section 2.7 below.

Experimental grouping for mammogenic studies

Few days prior to parturition, separate cages were provided for each dam and her pups. The rats were then reshuffled into 5 groups of 5 dams each after parturition. Each of the groups containing 5 dams and 5 pups per dam were put in one cage. Cai *et al*.[22] method was used with little modification.[22] Briefly, twenty-five mature nulliparous lactating female Wistar rats were divided into five groups of 5 dams each. The dams in groups I, II, III, IV and V were treated by gavage daily at 7:00 pm with distilled water (DW, 2 ml/kg), metochlopromide (MET, 15 mg/kg), resveratrol (RES, 5 mg/kg), hexane fraction of the *Launaea taxacifolia* (HEXF, 333 mg/kg) and the combination of RES and HEXF (CO, 5 + 333 mg/kg), respectively. The treatment was done daily at 7.00 pm for 14 days, starting from day 2 to 16 of lactation. Milk yield and weight gain of pups were recorded daily (18 hours after gavage), using an electronic balance accurate to 0.01g.[23] Treatment was done via oral route.

Management of pups
The pups were individually weighed three times daily, using an electronic balance accurate to 0.01 g.[23] At 8.00 am, the first weight (W_1) was taken and the pups were subsequently isolated from the dams for four hours. By 12.00 noon, they were weighed again (W_2) and then taken back to their dams to suckle for another 1 hour. Finally, at 1.00 pm, they were weighed again (W_3) and then allowed to stay with their respective dams for the rest of the day.

Determination of Milk Yield
Milk yield 18 hours after the gavage was estimated as W_3-W_2.

Histology of Mammary Gland
At the end of 14-day experiment, 3 dams were sacrificed from each group and sections of the mammary gland tissue of each dam were taken for histology and mammary cell count. After euthanasia which was achieved by using ketamine at a dosage 3x its anaesthetic dose followed by cervical dislocation, the dam was placed on dorsal recumbency and a midline incision was made on the ventral aspect starting from the base of the neck down to the umbilicus. The ventral skin was flapped side ways to expose the area of the mammary gland. Individual glands were nipped from behind using a digital pressure to make the gland prominent from the inside. With the aid of an assistant, a small incision was made near the gland which then popped out and was cut out. This was immediately fixed in Bouin's fluid for 48 h and then dehydrated through a graded series of ethanol and embedded in paraffin. Paraffin sections of the mammary glands were sliced in cross sectional sections at 5 mm thicknesses and mounted onto coated slides. Before staining, sections were dewaxed in xylene and rehydrated through a decreasing series of ethanol. Then, sections were stained with hematoxylin and eosin according to standard procedures. Tissue preparations were observed and microphotographed under a light microscope.[24]

Statistical Analysis
Data obtained was expressed as mean ± standard error of the mean (±SEM). One-way Analysis of variance (ANOVA) was used, followed by Tukey post-hoc test for multiple comparisons of groups. The statistical package, Graph Pad Prism version 5.1 was used for analysis. Values of $P< 0.05$ was considered significant.

Results and Discussion

There were no signs of toxicity and mortality throughout the two-day observation period for the limit dose of 5,000 mg/kg administered to the rats. The absence of any sign of toxicity at this dose implies that the extract is acutely safe. This observation supports wide range of doses of infusion of leaf of *L. taraxacifolia* used by lactating women. Our finding agrees with earlier reports that any chemical substance devoid of acute signs at 5000 mg/kg in a test population can be regarded as acutely safe and can be used as therapeutic agent.[25,26] Therefore, n-hexane extract of *L. taraxacifolia* is unlikely to be associated with acute hazardous effects in normal usage.
The specific TLC chromatogram was positive for all sprays. With ferric chloride, blue-black coloration was seen under daylight which indicates the presence of phenolic compounds. Aluminum chloride spray gave yellow coloration under ultraviolet light indicating the presence of flavonoids. Following dragendoff spray, orange spots were seen indicating alkaloids. Also, Liebermann-Burchard spray revealed seven spots under day light. Four of the spots turned green while the remaining three turned purple indicating the presence of steroids and triterpenes. Lastly bontragers spray showed pink coloration on the TLC plate which indicates the presence of anthraquinones.
The results of milk yield in the preliminary study shows that hexane fraction of *Launaea taraxacifolia* (HEXF) had significantly higher ($P < 0.05$) milk yield on days 2, 4 and 9 of administration (Figure 1). Furthermore, HEXF had higher milk yield on days 3, 7, 8 and 10 though not statistically significant ($P > 0.05$). Crude methanol extract (CME) of the plant however gave significantly ($P < 0.05$) higher milk yield on day 1 of treatment. The milk yield of CME was also higher than that of HEXF on day 6 but with no statistical significance ($P > 0.05$). HEXF yielded significantly higher ($P < 0.05$) total mean cumulative milk yield when compared to the ethyl acetate fraction (Figure 2).
The effect of treatment with n-hexane fraction of HEXF, resveratrol and their combination in the mammogenic studies on mean daily milk yield is shown in figure 3. Rats in the combination (HEXF and resveratrol; CO) and HEXF groups had higher milk yield than Distilled water (DW), metochlopramide (MET) and resveratrol (RES) groups, though not statistically significant ($P > 0.05$). Furthermore, milk yield of dams in the CO, HEXF and RES groups peaked at day 11 of treatment before gradual decline and then maintained steady levels; while, dams in the MET and DW groups peaked on day 10. However, all the groups showed decreased milk production on day 5 with RES and DW groups having lowest values. Nonetheless, the CO and HEXF still exhibited significantly higher ($P < 0.05$) milk production despite the general decline in milk production after day 11.
There was proliferation of myoepithelial cells, and ducts branching into ductile in all the groups. However, HEXF group had more alveolar bud development and highest number of active secretory cells indicated by increased basophilic stained alveolar cells and interlobular ducts (Figure 4 A). Mammary gland of DW group showed fibroblast and dense connective tissue (Figure 4 E).
Although the estimation of milk yield in rats is somewhat difficult compared to large animals, weight of pups has been reliably used to estimate milk yield from dams.[27] The n-hexane fraction of *Launaea taraxacifolia* (HEXF) significantly increased milk yield on days 4 and 9 of the treatment (figure1). It also had highest milk yield on days 3, 7, 8 and 10 when compared to other treatment groups though not statistically significant ($P > 0.05$). Crude methanol extract of the plant (CME) yielded significantly higher milk on day 1 of treatment. On the over all, HEXF had significantly highest milk yield (figure 2). HEXF thus improved secretory activity of mammary gland and increased milk yield of the rats. In another study, enhanced milk production in lactating rats was attributed to the possible stimulatory effect of *Musa paradisiaca* extract on cell proliferation of the mammary gland.[28] Plants with galactopoietic activities have been reported to affect mammary gland secretory cell proliferation and cellular activity with resultant increased milk production.[29,30] Cai *et al*.[22] observed significant relationship between lobolaralveoli development and increased milk production in Sprague Dawley rats treated with hydrolysate from *Octupus vulgaris* and *Carica papaya*. On the other hand, significant architectural development of mammary glands was reported to commence at birth and reaches peak during puberty and pregnancy.[31] Since lactation is an energy demanding period,[32] these components might have played significant roles in elevating the milk yield of the experimental rats. Although the mechanism of action by which herbal galactogogues increase milk secretion is not known, antagonism of dopamine receptors has been identified as putative mechanism of herbal galactogogue.[33] The HEXF may have acted centrally to increase the antagonism of dopamine receptors and locally to cause myoepithelial contraction.
The myoepithelial cell proliferation, lobulo-alveolar bud development and basophilic secretion seen in the histological section of mammary gland of HEXF group showed that the plant has the potential to augment secretory differentiation and secretory activation of lactating mammary gland. Lactation-promoting medicinal plants demonstrated stimulatory effect on lobulo-alveolar development of lactating mammary gland.[22,27] Although Truchet and Honvo-Houéto [31]opined that complete mammogenesis (secretory differentiation) occurs during pregnancy and become fully functional at parturition, our findings showed that secretory differentiation may be augmented during lactation. Nevile *et al*.[10] observed that secretory differentiation is regulated by lactogenic hormones (eg, prl, oestrogen and progesterone) alongside with metabolic hormones and growth factors. Secretory differentiation is the differentiation of mammary gland epithelial cells into milk-producing lactocytes that are organized into lobulo-alveolar units and maintained by lactocyte renewal.[34] Secretory activation on the other hands takes place at parturition and involves initiation of secretion of milk constituents into the lumens of alveoli. It

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is triggered by the withdrawal of progesterone and surge in the circulating levels of Prl and cortisol.[11] Furthermore, the HEXF was the most potent lactogenic fraction among crude methanol and ethyl acetate fraction of the same plant. Lactogenic/galactopoietic effects of the HEXF observed in this study could be attributed to steroids and triterpenes detected in this fraction.[35] TLC derivatization chromatogram was positive for only these phytochemicals. The steroid, shatavarin isolated from *Asparagus racemosus* enhanced lactogenesis by potentiating oestrogen and prolactin secretion.[36]

Conclusion

The crude methanol extract of *Launaea taraxacifolia* revealed the presence of phenolic compounds, flavonoids, alkaloids, steroids, triterpines and anthraquinones. This study showed that the hexane fraction of the methanol leaf extract of *L. taraxacifolia* gave the highest milk yield than the methanol extract and ethyl acetate fraction. It also had the potential to enhance mammogenesis (secretory differentiation) and lactogenesis (secretory activation); thus justifying its use locally by breast feeding women in Nigeria to enhance milk production.

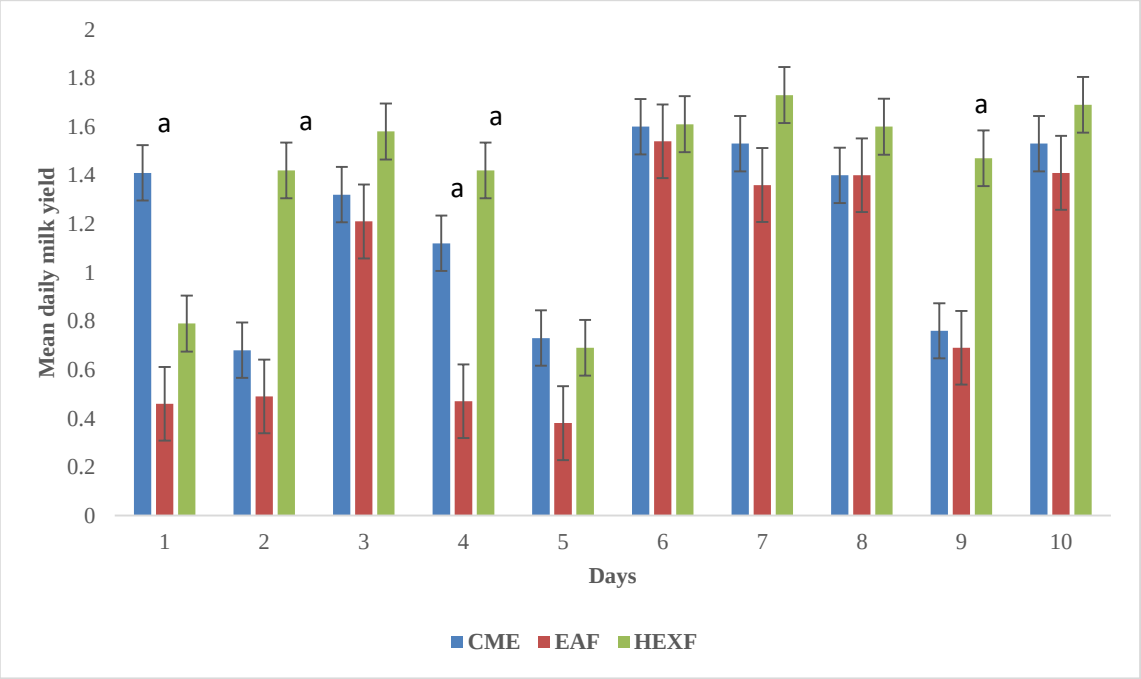

Figure 1: Effect of treatment with the crude methanol extract and fractions of *Launaea taraxacifolia* on mean daily milk yield. Superscript "a" means statistical significance ($P < 0.05$). CME = crude methanol extract; EAF = ethyl acetate fraction; HEXF = n-hexane fraction.

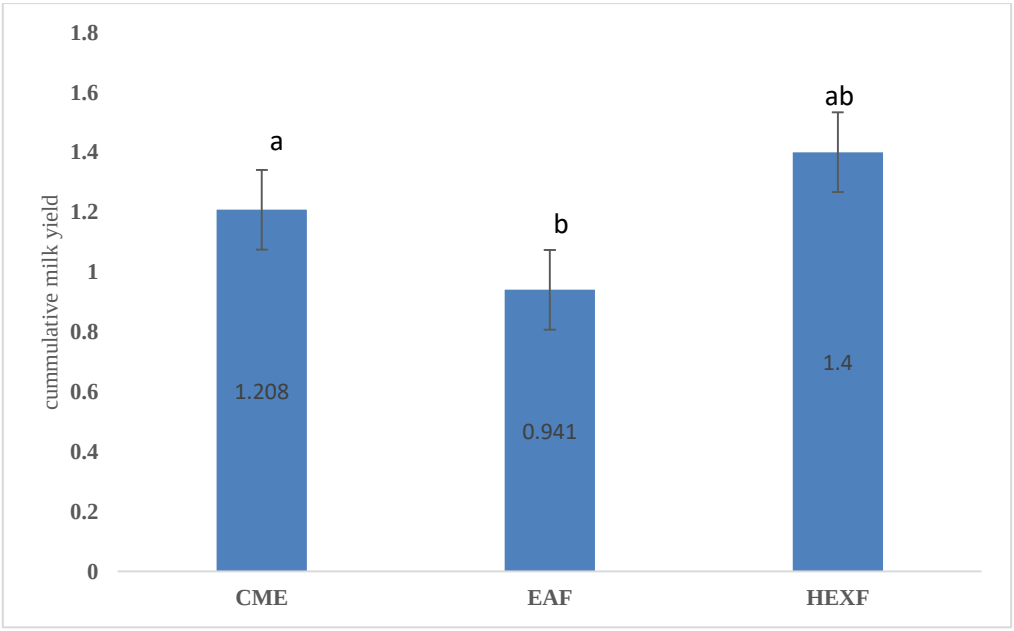

Figure 2: Effect of treatment with the crude methanol and fractions of *Launaea taraxacifolia* on average cumulative milk yield. Different superscript means statistical significance ($P < 0.05$). CME = crude methanol extract; EAF = ethyl acetate fraction; HEXF = n-hexane fraction.

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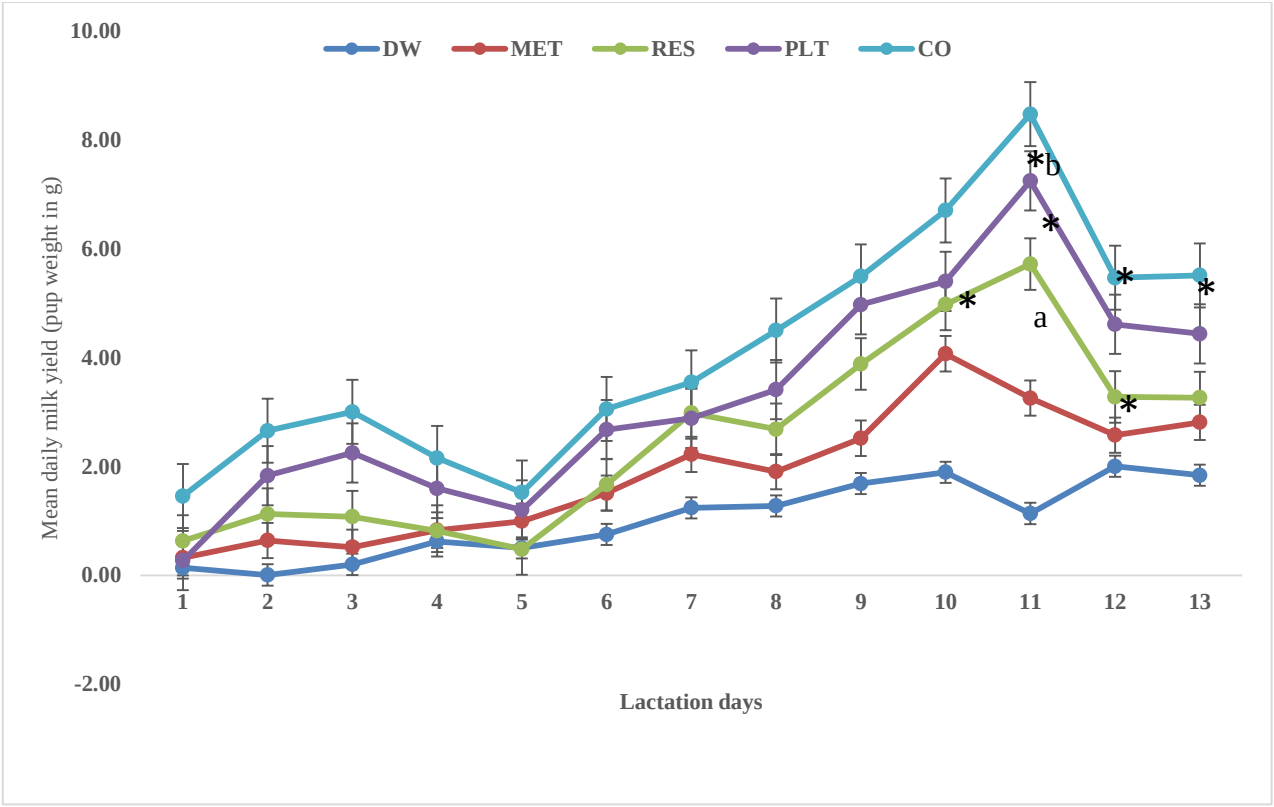

Figure 3: Mean daily milk yield of rats treated with n-hexane fraction of the leaf extract of *Launaea taraxacifolia*, resveratrol and their combination. DW: distilled water, MET: metoclopramide, RES: resveratrol, PLT: *Launaea taraxacifolia* and CO: combination of resveratrol and *L. taraxacifolia.* Points with different alphabets indicate significance ($P < 0.05$) between groups, while points with asterisks indicate significance ($P<0.05$) within groups when compared to day 1.

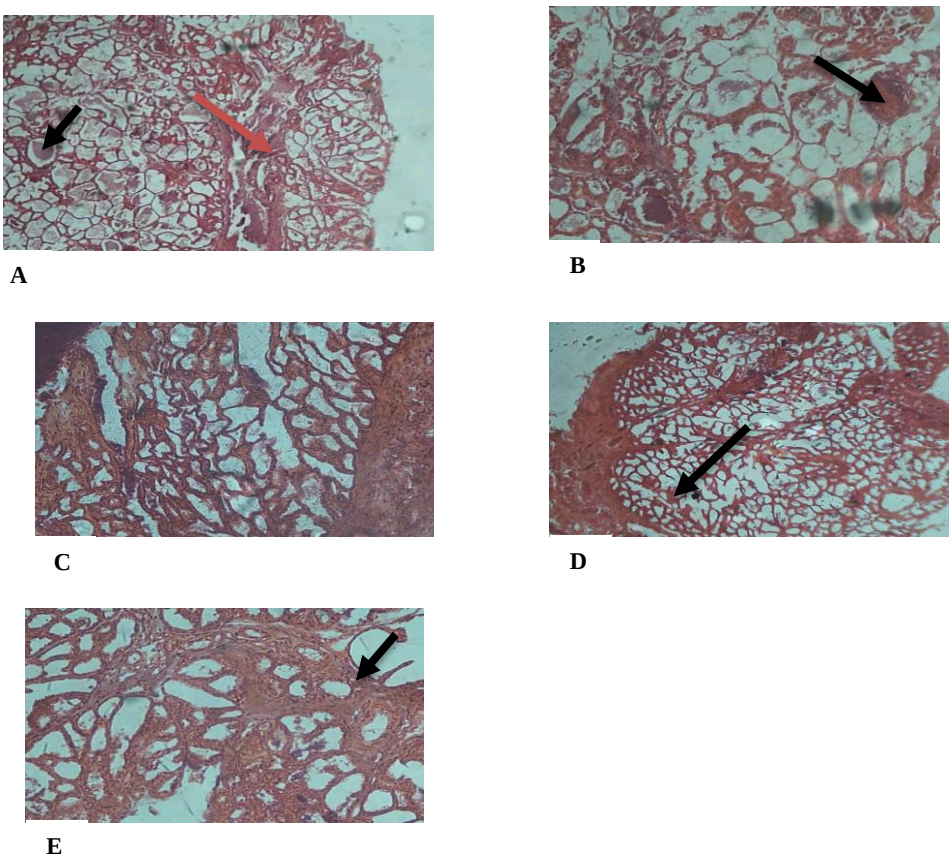

Figure 4: H and E staining of mammary gland and mophometry. Note the high number of secretory cells with basophimic staining (arrow with **A**), filled interlobular alveolar duct (arrow with **B**) and dense adipose tissue (arrow with **C**). A = HEXF group, B = RES group, C = CO group, D = MET group and E DW group.

Conflict of interest
The authors declare no conflict of interest.

Authors' Declaration
The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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