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Composition, nutritive value of *Moringa oleifera* leaves and its clinico - nutritional role in conserving bone integrity of albino rats fed on purified diet

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Abstract

Moringa oleifera leaves (MLS) is a medicinal phytogenic feed additive that commonly fortified to human and animal feeds due to its higher content of many biologically active compounds used to overcome numerous nutrition-related health condition. In this context, two steps feeding trial was conducted, the first was to determine the proximate chemical composition of dried leaves (MLS) and also to assess its contents of some biologically active principles including; total antioxidant activity (TAA),total phenolic contents (TPC) and total flavonoids as well as detecting and quantitating some commonanti-nutritional factors including (Total oxalate, Phytic acid and Total Tannins).Results of proximate composition revealed that the MLS contains 91.3Dry Matter (DM),25.2 Crude Protein (CP), 6.77 Ether Extract (EE), 7.99 Crude Fiber (CF),10.7total ash and 39.64 Nitrogen Free Extract (NFE) % and(1.98%)Ca% .In addition, results indicated that, the total antioxidant activity was (1.360%),the total Phenolic compounds were (1.459%) and the Total flavonoids were (0.971%), while anti-nutritional factors namely oxalate, phytic acid and tannins were (0.35%,0.014% and 2.24%), respectively. The second step in the feeding trial was planned to visualize the impact of dietary fortification of different levels of (MLS) onsome selected serum parameters, femur bone density, Strength and mineral content of albino rats fed on purified diets in comparison to calcium carbonate ($CaCO_3$) as the most common inorganic Ca source. Consequently, a total of 30 apparently healthy male albino rats with an average weight of (80-90 g) were assigned into 5 equal experimental groups. The first group(G1) was fed on modified AIN-93G purified diet supplemented with 1.25% $CaCO_3$ and served as control, the Second (G2) was fed on the same diet supplemented with only 0.06% $CaCO_3$(low Ca diet), the third (G3) was supplemented with $CaCO_3$ (0.6%) and the diet was fortified with MLS (1.26%), the fourth (G4) was fed on the diet fortified with 2.5% MLS and the fifth (G5) was supplemented with $CaCO_3$ (1.25%) and the diet was fortified with MLS (2.5%) respectively. The experimental trial lasted for 11 weeks of which one week was considered as acclimatization period. Results revealed that rats in G2 significantly surpassed all groups in terms of final body weight; meanwhile G5 had the lowest final weight. Animals in G2 showed the highest serum cholesterol and triglycerides levels in comparison to the other experimental groups. Results of the tested biochemical parameters in serum (cholesterol, triglyceride, alkaline phosphatase, total calcium, total phosphorus and parathyroid hormone) and in bones (Total calcium, total phosphorus, density, integrity, mineral density and mineral content) indicated that, serum cholesterol and triglycerides values were higher in G2 and the lowest values were obtained in G5. No significant differences were recorded in the obtained values of bone parameters between G1, G3 and G4 while G2 had the lowest values of bone characteristics evaluation parameters and G5 showed the best characteristics concerning density, strength, mineral density and mineral content. It was concluded that, MLS could be considered as a reliable dietary fortification additive which has a perfect effect on serum cholesterol, serum triglycerides, bone integrity, strength and bone mineral content and density.

Keywords: *Moringa oleifera* leaves, Total flavonoids, antioxidant activity, Total phenolics, Bone mineral density, Rats, physical parameters and biochemical parameters.

Introduction

Medicinal plants have been used to prevent and/or control of some physiological, nutritional and health related problems of both human and animals (**Tilford and Wulff, 2009***)*. They hold a great promise in clinical nutrition and therapy as they possess minimal side effects that are usually associated with chemotherapy or radiotherapy; they are also comparatively cheap and thus significantly reduce health care cost (**Gupta, 2017).** *Moringa oleifera* is a plant of the family *Moringaceae*, grows in tropical and sub-tropical climates **(Foidl *et al.*, 2001, Allen *et al.*, 2014)**. Due to its highnutritivevalue and its content of many biologically active compounds *Moringa oleifera* has been fortified to human and animal feeds either as growth stimulant and/or medicinal purposes **(Richter *et al.*, 2003; Sanchez *et al.*, 2006; Nkukwana *et al.*, 2014; Babiker *et al.*, 2017; Caturao *et al.*,2017)**. Nutritionally, *Moringa oleifera* leaves (MLS) are a good source of protein, vitamin A, B complex and C and minerals such as calcium and iron **(Dahot and Memon, 1985; Foidl *et al.*, 2001; Aregheore, 2002; Ferreira *et al.*, 2008; Mustapha and Babura, 2009; Nuhu, 2010; Madukwe *et al.*, 2013; Gakuya *et al.*, 2014)**. MLS (w/w) has vitamin C content seven times that of orange, potassium content fifteen times that of bananas, 25 times that iron of spinach, ten times the amount of vitamin A in carrots and nine times that of protein in yogurt **(Olson, 2002; Onyeze *et al.*, 2013; Oduro *et al.*, 2008; Manzoor *et al.*, 2007; Mahatab *et al.*, 1987)**. MLS can be used fresh, cooked, or stored as dried powder for many months without refrigeration, and reportedly without loss of nutritional value **(Tesfay *et al.*, 2011)**.On the other hand, Calcium is the most abundant mineral in the body that plays a multiple nutritional roles, as structural constituent of bone and teeth **(Matkovic and Heaney, 1992 and Goodman et al., 1996)** and/or regulatory constituent element specially in regulation of normal neuro-muscular irritability through maintenance of intracellular and extracellular calcium levels **(Thiel *et al.*, 2017)**. In bone, calcium provides skeletal strength and, concurrently, provides a dynamic store to maintain intra- and extracellular calcium pools. Low calcium intake, poor calcium absorption, excessive calcium losses, higher phosphorous intake, vitamin D deficiency or combinations of these factors contributes to calcium deficiency diseases. Numerous health problems such as osteoporosis, hypertension, bone deformity, rachitic bone changes and increased bone resorption and colon cancer are the most prevalent conditions that associated with calcium deficiency **(Feik and Storey, 1979)**. In rats, a low calcium diet (0.03%) found to decrease the level of the ionic calcium in serum and has been accepted as a process to initiate bone resorption and as one of the experimental models to reduce bone mass **(Salomon, 1972; Sissons *et al.*, 1984; Stauffer *et al.*, 1973; Persson *et al.*, 1993; Chen *et al.*, 2001a)**. It has been proven that, a diet containing 0.5% calcium has found to exhibit normal bone growth and development **(Persson *et al.*, 1993)**. Under normal condition milk and its by-products are the most common sources of calcium in human diets. The presence of some bioactive compounds in milk and dairy products may interfere with calcium utilization. Plants may be good alternative sources of calcium; however, calcium bioavailability may be low as it forms complexes with oxalates, phytate and other competing minerals **(Allen *et al.*, 2014).**

Research activities on *Moringa oleifera* leaves (MLS) as phytogenic additive exerting various pharmacological actions are numerous, however the exact proximate composition and nutritive value as well as its content of biologically active compounds and anti-nutritional factors and the possibility of its use to conserve bone integrity under Egyptian condition are scanty and not well documented. That is why the current study was conducted in two steps, the first was to determine the proximate chemical composition of dried leaves (MLS) and also to assess its contents of some biologically active principles including; total antioxidant activity (TAA), total phenolic contents (TPC) and total flavonoids as well as detecting and quantitating some commonanti-nutritional factors including (Total oxalate, Phytic acid and Total Tannins). The second step was planned to visualize the impact of dietary fortification of graded levels of (MLS) onsome selected serum parameters, femur bone density, Strength and mineral content of albino rats fed on purified diets in comparison to calcium carbonate ($CaCO_3$) as the most common inorganic Ca source.

Materials and Methods

The current study was carried out at Regional Centre for Food and Feed,Agricultural Research Center, Giza, Egypt. (From 2018 to 2019).

A. Phase (I)

a. 1-Preparation of *Moringa oleifera* leaves powder

Fresh MLS were collected from medicinal plants farm located at El-Fayoum- Egypt. The leaves were washed with distilled water to remove the residual soil, completely dried firstly under shed then oven dried at 60º C overnight, until constant weight, ground to fine powder, and stored at -20°C in tightly closed plastic containers until use.

a. 2- Determination of proximate chemical composition of MLS

Proximate analysis for MLS powder including moisture, total protein, fat in the form of Ether Extract (EE), ash, minerals and dietary fibre were carried out according to**(AOAC, 2005 and 2007)**. Carbohydrate (NFE) content was calculated by difference. Minerals determination was carried out using Optima 2000DV inductively coupled plasma spectrometer with full PC control (Perkin Elmer). Concentrations were obtained based on calibration curves developed by using (Merck) ICP standards.

a.3- Determination of total antioxidant activity (TAA) of MLS

Total antioxidant activity of MLS powder was determined using the phosphor-molybdenum method according to the procedure described by **Prieto *et al.* (1999)**.

a.4- Determination of total phenolic contents (TPC) of MLS

Contents of total phenolics of MLS were estimated spectrophotometrically using the Folin–Ciocalteu assay **(Singleton *et al.*, 1999)**.

a. 5- Determination of total flavonoids of MLS

Total flavonoid contents were determined using the method of **Ordon *et al.*, 2006**.

a.6-Citrate: was determined according to (AOAC, 2000) using reversed phase HPLC Dionex Ultimate 3000 coupled with ultimate 3000 variable wavelength UV-VIS Detector and C18 150x 4.6 mm x 5 µ column.

a.7-Determination of anti-nutritional factors:

a.7-1 Total oxalate: Determined using titration methods according to **Day and Underwood, 1986**.

a.7-2 Phytic acid: Determination based on precipitation of phytate according to the procedure of **Wheeter and Ferrel, 1971** using Iron (III) nitrate calibration curve.

a.7-3 Total Tannins: Tannins were determined by spectrophotometric method of **Makkar**, **2003** using tannic acid as a standard

B. Phase (II)

b.1-Animals and husbandry

Thirty male rats (80-90 gm) were purchased from National Research Center (NRC) animal housing department, Giza, Egypt were utilized throughout the current study. Rats were housed in stainless steel cages in animal house at Regional Center for Food and Feed, Agricultural Research Center, Ministry of Agriculture, Giza, Egypt, under controlled light and temperature conditions (12/12 h light/dark cycles at 23±3 ºC). During the acclimation period (1 week) and experimental period (10 weeks),

b.2- Experimental diets and Nutritional Management Protocol

Rats were fed on standard diet (Table 1) based on AIN-93G purified diet (**Philip *et al.* 1993**), mineral mix AIN-93G and vitamin mix AIN-93-VX. The diets were isocaloric in nature and differed only in calcium content and source(s).Purified tap water was supplied ad libitum. Rats were assigned into 5 equal experimental groups. The first group (G1) was fed on modified AIN-93G purified diet supplemented with 1.25% $CaCO_3$ and served as control, the Second (G2) was fed on the same diet supplemented with only 0.06% $CaCo_3$ (low Ca diet), the third (G3) was supplemented with $CaCO_3$ (0.6%) and the diet was fortified with MLS (1.26%), the fourth (G4) was fed on the diet fortified with 2.5% MLS and the fifth (G5) was supplemented with $CaCO_3$ (1.25%) and the diet was fortified with MLS (2.5%).

b.3- Measurements, Observations and Statistical analysis

b.3-1 Growth performance parameters

Rats in different experimental groups were weighed individually at the onset of the feeding trial then weighed on biweekly basis to calculate body weight gain. The daily consumed amounts of food were recorded.

b. 3-2 Serum parameters

At the end of treatment period, all the animals were fasted overnight and were sacrificed on the next day. Blood samples were collected from the retro-orbital venous plexus under carbon dioxide anesthesia into no-anticoagulant coated tubes and were left to coagulate at 4°C and then centrifuged at 3000 x g for 15 minutes. The clear serum yields were collected and stored at −20°C for further biochemical analysis.

Table 1 Composition of the experimental diets (g/100 g dry matter) formulated according to AIN-93G:

Ingredients	G1	G2	G3	G4	G5
Diet Ingredients:					
Casein	20.00	20.00	19.68	19.36	19.36
Maltodextrin	13.20	13.20	13.20	13.20	13.20
Sucrose	10.00	10.00	10.00	10.00	10.00
Corn Starch	38.5	39.69	38.41	38.25	37.07
Cellulose	5.00	5.00	4.89	4.79	4.79
Corn Oil	7.00	7.00	6.91	6.83	6.80
*AIN-93G Mineral Mix	3.50	3.50	3.50	3.50	3.50
L-Cystine	0.30	0.30	0.30	0.30	0.30
**AIN-93-VX Vitamin Mix	1.00	1.00	1.00	1.00	1.00
Choline bitartrate	0.25	0.25	0.25	0.25	0.25
Calcium sources:					
Calcium carbonate	1.25	0.0625	0.625	-	1.25
Moringa	-	-	1.26	2.525	2.53

***Mineral Mix according to AIN-93G: **Monopotassium phosphate**(19.6%), **Potassium Citrate monohydrate** (7.078%), **Sodium Chloride (**7.4%), **Potassium Sulfate(**4.66%), **Magnesium Oxide (**2.4%), **Ferric Citrate (**0.606%), **Zinc Carbonate (**0.165%), **Manganese Carbonate**(0.063%), **Copper Carbonate** (0.03%), **Potassium Iodate**(0.001%), **Sodium Selenate, Anhydrous (**0.00103%), **Ammonium Molybdate 4H_2O** (0.000795%), **Sodium Metasilicate 9 H_2O**(0.145%), **Chromium Potassium Sulfate 12 H_2O**(0.0275%), **Boric acid**(0.00815%), **Sodium Floride(**0.00635%), **Ammonium Vanadate** (0.00066%), **Powdered Sugar** (57.8%).

****Vitamin Mix according to AIN-93 VX: **Nicotinic Acid**(3.0 **gm/kg)**, **D- Calcium Pantothenate**(1.6 **gm/kg)**, **Pyridoxine HCl**(0.7 **gm/kg**), **Thiamine HCl**(0.6 **gm/kg)**, **Riboflavin(** 0.6 **gm/kg**), **Folic Acid(**0.2 **gm/kg)**, **D-Biotin(**0.02 **gm/kg**), **Vitamin B_{12}** (2.5 **gm/kg)**, **α-Tocopherol Powder (250 U/gm)**(30 **gm/kg)**, **Vitamin A Palmitate (250,000 U/gm)**(1.6 **gm/kg)**, **Vitamin D3 (400,000 U/gm)**(0.25 **gm/kg)**,**Pylloquinone(**0.075 **gm/kg)**,**Powdered Sucrose** (959.655 **gm/kg).**

Serum biochemical analysis:

Biochemical Blood Analyzer (Alfa Wassermann Diagnostic Technologies, Llc, Ace, Alera, USA) was used to measure the following parameters: Alkaline phosphatase (ALP), total cholesterol (TC), triglycerides (TG)high-density, total calcium and phosphorus using standard commercial kits Diagnostic Systems GmbH (DiaSys) Holzheim, Germany.PTH was measured from serum using chemiluminescent immunoassay for the quantitative determination of intact parathyroid hormone (PTH) levels using the Access Immunoassay Systems, Beckman Coulter, USA) according to the manufacturer's instruction.

b.3-3 Determination of minerals in bone:

At the end of the experiment, Femoral bones from both limbs were dissected out and dried in fresh air for one day then preserved in plastic bags for further investigations.

Calcium and phosphorus were determined in bone by using inductively coupled plasma (optima 2000, Perkin Elmer).Test portion was dry-ashed, treated with nitric acid 70% and dissolved in hydrochloric acid 36-37% then measurements were done at 317.9 nm for calcium and 214.9 nm for phosphorus **(AOAC, 2016)**. Duplicate of each sample have been analyzed and average were taken.

Physical measurement of rat femur bone:

- **Bone density:** Bone density of femur has been measured according to **(Iwamoto *et al.*, 2004)**. The femur was weighed by digital sensitive balance (0.1 mg resolution). The femur was weighed again after submersion in deionized water in volumetric flask. The difference between the weights of the flask before and after placing the bone was divided by the density of water, resulted in the volume of the bone. The bone density was calculated by equation:

  Bone density (g/ml) = bone weight/ bone volume

- **Strength:** The strength of hardness of rat's femur was performed by measuring the breaking force of the femur according to **Tamaki *et al.*, 2003** using Gauge (FGN-50).

- **Bone mineral density and bone mineral content:** The bone mineral content (BMC) and the bone mineral density (BMD) were measured by Dual Energy X-ray Absorptiometry (DXA) using a Dexa NORLAND XR-46.

b.3-4 Statistical analysis:

All obtained results were expressed as mean ± SE and were statistically analyzed using the least significant difference test (LSD) at the 5% level of probability, as described by **Snedecor and Cochran, 1980**. Statistical analysis was performed by computer Duncan test institute program.

Results and Discussion

Due to its high nutritional value, MLS has been found to be used for various purposes such as to overcome human malnutrition, as growth promoter in animal nutrition and as a source of active ingredients for many medicinal purposes **(Richter *et al.*, 2003; Sanchez *et al.*, 2006; Nkukwana *et al.*, 2014; Babiker *et al.*, 2017; Caturao*et al.*, 2017).**

Table 2: Chemical composition of dried MLS:

MLS Chemical composition	Protein	EE	Ash	NFE	Dietary fiber	Moisture
Value (gm/ 100gm) DM	25.2±0.25	6.77±0.22	10.7±0.44	39.64±0.74	7.99±0.15	9.7±0.45

Values are expressed as mean ± standard deviation (n = 3).

In the present study the nutritional constituents of MLS powder (protein, fat (EE), dietary fiber, ash, moisture and carbohydrate (NFE) illustrated in table (2) were found to be in accordance with that represented by **Raimunda *et al.*, 2017; Moyo *et al.*, 2011; Makkar and Becker,1996; Richter *et al.*, 2003; Oduro *et al.*, 2008 and Ferreira *et al.*, 2008**.Among the several nutrients found in MLS powder, protein was the second most abundant content with 25.2 percent which was in accordance with **Anwar *et al.*, 2007**who reported that, MLS contains approximately 25% protein of its dry weight. Also, the carbohydrates content which is considered as the main source of energy for cells **Eze and Ernest, 2014** was found to be close to that obtained by **Okiki *et al.*, 2015**(37.87%) and **Marwa A. Roushdy *et al.*, 2017** (33.48%).

Table 3: Minerals content of MLS powder:

Minerals	Na	K	Br	Cd	Mg	Se	Zn	Fe	Mn	Ca	Ph
Value (%) DM	0.22± 0.027	1.91± 0.17	0.01± 0.003	0.00016± 0.00002	0.3± 0.036	0.0033± 0.0004	0.0029± 0.0004	0.08627± 0.0018	0.007± 0.0004	1.98± 0.172	0.28± 0.019

Values are expressed as mean ± standard deviation (n = 3).

Minerals contents of MLS powder presented in (Table 3)showed that, calcium concentration was 1.98% which was almost not far from what was obtained by **Donkor *et al.*, 2013,** 1.47%, **Marwa A. Roushdy *et al.*, 2017**(2.23%)and **Mahfuz and Piao 2019** (2.19%). Calcium is essential for keeping the integrity of human and animals' skeleton as it regulates many humeral and cellular functions such as blood clotting, muscle contraction and exocytosis. Potassium content in MLS powder percent was 1.91% which was higher than that reported by **Donkor *et al.*, 2013**1.37%. This discrepancy may be attributed to the difference in the origin of the plant and also the time of its harvesting. Potassium has a vast role in human body such as transporting nerve impulse, lowering blood pressure and controlling with sodium the water balance in the body.

Table 4: Antinutritional materials of MLS powder:

Anti-nutritionals	Oxalate	Phytic acid	Tannins
% in 100 gm	0.35±0.026	0.014±0.0017	2.24±0.1

Values are expressed as mean ± standard deviation (n = 3).

Data obtained from Table 4 showed that, the tannin content in the MLS powder was 2.24% and the phytic acid content was 0.014%.**Giner-Chavez, 1996** reported that levels of tannin from 0.5% to 2.0% can cause depression in growth and levels of tannins above 5.0% in the diet are often lethal. High dietary phytic acid (2.5%) dramatically depressed the growth rate in salmon fish **(Richardson *et al.*, 1985)**and it has been reported that levels of 0.5% - 0.6% can impair the growth of rainbow trout **(Spinelli *et al.*, 1983)** and common carp **(Hossain and Jauncey, 1993)**. Therefore, the use of plant materials in animal feed should take into account the toxic chemicals contained in raw materials for animal feed **(Siddhuraju *et al.*, 2000)**.

Oxalate was reported as anti-nutrient factors in Moringa leaves **(Freiberger *et al.*, 1998)**. MLS powder contained very low value of oxalate (0.35% dw) if compared to spinach (25−45%dw). The data indicated that oxalate is not a significant anti-nutrient factor in Moringa. In this study, the inclusion rates of MLS in the diet of the examined groups were determined taking into account the facts reported by **Spinelli *et al.*, 1983, Richardson *et al.*, 1985, Hossain and Jauncey, 1993, Giner-Chavez, 1996 and Siddhuraju *et al.*, 2000**, who clarified the toxic effect of high inclusion rates of MLS due to its anti-nutritional factors content.

Antioxidant Contents of Moringa:

The presence of bioactive compounds in plants makes ita safe choice as food and feed additives. It is well known that plants contain many phenolic compounds which contain a hydroxyl group on an aromatic ring. These phenolic compounds interrupt chain oxidation reactions by donation of a hydrogen atom or chelating metals. So,it acts as reducing agents and antioxidants **(Bursal and Ko¨ ksal, 2010)**.Concentrations of natural antioxidants total phenols, total flavonoids and total antioxidant activity have been analyzed on a dry weight basis in many studies and these secondary metabolites act as bioactive compounds which have nutritional, pharmaceutical and/or antimicrobial properties **Singh *et al.*, 2009; Lako *et al.*, 2007 and Mbikay, 2012**.

Table 5: Antioxidants compounds of MLS powder:

Item	Total Phenols	Total Antioxidants activity	Total Flavonoids	Citric
Amount mg/100 gm	1459±15.87	1359.9±12.52	970.5±10.62	1.45±0.062

Values are expressed as mean ± standard deviation (n = 3).

In this study, total phenols recorded 1459 mg/100gm gallic acid equivalent, total antioxidant activity was 1359.9 mg/100gm in term of ascorbic acid, and total flavonoids measured 970.5 mg/100gm quercetin equivalent, as illustrated in Table 5. MLS powder natural antioxidants are high even when compared to vegetables and fruits known for high antioxidant contents such as strawberry which is high in phenols (330 mg gallic acid (GA)/100g dw, or ~190 µmol GA/g dw)**Yang *et al.*, 2006**. MLS is an excellent source of a wide spectrum of dietary antioxidants with low oxalate contents. Also, it was found among the most promising species according to their high antioxidant activity, and high contents of micronutrients and phytochemicals. From the same table, the amount of citric acid present in MLS powder was 1.45%. Citric acid acts as a chelating agent that make soluble complexes with trace metals like iron, calcium, and magnesium **Gupta, 2017**.

Table 6: Initial, final and body weight gain of rats in the experimental model:

Body weights	Initial Body weight (g)	Final Body weight (g)	Body weight Gain (g)
G1	90.90 ± 4.66^{a}	324.83 ± 31.26^{b}	233.93 ± 33.02^{b}
G2	90.08 ± 3.70^{a}	383.55 ± 20.82^{a}	293.47 ± 22.05^{a}
G3	87.25 ± 3.32^{a}	336.44 ± 24.64^{b}	249.19 ± 25.64^{b}
G4	89.73 ± 3.46^{a}	317.85 ± 18.43^{b}	228.12 ± 16.44^{b}
G5	88.43 ± 4.79^{a}	270.72 ± 20.52^{c}	182.28 ± 21.80^{c}
LSD	4.79	28.03	29.23

Values are expressed as mean ± standard deviation (n = 6).

Data presented in table (6) revealed that, body weight increased throughout the entire period of the experiment. Initial body weight did not significantly differ among all groups however rats fed on low calcium G2 had a significant increased body weight gain compared to those in groups (1,3 and 4), whereas the least significant difference was found in high calcium diet group (G5). This result agreed with that reported by **Sandeep and Dipayan, 2017**, which revealed that, dietary calcium played a significant role in regulating adiposity and body weight in rats exposed to different levels of calcium. They also reported the inverse association of dietary calcium with body weight gain and adipose tissue mass as what was obtained in this study. Low calcium diets led to an increase in 1,25 dihydroxy vitamin D_3 [$1,25(OH)_2D_3$] which in turn stimulates calcium influx into adipocytes, resulting in stimulation of lipogenesis, inhibition of lipolysis and expansion of adipocyte triglycerides stores. Suppressing 1, $25(OH)_2D_3$ levels by increasing dietary calcium would be predicted to inhibit adiposity and promote weight loss **(Parikh and Yanovski, 2003; Shapses *et al.*, 2004; Zemel, 2004; Thompson *et al.*, 2005)**.

Table 7: blood biochemical parameters:

Item /Group	Cholesterol mg/dl	Triglyceride mg/dl	AL Pu/l	Calcium mg/dl	Phosphorus mg/dl	PTH pg/ml
G1	99.35±8.66^{b}	89.15±3.50^{b}	188.50±15.58^{b}	13.43±0.83^{b}	10.10±0.52^{b}	46.82±4.82^{b}
G2	124.18±6.31^{a}	102.83±4.54^{a}	286.83±31.52^{a}	11.77±0.45^{c}	11.93±0.81^{a}	62.38±4.58^{c}
G3	86.75±4.15^{c}	80.48±3.03^{c}	197.50±19.21^{b}	13.20±0.54^{b}	10.14±0.66^{b}	45.75±3.19^{b}
G4	77.27±4.26^{d}	74.90±3.83^{d}	187.50±20.28^{b}	13.33±0.65^{b}	10.35±0.51^{b}	46.13±4.29^{b}
G5	65.85±6.18^{e}	68.58±4.20^{e}	161.33±10.07^{c}	14.37±0.95^{a}	7.82±0.69^{c}	29.42±3.29^{a}
LSD	7.29	4.60	24.47	0.84	0.77	4.89

Values are expressed as mean ± standard deviation (n = 6).

As lipids constitute a major class of hydrophobic constituents of the body, their main forms are cholesterol, phospholipids, and triglycerides. Lipids are involved in a variety of biological processes, including membrane formation, intracellular and intercellular signaling, as well as energy storage and production **(Horton, 2002)**. **Table 7** illustrates the results of blood biochemical parameters of experimental rats. It was clear from the obtained data that, groups fed on MLS powder (G3, G4 and G5) showed a beneficial effect on reducing total cholesterol and triglycerides than that in normal control especially group 5 which was fed a high calcium level. This result was supported by that reported by **Sandeep and Dipayan 2017** which mentioned that, groups fed with both MLS powder and $CaCO_3$showed a mimic effect in decreasing lipid profile. However, the levels of cholesterol and triglyceride increased in rats fed with low calcium diet (G2) when compared to normal control group which was explained by **Ditscheid *et al.*, 2005** who reported that, the saponification of free fatty acids in the presence of calcium decreases the intestinal absorption of lipids in gut thereby regulating the lipid profile which subsequently reduce the serum cholesterol level. Calcium may also bind to the bile acids increasing the fecal excretion through conversion of cholesterol into bile acids **Vaskonen, 2003**. Since the lipolysis pathway is inhibited in the low calcium diet fed rats, which in turn may reduce the amount of free fatty acids causing a stable serum triglyceride concentration. **Mbikay, 2012** referred to the therapeutic potential of *M. oleifera* in chronic hyperglycemia and dyslipidemia. He stated that, MLS contain phytosterols such as β-sitosterol which can reduce intestinal uptake of dietary cholesterol which lead to decrease of plasma cholesterol and the increase of fecal cholesterol observed in rodents treated with MLS**(Lin *et al.*, 2010; Mehta *et al.*, 2003 and Jain *et al.*, 2010)**. It was clear from the same table that, dietary calcium helped in regulation of PTH secretion, as the rats fed with low calcium diet were found to have lower serum calcium level and which in turn raised the PTH level in blood, while the high calcium diet fed rats showed the opposite effect.ALP has the least value in serum of rats fed with high calcium more than that in G1, G3 and G4, whereas the highest concentration was found in G2. The serum phosphorus concentration was lowered by the highest dose of calcium.

Table 8: Calcium and phosphorus concentration, BMD, density and Strength of rat's femur:

Groups	Ca%	Ph%	BMD (g/cm^2)	Strength (Newton)	Density (gm/cm^3)
G1	21.15±1.68^{b}	6.42±0.63^{b}	0.10±0.01^{b}	97.33±4.18^{b}	1.13±0.06^{b}
G2	14.48±0.96^{c}	8.93±0.67^{a}	0.08±0.01^{c}	56.50±4.85^{c}	0.93±0.08^{c}
G3	20.84±1.93^{b}	6.84±0.96^{b}	0.10±0.01^{b}	96.67±5.35^{b}	1.16±0.07^{b}
G4	20.17±1.55^{b}	6.71±0.70^{b}	0.11±0.01^{b}	99.17±5.00^{b}	1.18±0.09^{b}
G5	24.21±0.70^{a}	4.07±0.53^{c}	0.13±0.01^{a}	105.17±5.27^{a}	1.38±0.10^{a}
LSD	1.71	0.85	0.01	5.88	0.095

Values are expressed as mean ± standard deviation (n = 6).

Data in Table (8) and Fig (1) illustrated the effect of different Calcium sources and inclusion rates in rats' diet at the end of the experiment on bone characteristics. It is clear from the obtained data that, the highest values of Ca content, BMD, strength and bone density were in individuals belonged to G5 as it was offered the highest inclusion rate of Calcium content in the diet from both sources. The obtained values of the same parameters in G3 and G4 were similar and had no significant differences if compared to the control group and if compared to each other as well, indicating that there was a synergetic effect of MLS in Calcium absorption due to its content od active ingredients beside it was clear that, no adverse effect of the used sources when used in the previously specified inclusion rates in the diet of these groups. The lowest values of Calcium content, BMD, strength and density were recorded in individuals of G2 which were fed low Calcium diet. Also, from the same table, it was clear that the opposite behavior was obtained concerning Phosphorus content in the examined bones as the highest value was obtained from individuals of G2 and the lowest value was obtained from individuals in G5. Values of Phosphorus content in the bones of individuals in groups G1, G3 and G4 showed similar values with non-significant differences.

These data were supported by that of **Rehman *et al.*, 2018 and Mahfuz and Piao, 2019** who reported that using MLS at an inclusion rate of 1.2% in broilers' diet caused significant improvement of bone parameters indices. All data obtained in this research work demonstrated that, the inclusion of MLS alone or in combination with Calcium Carbonate in the tested levels in the diet had no adverse effect on BW, BWG, blood biochemical parameters as well as bones' strength, density and other bone characteristics of the experimental animals under study. Also, it was clear that the inclusion of MLS revealed the highest Calcium content in blood and bones and the best score in the evaluation of bone characteristics as well as the lowest lipid profile among all groups. These findings are explained by the outcome of many researches that studied the bioavailability, accessibility and digestibility of different Calcium sources into the living body. **Bronner and Pansu, 1998**and **Tondapu *et al.*, 2009** reported that, the bioavailability and the absorption of Calcium from MLS is much more better if compared to that of mineral salts due to many reasons including the effect of gastric juice on the digestibility of $CaCO_3$, as the low pH in the stomach followed by the increased pH in the dedendum causes precipitation of $CaCO_3$ as it has an alkaline part (CO_3) preventing proper absorption of Calcium. In contrast, this is not the case of Calcium originated from MLS as it is present in the form of Calcium citrate that contains an acidic part (citrate) which could survive the change in the pH along the gastro-intestinal tract (GIT) making it more available. Also, **Nehal Al Fky *et al.*, 2017** mentioned that the nature of the chemical composition of MLS which consists of many other nutrients, improves the utilization of Calcium. These constituents include Vitamin E which is present in MLS in a concentration of 10.8 mg/100 g dry matter (**Gopalakrishnan *et al.*, 2016**) which was found to be synergetic in the digestion and deposition of Calcium in bones preventing osteoporosis. Also, **Morcos *et al.*, 1976 and Kim and Lee, 2016** reported that, Vitamin C (15.8 mg/100 g dry matter of MLS as reported by **Gopalakrishnan *et al.*, 2016**) is a crucial factor that strongly affects the absorption of Calcium along GIT increasing its level in serum and its deposition in bones. On the other hand, **Mahfuz and Piao, 2019** highlighted the important role of the phytochemicals present in MLS mentioning its synergetic effect on Calcium utilization in animal body. **Parikh *et al.*, 2015** reported that, the phytochemicals present in MLS caused decrease in Calcium excretory rate suggesting that the components were helpful in keeping Calcium load in the body. From the overall data it could be concluded that, MLS can be considered as a reliable food/feed additive which can be used as for improvement of bone integrity with no reverse effect on all tested physiological and bone parameters indices. Also, it can be concluded that, dietary Calcium deficiency causes higher Cholesterol, Triglycerides, AlP, Phosphorus in serum and bone and Body Weight Gain while increased dietary supplementation with Calcium controlled the Cholesterol and Triglycerides levels and caused higher rates of Calcium concentration in the examined femur bone.

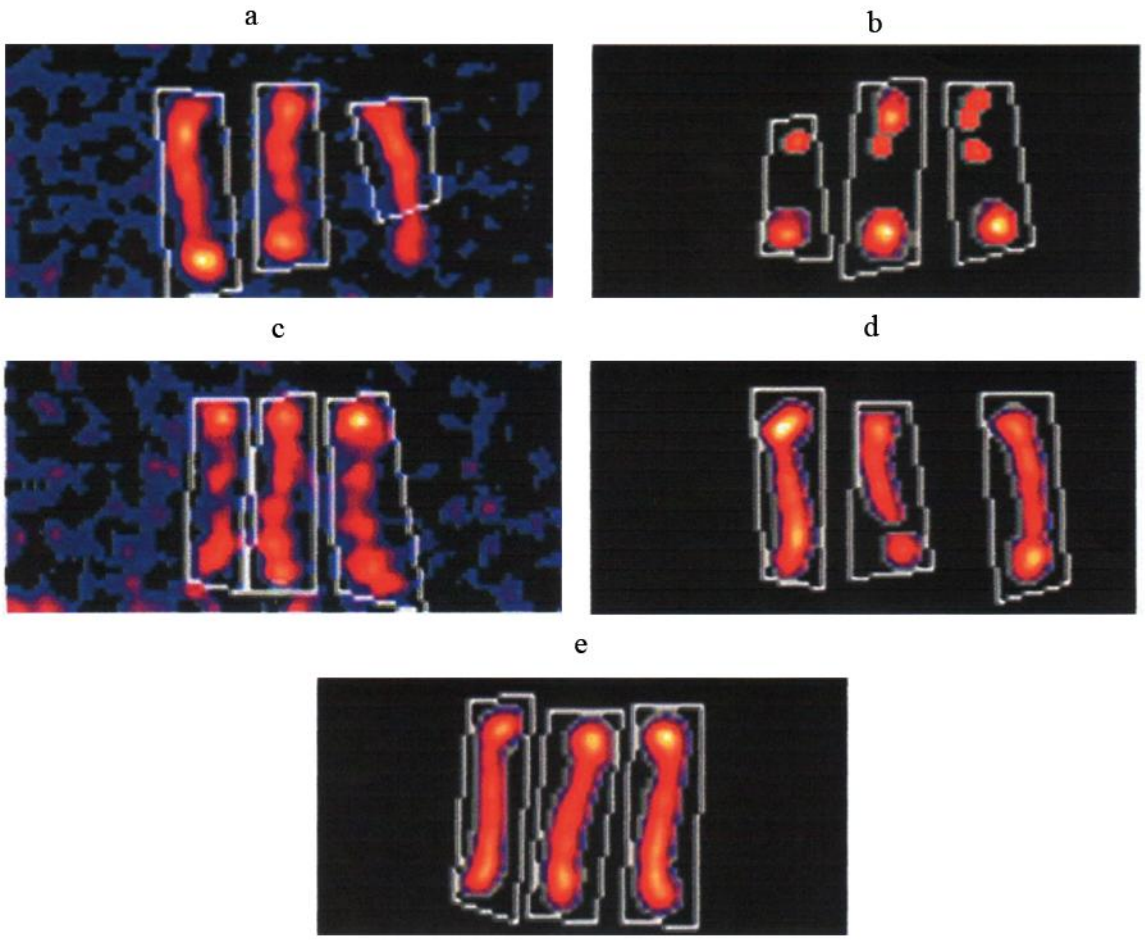

Figure (1) Results of BMC and BMD in femur using Dual Energy X-ray Absorptiometry (DXA) a) normal appearance of femur bone in G1, b) severely affected femur of G2, c) no adverse effects on femur of G3, d) better appearance of femur of G4 and e) The best score obtained in G5.

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