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# Nutritive Composition and Antimicrobial Activity of *Moringa stenopetala* (Baker f.) Cufod

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***Authors' contributions***

*This work was designed and planned by authors HLR and TRPK and experimental work was carried out in collaboration with all the authors. Authors TRPK, DD and TS were involved in collection and antimicrobial activity of the plant material. The proximate and metal analysis was performed by authors BNV and HLR. The authors TRPK and HLR wrote the first draft of the manuscript. All authors read, critically reviewed the article and approved the final manuscript.*

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## ABSTRACT

**Aims:** The present study was carried out to determine nutritive composition and to screen antimicrobial activity of leaf extract of *Moringa stenopetala* (Baker f.) Cufod.
**Place and Duration of the Study:** The study was performed at Wollega University, Ethiopia, BGK Life Technologies Pvt. Ltd., Bagalkot and Department of Microbiology, S.R.N.M.N College of

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Applied Sciences, Karnataka, India between May-2016 to July-2016.
**Methodology:** The powdered leaf material was subjected for determination of various proximate parameters namely moisture, ash, crude fibre, crude fat, protein and carbohydrate content. Mineral analysis was carried out using ICP-OES technique. For determining antimicrobial activity, extraction of shade dried and powdered leaf material was carried out by maceration process. Antibacterial activity of extract was assessed against a panel of Gram positive and Gram negative bacteria by Agar well diffusion assay. Antifungal activity was tested against three phytopathogenic fungi by Poisoned food technique.
**Results:** Leaf material of *M. stenopetala* was found to contain appreciable quantity of carbohydrates (42.20%), crude fibre (07.34%) and protein (26.33%) while the crude fat (05.25%) content was lesser. Among minor elements, the iron content was found to be 962.50 ppm and nickel content was found to be 2.89 ppm. In case of major elements, potassium (18426.75 ppm) content was found to be highest while the content of sodium (1519.68) was less when compared with other major elements. Extract caused dose dependent inhibition of test bacteria with marked activity against Gram positive bacteria. Extract was not effective against *Ralstonia solanacearum* and showed no activity when tested up to 50 mg/ml. A dose dependent suppression of mycelial growth of test fungi was observed in plates poisoned with different concentration of extract.
**Conclusion:** The leaf of *M. stenopetala* is a good source of proteins, carbohydrates and minerals needed for normal physiology of the body and hence, the leaves can be used as food or feed supplement to enhance growth and health. The plant can be used against infectious microorganisms as the extract exhibited marked antibacterial and antifungal activity.

*Keywords: Moringa stenopetala; proximate; minerals; maceration; agar well diffusion; poisoned food technique.*

## 1. INTRODUCTION

The genus *Moringa* belonging to the family *Moringaceae* consists of tropical plant species and consists of about 10 species. Of the species of *Moringa*, only *M. oleifera* has got research and development attention. *Moringa* is a multipurpose tree with significant economic value due to its industrial, nutritional and medicinal applications. *Moringa stenopetala* (Baker f.) Cufod (called cabbage tree) is an underutilized, fast growing, domesticated *Moringa* species in East African lowlands and indigenous to southern Ethiopia and is an important indigenous vegetable food crop in south western Ethiopia. It is popularly called Shifara or Shalchada in Ethiopia. Tribes such as Gofa, Konso, Burji and Gamo consume the leaves as vegetable especially during the dry season. The plant is well adapted to semi-arid areas. The seeds contain edible oil that can be used for cooking and as salad dressings. The leaves and fruits are consumed as vegetables as they are rich in proteins, essential amino acids, calcium, iron, phosphorous as well as vitamin A and C. The leaves and pods are often used as animal fodder. The high protein content appears to be the promising use of the plant. The plant is grown as boundary or barrier as it serves as a live fence. The species is also grown as intercropping and grown in mixed multi-storey stands with other food crops. The seeds have flocculating property and hence promising as purifying agent in turbid water. Also, the plant appears to be more resistant to insect pests and tolerates draught. The wood is soft and is not usually used for fuel purpose [1-4].

The potential roles of *M. stenopetala* have been investigated by several researchers. Various parts of the plant have been used traditionally as food and to treat malaria, hypertension, asthma, diabetes, common cold, wounds, retained placenta and stomach problem. The extract of leaf and root caused concentration dependent antileishmanial activity in terms of causing abnormal morphologies in promastigotes of *Leishmania donovani* [5]. The ethanol extract of fresh root wood and the acetone extract of dried leaves were found to be active against trypomastigotes of *Trypanosoma brucei* indicating antitrypanosomal activity [6]. The study of Melesse et al. [2] showed the potential effect of leaf meal of *M. stenopetala* on the nutrient intake and growth performance of Rhode island red chicks under tropical climate. The leaf meal was found to be a cheaper alternate for protein supplement. Extracts and fractions obtained from various parts of the plant have shown to exhibit antimicrobial activity [7,8]. The crude aqueous extract from leaves was found to cause significant fall in blood pressure of experimental animals indicating the potential blood pressure

lowering effect [9]. The seed powder was found to be effective in the removal of chromium from tannery wastewater [10]. The study of Sajidu et al. [11] showed the potential of seed powder of *M. stenopetala* to absorb metals namely cadmium, zinc, copper and chromium. It was found that the leaf powder and extracts of *M. stenopetala* exhibit protective effect in terms of anticoccidial activity against *Eimeria tenella* infection in broiler chickens [12]. The hydroalcoholic extract of leaves of *M. stenopetala* was shown to inhibit intestinal α-glucosidase, pancreatic cholesterol esterase and pancreatic lipase activities indicating the possible role in reducing hyperglycemia and hyperlipidemia [13]. It has been shown that, the crude extract and solvent fractions of leaves of *M. stenopetala* exhibits antihyperglycemic effect in mice [14]. The present study investigates the nutritive composition and antimicrobial activity of leaf and *M. stenopetala*.

## 2. MATERIALS AND METHODS

### 2.1 Collection of Plant Material

The fresh *M. stenopetala* leaves were collected from Ambo, Ethiopia. Ambo city is located 112 km south of Addis Ababa, the capital city of Ethiopia. It lies between latitude 8°59'N, longitude 37°51'E and an elevation of 2101 m above sea level.

### 2.2 Proximate Composition of Leaf

The powdered leaf was subjected to analyze various proximate parameters namely moisture, total ash, crude fibre, crude fat, protein and carbohydrates. The assay procedures employed by Indrayan et al. [15] and Kambar et al. [16] were followed for the proximate analysis of *M. stenopetala*.

### 2.3 Moisture Content

Weight difference method was employed to estimate the content of moisture in the leaf sample. In brief, a known quantity of powdered material taken in a pre-weighed dish (W1) was kept overnight in hot air oven maintained at 100°C. The dish was cooled and weighed (W2). The moisture content was calculated using the formula:

> Moisture content = ([W2-W1] / weight of material) x 100.

### 2.4 Total Ash

The 10 g of leaf material was placed in a pre-weighed silica crucible (W1) and the crucible was heated over a low flame (till complete charring) followed by heating at 600°C in a muffle furnace for about 3–5 hours. The crucible was cooled in desiccator and weighed again (W2). Heating of the crucible was repeated for half an hour and this was repeated consequently till the weight of ash become constant. Total ash content was calculated using the formula:

> Total ash content = (weight of ash [W2-W1] / weight of material) x 100.

### 2.5 Crude Fiber

The moisture free and fat-free leaf (2 g) material was added to 200 ml of 1.25% sulfuric acid and boiled for 30 minutes. The content was filtered and the residue was washed with boiling water till no acidic. The residue was treated with 200ml of 1.25% sodium hydroxide and boiled for 30 minutes. The content was filtered, washed with boiling water followed by 1% nitric acid and hot water. The residue thus obtained was taken in a pre-weighed silica crucible (W1), heated at 600°C until complete ashing and the crucible was again weighed (W2). Crude fibre content was calculated using the formula:

> Crude fibre content = (weight of residue [W2 -W1] / weight of material) x 100.

### 2.6 Crude Fat

The 2 g of moisture free leaf powder was subjected to extraction using petroleum ether (boiling point 40-60°C) in a Soxhlet extractor for 24 hours (or until a drop taken from the drippings left no greasy stain on the filter paper). After completion of extraction, solvent was transferred into a pre-weighed (W1) container. The container was kept in hot air oven (100°C) to evaporate solvent. The container was cooled in desiccator and weighed (W2). The difference in the weight was taken as fat content of leaf material. The crude fat (%) was calculated using the formula:

> Crude fat (%) = (Weight of fat [W2-W1] / Weight of sample taken) × 100.

### 2.7 Protein Content

The micro Kjeldahl method was used to estimate the crude protein content of leaf powder. 2 g of

oven-dried leaf material was placed in a Kjeldahl flask to which 30 ml of concentrated sulfuric acid, 10 g potassium sulphate and 1 g copper sulphate was added. The mixture was heated gently first and then strongly (once the frothing had ceased). The colorless or clear solution obtained was heated for another one hour, cooled and diluted with distilled water to the mark in a 100ml volumetric flask. 10 ml from the digest was measured into the decomposition chamber of the distillation apparatus and 15ml of 40% NaOH was added and the ammonia released was trapped into 20 ml of 2% boric acid solution containing mixed indicator. A colour change from pink to green was observed as the ammonia being trapped. Distillation was continued for 5 minutes and the boric acid-ammonia solution so obtained was titrated against 0.1N HCl. The nitrogen content (%) was calculated by using the formula:

% nitrogen = (T–B) x N x 0.014 x D x 100 / Weight of the sample x V,

Where T is sample titration reading, B is blank titration reading, N is normality of HCl, D is dilution of sample after digestion, V is volume of sample taken for distillation and 0.014 is milliequivalent weight of nitrogen. % crude protein was calculated using the formula:

% crude protein = 6.25* x % nitrogen, where * is correction factor.

## 2.8 Total Carbohydrate Content

The content of total carbohydrates (%) was calculated by using the formula:

Carbohydrate (%) = 100 – (% ash + % moisture + % fat + % protein)

## 2.9 Energy Value

The nutritive value was determined by using the formula:

Energy value = (4 x % protein) + (9 x % fat) + (4 x % carbohydrate)

## 2.10 Mineral Composition of Leaf

ICP-OES technique was employed to estimate the content of major minerals (Calcium, sodium, potassium, magnesium and phosphorus) and minor minerals (chromium, copper, iron, manganese, nickel and zinc) in the powdered leaf material. In brief, 1 g of powdered leaf material was digested using 10ml of ultrapure metal free nitric acid in a microwave digester, the content was filtered through Whatman filter paper No. 1 and diluted to 25 ml with distilled water. The digested leaf sample was aspirated into ICP-OES to estimate the content of major and minor elements. Instrument configuration and experimental conditions are shown in Table 1.

**Table 1. ICP-OES conditions**

| | |
|---|---|
| Power (kW) | 1.2 |
| Plasma flow (L/min) | 15.0 |
| Auxiliary flow (L/min) | 1.50 |
| Nebulizer flow (L/min) | 0.75 |
| Sample flow rate (L/min) | 1.5 |
| Replicate read time (s) | 3.00 |
| Instrument stabilization delay (s) | 15.0 |
| Sample uptake delay (s) | 10.0 |
| Pump rate (rpm) | 15.0 |
| Rinse time (s) | 10.0 |
| Spray chamber | Cyclonic type |

## 2.11 Preparation of Extract for Antimicrobial Activity

Maceration technique was performed for extraction of leaf of *M. stenopetala*. In brief, 10 g of powdered leaf material was added to 100 ml methanol taken in a clean conical flask. The flask was shaken well and left for two days with occasional stirrings. The content of the flask was filtered through Whatman filter paper No. 1. The filtrate was evaporated to dryness and used for determining antimicrobial activity [17].

## 2.12 Antibacterial Activity of Leaf Extract

Two Gram positive bacteria namely *Staphylococcus aureus* and *Bacillus subtilis* and three Gram negative bacteria namely *Pseudomonas aeruginosa*, *Escherichia coli* and *Ralstonia solanacearum* were used to assess their susceptibility to leaf extract. The test bacteria were inoculated into sterile Mueller-Hinton (M-H) broth tubes and incubated overnight at 37℃. Agar well diffusion assay was carried out to investigate antibacterial activity of leaf extract of *M. stenopetala*. The M-H broth cultures of test bacteria (0.5 McFarland standard turbidity) were swab inoculated on sterile M-H agar plates under aseptic conditions. With the use of sterile cork borer, wells of 6 mm diameter

were punched in the inoculated plates. 200 µl of leaf extract (25 and 50 mg/ml of DMSO), reference antibiotic (Chloramphenicol, 1 mg/ml of sterile distilled water) and DMSO were transferred into respective wells. The plates were left for 30 minutes and then incubated for 24 hours at 37℃. Zones of inhibition formed around the wells were measured using a ruler [18,17].

### 2.13 Determination of Minimum Inhibitory Concentration (MIC)

The MIC of leaf extract of *M. stenopetala* was determined by broth dilution method. The extract dilutions (ranging from 50 to 0.0 mg/ml) were tested against all test bacteria except *R. solanacearum*. Two-fold dilutions of extract were prepared in sterile M-H broth tubes, the tubes were inoculated with test bacteria and incubated at 37℃ for 24 hours. The MIC of extract was determined by observing the visible growth of the test bacteria after incubation. The dilution showing no visible growth of bacteria was considered as the MIC of extract [19].

### 2.14 Antifungal Activity of Leaf Extract

Poisoned food technique was performed to investigate the potential of leaf extract of *M. stenopetala* to inhibit three phytopathogenic fungi namely *Colletotrichum capsici*, *Fusarium oxysporum* f.sp. *zingiberi* and *Bipolaris sorokiniana*. In brief, the control (without extract) and poisoned (0.5 and 1.0 mg extract/ml of medium) Potato dextrose agar plates were inoculated with test fungi by point inoculation technique aseptically followed by incubating the plates in upright position at room temperature. The size (diameter, in cm) of fungal colonies in mutual perpendicular directions was measured after an incubation of 72 hours. Antifungal activity was calculated using the formula:

$$\text{Inhibition of mycelial growth (\%)} = (C - T / C) \times 100$$

Where C and T refers to diameter of fungal colonies on control and poisoned plates respectively [17].

## 3. RESULTS AND DISCUSSION

### 3.1 Proximate Composition of *M. stenopetala* Leaf

It has been shown that the leaves of *M. stenopetala* are used traditionally as food as well as medicine for treatment of various ailments [1,5]. The result of proximate analysis of leaf of *M. stenopetala* is shown in Table 2.

**Table 2. Proximate composition of *M. stenopetala* leaf**

| Parameter | Content |
|---|---|
| Moisture | 08.10% |
| Ash | 10.76% |
| Fat | 05.25% |
| Protein | 26.33% |
| Carbohydrates | 42.20% |
| Crude fibre | 07.34% |
| Energy value (Kcal/100 g) | 321.45 |

The moisture content of leaf of *M. stenopetala* was found to be 8.10%. In the present study, the leaf of *M. stenopetala* was found to contain an appreciable amount of ash (10.76%) and the ash content indicates the mineral composition of the sample. The ash content of leaf (12.6%) and leaf meal (11.8%) of *M. stenopetala* was high as revealed by the study of Abuye et al. [1] and Melesse et al. [2] respectively.

Carbohydrates serve as the structural and storage components in cells. They are most prevalent in plant kingdom. Carbohydrates such as sugars are used as energy sources by cells. Carbohydrate supplies energy to brain and muscle cells and contributes to fat metabolism and spare proteins as an energy source [16]. In the present study, the leaf of *M. stenopetala* was found to contain an appreciable quantity of carbohydrates (42%). In an earlier study, higher carbohydrate content (51%) was observed in the leaves of *M. stenopetala* [1].

Like carbohydrates, proteins are essential macronutrients in diet. Proteins serve as source of energy and adequate amount of required amino acids. Proteins serve several roles such as enzymes, hormones, carriers and antibodies. The deficiency of proteins leads to various ill effects like growth retardation, edema, muscle wasting and collection of fluids in the body of children [20,16]. In the present study, the content of proteins in the leaf of *M. stenopetala* was found to be 26.33%. The crude protein content of leaf meal of *M. stenopetala* was found to be 30.6% [2]. The study of Abuye et al. [1] revealed a low content of proteins (9%) in the leaves. The seeds were shown to contain a higher concentration of protein in the range 40.5 to 44.4% [3].

Estimation of crude fibre is based on treating the moisture and fat-free leaf sample with dilute acid (1.25%) and then with dilute alkali (1.25%) which is similar to the gastric and intestinal action in the process of digestion. The fibre content of food aids in digestion and absorption of water from the body and bulk stool. Fibre in food helps in softening of stool and hence, prevents constipation [20,16]. The crude fibre content of leaf of *M. stenopetala* was found to be 7.34%. In an earlier study, Melesse et al. [2] found slightly higher content of crude fibre in leaf meal of *M. stenopetala* (8.3%). However, the study of Abuye et al. [1] showed high content of crude fibre (20.8%) in the leaves of *M. stenopetala*. The crude fibre content of seeds was found to be lesser (4.7-5.4%) as revealed by the study of Seifu [3].

The term crude fat is often used synonymously with ether extract and generally refers to free lipids that can be extracted into less polar solvents such as diethyl ether or petroleum ether. Among solvents, petroleum ether is generally used as it is low cost and is less hygroscopic and flammable than diethyl ether. In this study, the crude fat content of sample was estimated by weight difference method by extracting a known quantity of the sample with petroleum ether. The crude fat content was found to be 5.25%. The study of Abuye et al. [1] revealed a slightly higher fat content (5.8%) in the leaves of *M. stenopetala*. The content of crude fat in leaf meal of *M. stenopetala* was found to be slightly lesser (4.73%) when compared to the result of this study [2].

Energy value of *M. stenopetala* leaf was found to be 321.45 Kcal/100 g. The metabolizable energy of leaf meal of *M. stenopetala* was found to be 299.2 Kcal/100 g [2]. An energy value of 290.6 Kcal in the leaf of *M. stenopetala* was observed in the study of Abuye et al. [1].

### 3.2 Mineral Content of *M. stenopetala* Leaf

Minerals are one among the micronutrients and are inorganic substances that represent comparatively smaller portion of the diet when compared to major nutrients such as proteins, carbohydrates and fats. These minerals do not yield energy but are too important as they are needed for several metabolic processes. On the basis of daily requirement, mineral elements can be classified into major and minor elements. The deficiency of minerals leads to a variety diseases or disorders in plants as well as animals. Several methods are available to estimate mineral content of foods and other samples. One among those is ICP-OES technique which has an advantage over other analytic techniques as the technique can estimate several elements at a time. ICP-OES technique is widely used for elemental determination of a large variety of specimen types including plants [16]. The quantity of major and minor minerals in the leaf of *M. stenopetala* is shown in Table 3. Among minor elements, the content of iron and nickel was highest and least respectively. Next to iron, manganese was detected in high quantity. In case of major elements, potassium content was found to be highest while the content of sodium was least. The content of calcium was highest next to potassium. However, the study of Abuye et al. [1] revealed higher content of calcium when compared to potassium in the leaves of *M. stenopetala*. In an earlier study, the content of calcium was detected in high quantity when compared to the quantity of phosphorus in leaf meal of *M. stenopetala* [2] which is similar to result of our study. The study of Abuye et al. [1] showed the high content of iron in leaves when compared to zinc. Similar result was obtained in our study also.

**Table 3. Content of minerals in leaf of *M. stenopetala***

| Element | Content (ppm) |
|---|---|
| Manganese | 101.93 |
| Chromium | 5.22 |
| Copper | 5.95 |
| Iron | 962.50 |
| Nickel | 2.89 |
| Zinc | 27.32 |
| Potassium | 18426.75 |
| Magnesium | 2158.83 |
| Calcium | 9716.03 |
| Sodium | 1519.68 |
| Phosphorus | 3022.73 |

### 3.3 Antibacterial Activity of Leaf Extract of *M. stenopetala*

Interest in plants with antimicrobial activity has been tremendously increased in these years due to several drawbacks such as high cost, adverse effects and emergence of resistant bacterial strains that are associated with the use of antibiotics [21,17]. The result of antibacterial activity is shown in Table 4. The extract was effective in inhibiting all test bacteria except *R. solanacearum*. Extract exhibited dose

dependent inhibition of test bacteria. Susceptibility to extract was marked in case of Gram positive bacteria when compared to Gram negative bacteria. *Bacillus subtilis* and *Pseudomonas aeruginosa* were inhibited to high extent among Gram positive and Gram negative bacteria respectively. Inhibition of test bacteria by reference antibiotic was marked when compared to extract. Here also, Gram positive bacteria showed marked susceptibility. DMSO did not show inhibition of test bacteria. The observed results were also supported by MIC values. The MIC of extract, as determined by broth dilution technique, was least for *B. subtilis* and highest for *E. coli*. Extracts and purified compounds from *M. stenopetala* were shown to exhibit antibacterial activity. In an earlier study, Biffa [22] showed marked antibacterial efficacy of aqueous and methanolic extracts of leaf and bark of *M. stenopetala* against mastitis causing organisms namely *Staphylococcus aureus*, *Streptococcus agalactiae* and *Streptococcus dysagalactiae*. The study of Walter et al. [7] showed the potential of methanol and n-hexane extract of seeds of *M. stenopetala* to inhibit bacteria implicated in causing waterborne diseases. The acetone extract and the purified compounds from root wood of *M. stenopetala* exhibited marked activity against *E. coli* [23]. More recently, Chekesa and Mekonnen [8] evaluated antibacterial activity of solvent extracts of various parts of *M. stenopetala*. Seed and root bark extracts displayed inhibitory activity against test bacteria. While leaf extracts did not show any activity. However, in the present study, the leaf extract of *M. stenopetala* showed marked activity against test bacteria.

### 3.4 Antifungal Activity of Leaf Extract of *M. stenopetala*

The control of plant diseases caused by phytopathogenic fungi is often achieved with the use of synthetic fungicides. However, their use is associated with several drawbacks such as high cost, environmental pollution and emergence of resistant fungal strains. This triggered immense interest in searching possible alternatives for plant disease control. Extracts and metabolites from higher plants appear to be potential alternatives for chemical agents. Studies have shown the inhibitory effect of several plant species against pathogenic fungi [24,17]. In the present study, the inhibitory effect of leaf extract against three phytopathogenic fungi was assessed by Poisoned food technique in which the reduction in the mycelial growth of test fungi in poisoned plates is considered positive for antifungal activity. The method has been extensively used for evaluating antifungal activity of various kinds of samples including plant extracts. The extract caused concentration dependent inhibition of mycelial growth of test fungi. At 0.5 mg/ml concentration, only *B. sorokiniana* was inhibited to >50%, however, at concentration 1.0 mg/ml, all test fungi were inhibited to >50%. Among the fungi, marked susceptibility was observed in case of *B. sorokiniana* followed by *C. capsici* and *F. oxysporum* (Table 5).

**Table 4. Antibacterial activity of extract of *M. stenopetala***

| Test bacteria | Zone of inhibition in cm | | | | MIC |
|---|---|---|---|---|---|
| | Extract 25 mg/ml | Extract 50 mg/ml | Antibiotic | DMSO | (mg/ml) |
| *S. aureus* | 1.7 | 2.2 | 3.6 | 0.0 | 1.53 |
| *B. subtilis* | 1.9 | 2.4 | 3.6 | 0.0 | 0.76 |
| *P. aeruginosa* | 1.4 | 1.7 | 2.8 | 0.0 | 3.06 |
| *E. coli* | 1.3 | 1.6 | 3.1 | 0.0 | 6.12 |
| *R. solanacearum* | 0.0 | 0.0 | 2.3 | 0.0 | ND |

*'ND'- Not determined*

**Table 5. Antifungal activity of extract of *M. stenopetala***

| Test fungi | Colony diameter in cm (% inhibition) | | |
|---|---|---|---|
| | *C. capsici* | *F. oxysporum* | *B. sorokiniana* |
| Control | 3.2 | 4.8 | 3.5 |
| Extract 0.5 mg/ml | 1.8 (43.75) | 2.8 (41.66) | 1.2 (65.71) |
| Extract 1.0 mg/ml | 1.2 (62.50) | 2.1 (56.25) | 0.8 (77.14) |

## 4. CONCLUSION

The result of proximate analysis and mineral estimation of the plant *M. stenopetala* highlighted the potential use of the plants as food and feed supplement. The result of nutritive composition of leaf justifies utilization of the leaf as food. Incorporation of leaf of *M. stenopetala* in the food of poultry and livestock may prove to be useful in terms of adequate nutrition as protein supplement. The plant can also be used to prevent and treat diseases caused by pathogenic microorganisms. It is evident from the study that the leaf contains antimicrobially active principles which are to be isolated and subjected to antimicrobial activity. The future study warrants for the isolation and characterization of the different secondary metabolites and their efficacy evaluation.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

It is not applicable.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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