

Evaluation of Phytochemical Composition of Extractives and Proximate Analysis of *Tagetes minuta* Powdered Leaves and Flowers

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ABSTRACT

Tagetes minuta is an invasive plant in agricultural areas. However, the weed has found application in herbal tea and as feed additive in industrial applications. Commercial poultry feeds on the other hand are costly and synthetic antibiotics are always added to the feeds to increase the production and growth rate through reduced disease incidence. However, this unnecessary use of antibiotic feed additives in poultry feed is associated with development of antibiotic-resistant bacteria and increased risk of occurrence of antibiotics residues in human beings. Hence, valorization of *T. minuta* into feed additive is envisaged to be a readily available, ecofriendly and sustainable alternative. The aim of this study therefore was to screen the secondary metabolites present, determine the phytochemical composition and perform the proximate analysis of nutrients present in powders from *T. minuta* leaves and flowers. Extraction was performed using maceration method with ethanol as solvent followed by the phytochemical screening of the

extractives using standard qualitative methods. Determination of total phenolic content (TPC) and total tannin content (TTC) in the extractives followed the Folin-Ciocalteu method while total flavonoid content (TFC) was evaluated using the aluminum chloride colorimetric method. The proximate analysis study was done using the Association of official analytical chemists (AOAC) methods to evaluate moisture content, ash, crude fiber, crude protein, lipid content, and carbohydrate content. Phytochemical screening revealed the presence of phenols, flavonoids, alkaloids, tannins, saponins, glycosides, steroids and terpenoids in the ethanolic extracts of *T. minuta*, while the phytonutrients assayed in the powdered material were carbohydrates, lipids, and proteins. During proximate analysis it was observed that powdered leaves of *T. minuta* contained 8.463 ± 0.23 % moisture, total ash of 5.709 ± 0.22 %, and dry matter of 91.537 ± 0.30 % as compared to 12.285 ± 0.29 % moisture, total ash 8.794 ± 0.15 %, and dry matter of 87.715 ± 0.27 % observed in the flowers. Analysis of nutritive composition revealed that lipid content (28.167 ± 0.24 %), crude protein (12.194 ± 0.08 %) and carbohydrate content (14.088 ± 0.11 %), was high in the leaves as compared to 23.824 ± 0.32 % lipid content, 9.750 ± 0.15 % crude protein, and 11.317 ± 0.12 % carbohydrate content, observed in the flowers. Based on these findings, the current study affirms that powdered leaves and flowers of *T. minuta* contain phytochemicals and phytonutrients which could be valorized into phytogenic poultry feed additive.

Keywords: Phytochemical composition, proximate analysis, *Tagetes minuta*, Nutritive value.

INTRODUCTION

Tagetes minuta is an aromatic plant belonging to the sunflower family, Asteraceae, which comprises the largest family of vascular plants with 1535 genera and more than 23,000 species [1]. It is an erect, strong-smelling plant [2], and is commonly known as Mexican marigold, stinkweed, stinking roger and khaki bush [3]. *T. minuta* is native to South American Countries including Argentina, Chile, Bolivia, Peru, and Paraguay where it grows naturally from spring and practically disappears at the beginning of the winter having developed its complete life cycle [1,3,4-5]. However, it has now been introduced to several other parts of the world including Europe, Africa, and Asia as a noxious weed [6]. In India, *T. minuta* was introduced by the Portuguese and is commonly grown for its natural dye extraction, and essential oils production at an industrial scale [4]. Specifically, in Eastern Himalayas and Northern Indian plains, *T. minuta* has been domesticated as an essential oil-producing crop through integrated cropping system in *Zea mays* plantations [7]. In Kenya, *T. minuta* was first recorded as an alien weed during the 1920s and was primarily restricted to the higher altitude's regions of the Country [8]. Today, however, the plant is found in most parts of the Country having spread as a result of increased agricultural activities [5]. In Kisii County, in Kenya, for example, *T. minuta* is one of the weed species that have adapted to the agricultural zone which is endowed with fertile soils and sufficient rainfall that favors the growth of maize crops and weeds to thrive [9].

Ethnobotanically, *T. minuta* weed in Kenya is locally known as *Ol' bangi* (Maa), *nyanjaga/omotioku* (Kisii), *Muvangi/Kavangi* (Kamba), *Nyanjaga/ang'we/anyach* (Luo), *Mubangi* (Kikuyu), *Etakanyi/omubazi gwemhazi* (Luhya) and *Chemiasoriet* (Kalenjin) [10-12]. In Swahili, *T. minuta* is commonly known as "kwekwe" meaning "weed" which is controlled through mechanical removal by hoeing and hand pulling in the affected area and cultural

methods [9]. On the other hand, chemical control of *T. minuta* involves the use of synthetic pesticides like Roundup (glyphosate-based herbicide), for non-selective post-emergence weed control in agricultural intensive regions in Kenya [13]. In integrated weed management practices *T. minuta* has effectively been controlled using *Desmodium uncinatum* and *Desmodium intortum* leaves [14]. Despite the availability of several methods for the management of *T. minuta*, it is still an uncontrolled weed in the agricultural production systems leading to a reduction in crop yields [10]. It is a problematic weed for pasture and many crops such as maize and wheat in East and South Africa, [15-16]. In Kenya specifically, *T. minuta* has been identified as a major invasive alien species that could lead to loss of biodiversity and loss of crop yields in maize plantations if left uncontrolled [16]. Despite being a problematic wild weed in agricultural areas, *T. minuta* is also cultivated commercially for production of essential oils in Argentina, Australia, Brazil, France, Spain, Venezuela, Iran, Mexico, and in India, in the Himalayas region [3,17], and is known for producing essential oils and commercially useful bioactive compounds [18]. Extractives from *T. minuta* weed have found great industrial applications by being valorized as inks, plastics, cosmetic articles, electronics, and colorants on cellulosic fiber with good fastness properties [10,19-20].

The utilization of *T. minuta* plant material as a new-generation crop and the value addition of its extractives to various products is well-documented [5]. In this context, the value addition of noxious, invasive *T. minuta* weed is envisaged to meet the industrial demand in the pharmaceutical, food, and agrochemicals industries thereby improving crop yields, enhancing food security, and improving human livelihoods, particularly in undernourished communities of the world [1,5,21]. Commercial poultry feeds on the other hand are costly and susceptible to adulteration by synthetic prophylactic antibiotics with the aim of improving productivity through reduced risk of disease occurrence in poultry. However, this unwarranted use of synthetic antibiotics as additives in poultry feed has associated disadvantages such as development of antibiotic-resistant bacteria in poultry, transfer of antibiotic resistant genes from animals to human pathogens and increased risk of occurrence of antibiotics residues in higher trophic levels like human beings, thereby posing a potential threat to human health [22-23]. Additionally, the ever-increasing energy cost in poultry farming for egg production has created the need for utilization of supplemental fats that can meet the requirements of contemporary birds especially in broiler chicken [24].

The challenges facing production of indigenous poultry farming among smallholder farmers includes disease outbreaks, feed shortages and fluctuation of market prices [25]. In Kenya specifically, the rising cost of farm inputs inform of feeds and the occurrence of uncontrollable poultry diseases has hampered the growth of indigenous poultry farming especially in the rural areas [26]. Proteins for example is the most expensive ingredient in poultry diet and hence protein sources from plant material are envisaged to enhance the amino acid profile of indigenous chicken feed regimen [27]. As a result, the use of phytogenic feed additives has been fronted as a potential alternative to synthetic prophylactic antibiotics due to associated benefits such as stimulation of digestion, reduced disease incidence and higher feed conversion rate leading to increased production [22]. Previous studies have evaluated the proximate composition of *Moringa oleifera*, *Gmelina arborea* and *Tithonia diversifolia* leaf meals with high nutritive value which could be valorized as cheap and readily available feed supplement that could improve production with reduced side effects [28-29]. This research, therefore, evaluated

the phytochemical composition of *T. minuta* ethanolic extracts and investigated the presence of macronutrients including carbohydrates, proteins and lipids through proximate analysis of the powdered leaves and flowers.

MATERIALS AND METHODS

Materials

Boric acid, ACS reagent purity 99.5% was purchased from Sigma Aldrich, while selenium catalyst, 98% purity in powdered form was sourced from Loba Chemie. Sulphuric acid and hydrochloric acid were all purchased from Pyrex Laboratory suppliers. Folin–Ciocalteu reagent (99.5%), aluminium chloride (99%), potassium acetate (99%), and Sodium carbonate (99.5%) were all analytical grade reagents from Loba Chemie supplied by Gelsap Laboratory suppliers, Nairobi. Standards used for phytochemical composition including Gallic acid (99.5%) and tannic acid (99.5%), were all analytical grade reagents purchased from Labo Chemie while quercetin (anhydrous basis), purity $\geq 99.5\%$ was sourced from Sigma Aldrich. Analytical grade solvents (99%), including ethanol, diethyl ether, and chloroform were all sourced from Merck through Kobian Laboratory Suppliers in Kenya and used without any further purification.

Sample Collection

Tagetes minuta leaves and flowers were collected from Changach, Kesses Sub County in Uasin Gishu County, Kenya GPS location 0°15'37.6"N 35°13'32.8"E, during the dry season. Voucher specimens of the plant species were deposited at the laboratory of Biological Sciences of Moi University where the taxonomic identification and authentication of the plant specimens were conducted by a botanist. The collected plant material including leaves and flowers were chopped and air-dried for two weeks under a shade. Dried samples were ground into a fine powder using a laboratory grinder (Nutribullet NB-101B model), and subsequently sieved with a 200 mm sieve and stored in airtight sample bottles awaiting extraction and proximate analysis.

Extraction:

The ground plant materials from *T. minuta*, leaves, and flowers were extracted using the maceration according to the methods described by [30]. Briefly, 100g of samples were extracted for 72 hours using 300 mL of ethanol, at a solvent-to-liquid ratio of 1:3. At the end of the maceration process, the extractives were concentrated at reduced pressure using a Hahn timer HS-2005S vacuum rotary evaporator, at 40 °C and subsequently dried inside a desiccator for two weeks. The crude solvent extracts were then placed in brown amber sample bottles and stored at 4 °C awaiting further phytochemical analysis.

Phytochemical Screening

The samples were tested for the presence of nine phytochemicals including alkaloids, phenols, flavonoids, glycosides, steroids, terpenes, terpenoids, tannins, and saponins using the standard methods of Harborne [31] as described by Rajvanshi and Dwivedi [31]. Additionally, screening for the presence of phytonutrients of nutritive values including carbohydrates, proteins and lipids was performed following the procedures described by Mandadi *et al* [33], to identify the nutrients using qualitative phytochemical screening protocols.

Colorimetric Quantification of Phenolic Compounds

Colorimetric determination of total phenolic content (TPC) was performed using the Folin-Ciocalteu colorimetric methods of Singleton *et al* [34] as described by Kim *et al* [35]. Briefly, an aliquot of 0.5 mL (1 mg/mL) of the *T. minuta* ethanolic extracts were mixed with 2.5 mL Folin-Ciocalteu reagent (1:10 v/v distilled water) and 2.5 mL of 7.5% sodium carbonate after which the reaction mixture was incubated at room temperature for 30 minutes and absorbance measured at 765 nm using (UV- Vis's spectrophotometer, Beckham Coulter DU 700). TPC in *T. minuta* ethanolic extractives was then evaluated in mg gallic acid equivalent /g of dry weight (mg GAE/G DW), using gallic acid standard curve developed using absorbance measurements at 765nm in the concentration range 5, 10, 20, 40, 80, and 160 µg/mL. Evaluation of total flavonoid content was determined spectrophotometrically at 415 nm with a UV-Vis spectrophotometer (Beckham coulter DU 700) using the aluminium chloride Colorimetric methods of Chang *et al* [36], as described by Mobeen *et al* [37], using quercetin as standard (3.124, 6.25, 12.50, 25, 50 and 100 µg/L) and quantified as mg quercetin equivalent /g of dry weight (mg QE/g DW). Determination of total tannins content (TTC), was performed spectrophotometrically using tannic acid standard (concentration range 6.25, 12,25,50, and 100 µg/mL), by measurement of absorbance at 725 nm, following the Folin-Ciocalteu's colorimetric methods as described by [38-39]. TTC in the ethanolic extracts *T. minuta* leaves and flowers was then determined as mg tannic acid equivalent per gram of dry weight (mg TAE/g DW), using the equation obtained from the tannic acid calibration curve.

Determination of Proximate Composition of Powdered Leaves and Flowers of *T. minuta*

Proximate Analysis:

Proximate analysis of moisture and total ash was conducted according to the Association of the official analytical Chemists [40] method as described by Kookal *et al* [41]. The evaluation of dry matter in powdered samples of *T. minuta* was determined by difference using the moisture content percent while the organic matter was estimated using the total ash percent values. Proximate determination of acid-insoluble ash, water-soluble ash, and sulfated ash was performed according to the methods described by Salwaan *et al* [42].

Moisture Content:

Moisture content was determined by the loss of weight that occurred when the dried and powdered leaves and flowers of *T. minuta* were dried to a constant weight in a dry air oven. Briefly, 2g of sample was weighed using an Analytical balance and placed into a preweighed silica dish. The sample was then placed in an oven at 105° C for 4 hours. Upon completion of the oven drying process, the sample was cooled inside a desiccator having sodium sulfate, and weighed continually until a constant weight was achieved. The analysis was performed in triplicate upon which the moisture content was evaluated in percentage using the following equation:

$$\% \text{ Moisture content} = \frac{\text{Weight of sample before drying} - \text{Weight of sample after drying}}{\text{Weight of sample before drying}} \times 100 \quad (1)$$

Dry Matter:

Percentage dry matter was determined by difference using the percentage moisture content using the following equation:

$$\% \text{ Dry matter} = 100 - \% \text{ moisture content} \quad (2)$$

Total Ash:

Total ash content in the powdered leaves and flowers of *T. minuta* was determined by weighing of 2g of sample into a preweighed porcelain crucible. The sample was then heated inside a muffle furnace at 600 °C for 4 hours. The total ash content was then determined as a percentage of the weight loss that occurred when the dried and powdered leaves and flowers of *T. minuta* were cooled to a constant weight inside a desiccator having sodium sulfate. All analyses were performed in triplicate upon which the total ash content was evaluated in percentage using the following equation:

$$\% \text{ Total ash content} = \frac{\text{Weight of sample before furnace pyrolysis} - \text{Weight of ash after pyrolysis}}{\text{Weight of sample before furnace pyrolysis}} \times 100 \quad (3)$$

Organic Matter:

Percentage organic matter was determined by difference using the percentage total ash content using the following equation:

$$\% \text{ Organic matter} = 100 - \% \text{ Total ash content} \quad (4)$$

Acid Insoluble Ash:

Determination of acid-insoluble ash was performed using the resultant ash obtained during the determination of total ash content according to the methods described by [42], with a few modifications. Briefly, the resultant ash obtained from total ash content was weighed and placed inside a round-bottomed flask and boiled with 25 mL of 2N hydrochloric acid, inside the fume hood at 80 °C for five minutes and filtered. The acid-insoluble ash residue obtained was then collected on an ashless filter paper and washed with hot water. The insoluble ash was then transferred to a pre-weighed porcelain crucible and then heated inside a muffle furnace at 600 °C for 4 hours. Upon completion of the incineration process, the acid-insoluble ash was cooled inside a desiccator until a constant weight was obtained. The content of acid-insoluble ash in *T. minuta* powdered leaves and flowers was then determined as a percentage of the resultant total ash using the following equation:

$$\% \text{ Acid insoluble ash content} = \frac{\text{Weight of resultant total ash} - \text{Weight of acid insoluble ash}}{\text{Weight of resultant total ash}} \times 100 \quad (5)$$

Water-Soluble Ash:

The resultant ash obtained in the determination of the total ash was boiled for 5 minutes with 25 mL of distilled water and used to evaluate the amount of water-soluble ash according to the methods described by Salwaan *et al* [42], The water-insoluble ash residue was collected on an ashless filter paper and washed with hot water. The insoluble ash was then transferred into a porcelain crucible then heated inside a muffle furnace at 600 °C for 4 hours. The obtained ash was cooled inside a desiccator to a constant weight. The percentage water insoluble ash content was evaluated (equation 4), upon which water-soluble ash was then calculated by difference as indicated in equation 5 below:

$$\% \text{ water insoluble ash content} = \frac{\text{Weight of resultant total ash} - \text{Weight of water insoluble ash}}{\text{Weight of resultant total ash}} \times 100 \quad (6)$$

$$\% \text{ water soluble ash} = 100 - \% \text{ Water insoluble ash} \quad (7)$$

Sulfated Ash:

The determination of sulfated ash content in *T. minuta* powdered leaves and flowers followed the methods of Salwaan *et al* [42], with modifications on the duration and temperature of pyrolysis. Briefly, 1 g of dried and powdered plant materials were accurately weighed using an analytical balance and placed in a preweighed porcelain crucible. The samples were then ignited at 250 °C for 2 hours in a dry air oven until the powdered substance become thoroughly charred. The charred *T. minuta* powders from the leaves and flowers were cooled, and moistened with 1 mL of concentrated sulphuric acid and heated gently (80° C) in the fume hood until white fumes were no longer evolved followed by pyrolysis at 600 °C in a muffle furnace until all black particles disappeared. The obtained ash was allowed to cool inside a desiccator, followed by further addition of 1 mL concentrated sulphuric acid, and the pyrolysis process repeated further at 600 °C for 1 hour. The obtained ash was cooled inside a desiccator to a constant weight. The sulfated ash content was then determined as a percentage of the initial powdered sample using the following equation:

$$\% \text{ Sulphated ash content} = \frac{\text{Weight of sample before pyrolysis} - \text{Weight of sulphated ash after pyrolysis}}{\text{Weight of sample before pyrolysis}} \times 100 \quad (8)$$

Proximate Analysis of Nutritional Composition

The nutritive value of the powdered leaves and flowers of *T. minuta* were investigated using the methods of AOAC [43], as described by [44-45], to give the proximate value of total protein, lipid content, crude fiber, and carbohydrate content.

Lipid Content:

Determination of lipid content was carried out using the soxhlet extraction method with diethyl ether as solvent following the AOAC, [40], methods as described by Alain *et al* [44]. Briefly, 10 g of powdered plant material was weighed and placed inside a thimble followed by soxhlet extraction for six hours using diethyl ether as a solvent with a solid to liquid ratio of 1 to 25. Upon completion of the defatting process, diethyl ether was recovered using a rotary evaporator at 30° C to obtain a dried sample. Lipid content was then evaluated as a percentage of the initial plant material using the following equation:

$$\% \text{ Lipid content} = \frac{\text{Weight of diethylether extract}}{\text{Weight of sample}} \times 100 \quad (9)$$

Crude Fiber:

The organic residue left after extraction using diethyl ether was used for the determination of crude fiber according to the standard methods of AOAC, [43], as described by Alain *et al* [44]. Briefly, the lipid-free material was weighed and placed inside a heating flask followed by the addition of 200 mL of 1.25% H₂SO₄ and the solution was gently heated at 50 °C for 30 minutes. The digested sample was then filtered hot using Whatman filter paper number 1 and the

obtained residue was washed several times to obtain a neutral pH. The neutral residue was then placed inside a heating flask followed by the addition of 200 mL of 1.25% Na₂SO₄ and further heated at 50 °C for 30 minutes. The reaction mixture was then filtered under suction and washed with hot water and finally rinsed twice with absolute ethanol. The residue obtained was then dried in a dry air oven at 105 °C for 12 hours. The dried solid residue was then placed inside a preweighed porcelain crucible and pyrolyzed inside a muffle furnace at 600 °C for 4 hours. The obtained ash was cooled inside a desiccator until a constant weight was obtained upon which the crude fiber was obtained as a percentage using the following equation:

$$\% \text{ Crude fiber} = \frac{\text{Dry weight of residue before ashing} - \text{Weight of residue after ashing}}{\text{Initial Weight of sample}} \times 100 \quad (10)$$

Crude Protein:

Crude protein was determined by measuring the nitrogen content of the powdered leaves and flowers of *T. minuta* following the Kjeldahl methods of the AOAC, [43], as described by [44,46]. Briefly, 2g of powdered sample from *T. minuta* leaves and flowers were separately placed inside the Kjeldahl flask followed by the addition of 25 mL of concentrated sulphuric acid, 0.5g of copper (II) sulfate, 5g of sodium sulfate and 0.2g of selenium catalyst and the reaction mixture digested in the Kjeldahl apparatus for 45 minutes until the digestant become clear pale green color. Upon completion of the digestion process, the digestant was cooled followed by the addition of 100 mL of distilled water after which the digestion flask was rinsed three times and added to the bulk. During the distillation process, an aliquot of 10 mL of the digestant was then placed inside the distillation apparatus followed by the addition of 50 mL NaOH and 50 mL of 2% boric acid, and 3 drops of Mixed indicator having 2 volumes of 0.2% methyl red in 95% ethanol with 1 volume of 0.2% methylene blue in ethanol (AOAC Method 991.20). The obtained alkaline ammonium borate formed was then titrated against 0.1 N HCl after which the percentage nitrogen was determined using the following equation adopted from Chang [45]

$$\% \text{ Nitrogen} = \text{N HCl} \times \frac{\text{Corrected acid volume}}{\text{Weight of sample}} \times \frac{14\text{g}}{\text{mol}} \times \frac{100}{1000} \quad (11)$$

Where,

N HCl – Normality of HCl in mol/1000 mL

Corrected volume of acid- Volume of HCL for sample- volume of HCl for blank

14- Atomic weight for Nitrogen

Most proteins contain 16% nitrogen, [45], hence percentage crude protein in the powdered leaves and flowers of *T. minuta* was computed from the percentage nitrogen content by multiplying by a conversion factor of 6.25 [37,44-45], using the following equation:

$$\% \text{ Crude protein} = \% \text{ Nitrogen} \times 6.25 \quad (12)$$

Carbohydrate Content:

Estimation of percentage carbohydrate as Nitrogen free extract (NFE) was obtained by difference according to the methods of AOAC, [40], as described by [46] and reported in percentage using the following equation:

$$\% \text{ Carbohydrate content} = 100 - (\% \text{ moisture content} + \% \text{ Total ash content} + \% \text{ crude fiber} + \% \text{ crude protein}) \quad (13)$$

Statistical Analysis

All experiments on ethanolic solvent extraction, TPC, TFC, TTC, and proximate analysis were conducted in triplicate and the data obtained were expressed as means $\pm$ standard deviations of the replicates. All statistical analyses and graphical work were done using Excel software (version 9.1.0, Graph-Pad Software, California, USA). The difference between the means were analyzed using Analysis of Variance (ANOVA), and separated using Tukey's honestly significant difference test. p -values < 0.01 were considered statistically significant at 99% confidence interval.

RESULTS AND DISCUSSIONS

Extraction Yield

The percentage yields for extraction of *T. minuta* leaves and flowers using the maceration method with ethanol as solvent of extraction were analyzed and presented as illustrated in Figure 1. From the data, ethanolic extracts of *T. minuta* obtained yielded 3.65 ± 0.27 % w/w in the leaves and 2.91 ± 0.18 % w/w in the flowers, based on a dry weight basis. Statistical analysis using analysis of variance at 99% confidence level revealed that there was a statistically significant difference in the extraction yield of the leaves and flowers ($p < 0.01$) as illustrated in Figure 1.

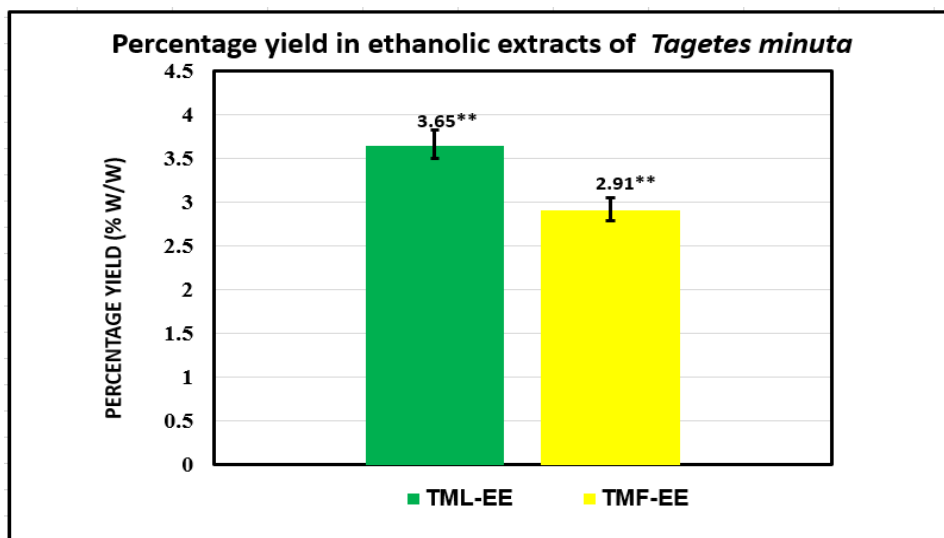


Figure 1: Percentage yield of ethanolic extracts of *T. minuta* leaves and flowers. Values are the mean of three replicates $\pm$ standard error. Values within bars followed by asterisks are significantly different at $P < 0.01$ according to ANOVA test. TML-EE: *T. minuta* leaf ethanolic extracts. TMF-EE: *T. minuta* flower ethanolic extracts.

Phytochemical Screening

During the phytochemical screening, the phytoconstituents were categorized as present in abundance (+++), moderately present (++), present (+), and completely absent (-), based on the intensity of precipitate formed upon the addition of the screening reagents. As depicted in table 1, eight assayed phytochemicals were reported present in the ethanolic extracts of *T. minuta*

leaves including, phenols, flavonoids, tannins, and saponins which were present in abundance; while alkaloids, steroids, glycosides and terpenoids were moderately present, suggesting that ethanol was a good solvent for extraction of the phyto compounds.

In the flower extractives of *T. minuta*, phenols, flavonoids, tannins, saponins, steroids, glycosides and terpenoids, were moderately present, alkaloids were indicated as present, while triterpenes were reported absent (table 1).

This observation could be attributed to the fact that polyphenols like tannins, flavonoids, and phenols are largely hydrophilic and are extracted using polar solvents like ethanol [47]. Additionally, in this current study, the use of maceration as a method of extraction gave ample time for interaction between the solvent and the solid for optimum extraction of phytoconstituents. Similar observations were reported by Mandadi *et al.*, [33] while investigating phytochemicals present in ethanolic extracts of *T. minuta* petals using the maceration method.

In a previous study, Rikisahedew [48] performed phytochemical screening of crude organic solvents and reported the presence of alkaloids, terpenoids, phenols in the methanolic extracts of *T. minuta* leaves. Saponins and sterols were reported to be absent. In another study, Grace *et al.*, [49] while analyzing squeezed crude extracts from *T. minuta* leaves reported the presence of phytochemicals in abundance including tannins, alkaloids, phenols, saponins and flavonoids except for steroids and terpenoids that were reported absent. This variation in phytochemicals present could be attributed to difference in extraction method, type of solvent used and sample preparation protocols.

As delineated in table 1, the present study revealed that powdered leaves of *T. minuta* possess nutrients like carbohydrates (moderately present), proteins (moderately present), and lipids (present in abundance), while in the powdered flowers, the lipids were assayed to be present in abundance while carbohydrates and proteins were assayed to be present.

The findings on presence of phytonutrients in *T. minuta* powdered leaves and flowers reported in this current study are consistent with the results of a previous study by Mandadi *et al.*, [33], who reported the presence of carbohydrates and lipids in petals of this species, except for proteins which were reported absent.

This variation in phytonutrients reported could be attributed to sample preparation protocols including size reduction and part of the plant assayed in addition to environmental conditions like drought, soil chemistry, and the season during plant collection [50-52].

Table 1: Phytochemical screening of ethanolic extracts of *T. minuta*

No.	Phytochemical	Test Reagent	<i>T. minuta</i> leaves ethanolic extracts	<i>T. minuta</i> flowers ethanolic extracts	<i>T. minuta</i> powdered leaves	<i>T. minuta</i> powdered flowers
1	Alkaloids	Wagner's test	++	+	-	-
2	Phenols	Ferric chloride test	+++	++	-	-
3	Flavonoids	Shinoda test	+++	++	-	-
4	Steroids	Salsowski's test	++	++	++	++
5	Glycosides	Bontrager's test	++	++	-	-
6	Terpenoids	Salsowski's test	++	++	++	++
7	Triterpenes	Liebermann-Buchard test	-	-	-	-
8	Tannins	Ferric chloride test	+++	++	-	-
9	Saponins	Frothing test	+++	++	-	-
10	Carbohydrates	Molisch's test	++	+	++	+
11	Proteins	Biuret's test	++	+	++	+
12	Lipids	Filter paper test	++	++	+++	+++

+++ indicates present in abundance; ++ indicates moderately present; + indicates present; - indicates completely absent

Phytochemical Composition

The total phenolic content in the leaves and flowers of *T. minuta* extractives were analyzed and expressed as milligram gallic acid equivalents per gram of dry weight (mg GAE/g DW) as presented in Table 2. From the tabulated data, TPC was 53.212 ± 0.04 mg GAE/g DW in the leaves and 38.907 ± 0.21 GAE/g DW in the flowers. Statistical analysis revealed that there was a significant difference between phenolic content in the leaves and flowers ($p < 0.01$), at a 99% confidence interval (Table 2). Total tannin content was evaluated as mg tannic acid equivalent per gram of dry weight (mg TAE/g DW) and was reported to be 37.002 ± 0.17 in the leaves and 32.218 ± 0.22 in the flowers, and the difference was not statistically significant ($p = 0.92$). Similar observations were reported by Macedo *et al.*, [53], while investigating extractives from decoction of *T. minuta* aerial parts refluxed for two hours and reported TPC of 45.2 GAE/g of DW and TTC of 34.6 GAE/g DW.

In another study, Babaei *et al.*, [51], while investigating morphological, physiological, and phytochemical responses of *T. minuta* leaves to drought stress in pot experiments reported TPC in the range (from lowest to highest), 8.19 ± 0.08 to 9.50 ± 0.31 mg GAE/g of fresh weight. This variation in phenolic composition could be attributed to the sample preparation protocols, extraction method, temperature of extraction and part of the plant assayed [47, 54].

Data from the quercetin standard was analyzed and the total flavonoid content of the ethanolic extracts of *T. minuta* were evaluated and summarized as shown in table 2 below. TFC in the leaves was found to be two-fold the TFC reported in the flowers as evidenced by observed results in the range (from highest to lowest), 40.890 ± 0.05 and 20.564 ± 0.02 mg Quercetin Equivalent/g of DW (mg QE/g DW) in the leaves and flowers respectively. Statistical treatment of TFC data revealed that there was a statistically significant difference between TFC reported in the leaves and TFC in the flowers ($p < 0.01$; 99% confidence interval). In a previous study,

Rezaei *et al.*, [52], evaluated the phytochemical composition of air-dried, aerial parts of *T. minuta* and reported that the methanolic extractives had TPC of 34.17 ± 0.6 mg GAE/g DW and TFC of 14.86 ± 0.4 mg QE/g. In another study, Al-Robai *et al.*, [50] investigated the amounts of total phenol, flavonoid, and tannin contents in the methanolic extracts of aerial and root parts of *T. minuta* and observed that the extractives had flavonoid content of 19.71 ± 0.51 mg/g. These variations of phenolic composition of *T. minuta* extractives reported in various studies [51-52], could be attributed to environmental conditions such as soil chemistry, topography, and the condition of plants' metabolic pathways for the production of bioactive compounds in different geographical locations [50].

Factors affecting the extraction of phytochemicals including structural stability of target compounds, the polarity of the phytochemical to be extracted, the plant matrix in which the phytochemical is present, extraction method, solvent used and time of extraction play a pivotal role in the phytochemical composition of extractives [47,54]. In this context, maceration as a method of extraction has been accentuated to give ample time for interaction between solvent and plant material intended to soften and destroy the cell wall of the plant and, as a consequence, to release soluble phytochemicals at room temperature [55]. Additionally, maceration has been shown to prevent the degradation of thermally labile phenols and flavonoids because the extraction is done at room temperature usually for 72 hours. Hence, in this current study, the reported phytochemical composition was attributed to the use of ethanol as a solvent of extraction and maceration as the method of extraction for the phytochemicals.

Table 2: Phenolic compounds in ethanolic extracts of *T. minuta* leaves and flowers

<i>T. minuta</i> ethanolic extracts	TPC (mg GAE/g DW)	TTC (mg TAE/g DW)	TFC (mg QE/g DW)
Leaves	53.212 ± 0.04^a	37.002 ± 0.17^a	40.890 ± 0.05^a
Flowers	38.907 ± 0.21^b	32.218 ± 0.22^a	20.564 ± 0.02^c

Values are the mean of three replicates $\pm$ standard error. Values within columns followed by different lowercase letters are significantly different at $P < 0.01$ according to Tukey's honestly significant difference test

Overall, the phytochemical composition of TPC, TTC, and TFC in the leaves and flowers of ethanolic extracts of *T. minuta* corroborates the phytochemical screening results obtained in this current study, which showed that phenols, flavonoids, and tannins were assayed to be present in abundance

Proximate Analysis

The results for the proximate analysis of moisture, dry matter, organic matter, total ash, acid insoluble ash, water soluble ash, and sulfated ash were analyzed in percentage and tabulated as delineated in table 3 below. As depicted in the table, the moisture content in *T. minuta* powdered leaves and flowers were $8.463 \pm 0.23\%$ and $12.285 \pm 0.29\%$ and the difference was statistically significant ($p < 0.01$). The results of the moisture contents of powdered material from *T. minuta* were generally lower which is an indication that the powders were dry and not susceptible to microbial spoilage during storage thereby having long shelf life [56]. Computation of dry matter revealed a high value of $91.537 \pm 0.30\%$ in the leaves as compared to the reported dry matter of $87.715 \pm 0.27\%$ reported in the flowers ($p < 0.01$). Ash content which is an index of mineral contents in plants, was in the range from low to high 5.709 ± 0.22

(leaves) and 8.794 ± 0.15 (flowers) with reported organic matter of 94.291 ± 0.21 and 91.206 ± 0.19 in the leaves and flowers respectively.

Table 3: Proximate analysis of powdered leaves and flowers of *T. minuta*

Parameter (%)	<i>T. minuta</i> powdered leaves	<i>T. minuta</i> powdered flowers
Moisture	8.463 ± 0.23^a	12.285 ± 0.29^b
Total ash	5.709 ± 0.22^a	8.794 ± 0.15^b
Dry matter	91.537 ± 0.30^a	87.715 ± 0.27^b
Organic matter	94.291 ± 0.21^a	91.206 ± 0.19^b
Acid -insoluble ash	0.998 ± 0.12^a	1.845 ± 0.10^b
Water-soluble ash	3.241 ± 0.26^a	4.087 ± 0.23^b
Sulfated ash	1.473 ± 0.18^a	2.618 ± 0.14^a

Values are the mean of three replicates $\pm$ standard error. Values within rows followed by different lowercase letters are significantly different at $P < 0.01$ according to Tukey's honestly significant difference test.

Proximate analysis revealed that powdered leaves of *T. minuta* contained 0.998 ± 0.12 % acid-insoluble ash and water-soluble ash of 3.241 ± 0.26 %, as compared to 1.845 ± 0.10 % acid-insoluble ash and water-soluble ash of 4.087 ± 0.23 %, reported in the flowers (Table 3). These results from proximate analysis of water-soluble ash suggest that powdered material from *T. minuta* leaves and flowers contain water-soluble organic matter an indication of the possibility of utilization as green manure. Sulfated ash on the other hand comprises the non-volatile residue on incineration in the presence of sulfuric acid and is an indication of the content of constituents or inorganic impurities in organic substances [57]. Hence, the sulfated ash content values of 1.473 ± 0.18 % reported in the leaves and 2.618 ± 0.14 % in the powdered flowers of *T. minuta* is an indication of the negligible presence of impurities of inorganic nature. Additionally, the reported acid insoluble ash of 0.998 ± 0.12 % in the leaves and 1.845 ± 0.10 % in the powdered flowers of *T. minuta* is within the specifications for compounded indigenous chicken feed.

Proximate Analysis of the Nutritional Composition:

Data on proximate analysis of crude fiber, lipid content, crude protein, and carbohydrate content in the powdered leaves and flowers of *T. minuta* were analyzed and graphically presented as shown in figure 2 below. From the proximate analysis of the diethyl ether defatted plant material, the percentage of crude lipids in powdered *T. minuta* plant material was 28.167 ± 0.27 % in the leaves and 23.824 ± 0.32 % (flowers), and the difference was statistically significant ($p < 0.01$). Using the defatted and pyrolyzed plant material, crude fiber was in the range high to low 34.032 ± 0.22 % (flowers) and 30.555 ± 0.14 % (leaves) ($p < 0.01$). Evaluation of crude protein in the powdered plant material revealed that crude protein was 12.194 ± 0.08 % and 9.750 ± 0.15 % in the leaves and flowers respectively, and the difference was not statistically significant ($p > 0.01$). Proteins from plant sources should comprise upto a maximum of 20% of the poultry diet in order to enhance amino acid profile for optimum growth [27]. Hence, the reported protein content in this current study, is within the range for the protein requirements for birds. Determination of carbohydrate content on the other hand revealed that powdered leaves of *T. minuta* had a high carbohydrate content of 14.088 ± 0.11 % as compared to the flowers which had a lower carbohydrate content of 11.317 ± 0.12 % ($p < 0.01$).

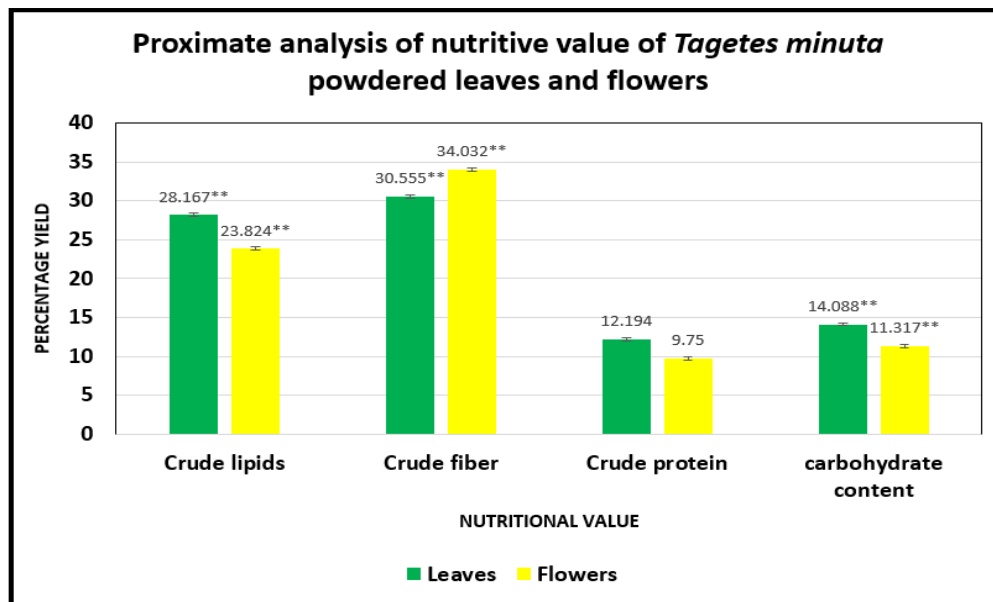


Figure 2: Graphical presentation of crude lipids, crude fiber, crude protein and carbohydrate content in powdered leaves and flowers of *Tagetes minuta*. Values are the mean of three replicates $\pm$ standard error. Means with asterisks are statistically different for the different plant parts ($p < 0.01$) using analysis of variance.

Comparative Analysis of the Nutritional Composition:

Comparative analysis of crude lipids, crude protein, crude fiber and carbohydrate content of powdered leaves and flowers of *T. minuta* was performed in comparison with different plant species used as poultry and animal feeds (Table 4). Data on lipid content (28.167 ± 0.24 %), as delineated on table 4, suggest that powdered leaves of *T. minuta* are rich in lipids which could act as a source of energy and essential omega-3 and omega-6 essential oils during hatching of eggs [58]. Additionally, the omega-3 and omega-6 essential oils play a significant role in the progeny of meat type chicken by enhancing growth, immune health and development of the central nervous system [58].

Compared to carbohydrates and proteins, absorption of lipids is a complex process involving breakdown to fat droplets, emulsification, lipolysis and micelle formation which slows down the rate of passage of feeds through the digestive track allowing more time for better digestion and absorption of nutrients [24,59]. In this context, fats and lipids are the preferred ingredients in feed supplements for poultry because the energy value is at least twice as high as those of carbohydrates and protein [60]. Additionally, poultry feed supplements having lipids has associated advantages such as reduced dustiness, lower particle separation in mash diets, improved palatability, carriers for fat soluble vitamins and continuous supply of the essential fatty acids [24]. Most poultry species are omnivorous and need to be fed with well-balanced diet dense in protein and energy content [61]. In this context, the recommended maximum crude protein nutrients requirements of meat chicken (3-6) weeks old is 20% while for egg laying hens it is 15% of the dry matter [60], and hence the reported values of 12.194 ± 0.08 % in the powdered leaves of *T. minuta* and 9.750 ± 0.15 % (flowers), is within the range of the nutritional requirements as poultry feed additive. It is noteworthy that comparative analysis of crude fiber revealed that the content was high in *T. minuta* flowers (34.032 ± 0.22 %),

suggesting that the powdered material could also be used as animal feed additive as rich source of fiber for ruminants.

Table 4: Comparative analysis of nutritional composition in different plant species

Parameter	Plant material	Nutritive composition	Type of proposed feed	Reference
Crude lipids (Diethylether extract)	<i>T. minuta</i> powdered leaves	28.167 ± 0.24 % ^a	-	This current study
	<i>T. minuta</i> powdered flowers	23.824 ± 0.32 % ^a	-	This current study
	<i>T. diversifolia</i> leaves	3.17 ± 0.16 % ^a	Lamb feed	[62]
	<i>M. oleifera</i>	4.52 ± 0.01 g/100g ^b	Ruminant animals	[29]
	<i>G. arborea</i>	4.80 g/kg ^c	Quail feed	[28]
	<i>Bidens pilosa</i>	7.49 g/100g ^c	Non-ruminants	[63]
Crude protein	<i>T. minuta</i> powdered leaves	12.194 ± 0.08 % ^a	-	This current study
	<i>T. minuta</i> powdered flowers	9.750 ± 0.15 % ^a	-	This current study
	<i>T. diversifolia</i> young flowers	195.8 g/Kg ^c	Ruminant animals	[64]
	<i>T. diversifolia</i> mature leaves	235.6 g/Kg ^c	Ruminant animals	[64]
	<i>T. diversifolia</i>	26.72 ± 0.62% ^a	Lamb feed	[62]
	<i>M. oleifera</i>	12.83 ± 0.03 g/100g ^b	Ruminant animals	[29]
	<i>G. arborea</i>	17.30 g/kg ^c	Quail feed	[28]
	<i>Bidens pilosa</i>	15.86 g/100g ^b	Non-ruminants	[63]
Crude fiber	<i>T. minuta</i> powdered leaves	30.555 ± 0.14 % ^a	-	This current study
	<i>T. minuta</i> powdered flowers	34.032 ± 0.22 % ^a	-	This current study
	<i>T. diversifolia</i>	18.8 ± 0.01 % ^a	Animal feed	[46]
	<i>T. diversifolia</i>	11.19 ± 0.62% ^a	Lamb feed	[62]
	<i>M. oleifera</i>	5.82 ± 0.04 g/100g ^b	Ruminant animals	[29]
	<i>G. arborea</i>	7.70 g/kg ^c	Quail feed	[28]
	<i>Bidens pilosa</i>	18.13 g/100g ^c	Non-ruminants	[63]
Carbohydrate content (Nitrogen free extract)	<i>T. minuta</i> powdered leaves	14.088 ± 0.11 % ^a	-	This current study
	<i>T. minuta</i> powdered flowers	11.317 ± 0.12 % ^a	-	This current study
	<i>T. diversifolia</i>	47.63 ± 0.40 g/100g ^b	Ruminant animals	[29]
	<i>M. oleifera</i>	40.03 ± 0.02 g/100g ^b	Ruminant animals	[29]
	<i>G. arborea</i>	55.90 g/kg ^c	Quail feed	[28]
	<i>Bidens pilosa</i>	49.11 g/100g ^b	Non-ruminants	[63]

^a-Percentage dry matter ^b-g/100g of dry matter ^c-g/Kg of dry matter

Carbohydrate content evaluated as nitrogen free extract (14.088 ± 0.11 %) in the leaves and (11.317 ± 0.12 %) in the flowers, revealed that powdered leaves of *T. minuta* contain nutrients which could act as a source of energy requirements for poultry. Proximate composition of carbohydrates, lipids, fiber and proteins is an indication of the effects of dietary treatments on animals in terms of type and amount of feed digested [28]. Hence, given the nutritional content of *T. minuta* reported in this current study, the powdered leaves and flowers could be used as a rich source of nutrients to be used as feed additive in indigenous poultry farming.

CONCLUSIONS

The present study verified the presence of phytochemicals including alkaloids, phenols, flavonoids, tannins, saponins, glycosides, steroids and terpenoids in the ethanolic extracts of *T. minuta* leaves and flowers. Additionally, phytochemical composition of the extractives revealed that *T. minuta* leaves contain total phenols (53.212 ± 0.04 mg GAE/g DW), flavonoids (40.890 ± 0.05 mg QE/g DW), and tannins (37.002 ± 0.17 mg TAE/g DW) as compared to total phenols (38.907 ± 0.21 mg GAE/g DW), flavonoids (20.564 ± 0.02 mg QE/g DW), and tannins (32.218 ± 0.22 mg TAE/g DW) reported in the flowers. The presence of these phytochemicals shows the potential application of extractives from *T. minuta* as phytogenic poultry feed additives. During proximate analysis, the current study reported for the first time that powdered plant material from *T. minuta* leaves contain nutrients in terms of percentage lipids (28.167 ± 0.27 %), crude protein (12.194 ± 0.08 %) and carbohydrate content of 14.088 ± 0.11 %, while in the flowers, the reported percentage nutrients was in the order 23.824 ± 0.32 % lipid content, 9.750 ± 0.15 % crude protein, and 11.317 ± 0.12 % carbohydrate content. Based on these findings, the data on nutritional composition of powdered leaves and flowers from *T. minuta* suggest the possible exploitation of the weed in the formulation of value-added products such as poultry feed additive.

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