Doses and sources of organic fertilization influence biomass, volatile chemical composition, and phenolic compounds in *Lippia gracilis*

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ABSTRACT

Lippia gracilis has emerged as one of the most important medicinal plants of the genus. Organic farming systems are currently growing substantially, aiming to obtain sustainable and economically viable plant production. Thus, the objective of this study was to assess the effect of different doses and sources of poultry manure on biomass production, photosynthetic pigment levels, phenolic content and volatile chemical composition in *L. gracilis*. The experiment was conducted in a completely randomized design in a 2 × 4 factorial arrangement with two sources of poultry manure (quail and chicken) and four doses (0.0, 1.5, 3.0, and 6.0 kg/m^2) in 10 l pots. Plants fertilized with quail manure at a dose of 1.5 – 3.0 kg/m^2 accumulated more total dry weight, while for chicken manure, the highest total dry weight gains were observed at doses of 3.0 and 6.0 kg/m^2. In the chemical analyses of the essential oil, the major chemical constituent identified in all treatments was carvacrol. However, the highest carvacrol content was observed in the control treatment. *L. gracilis* plants increase their biomass when grown with 1.5 kg/m^2 of quail manure or 3.0 to 6.0 kg/m^2 of chicken manure. However, fertilization reduces carvacrol levels.

Keywords: Alecrim da chapada, Organic fertilization, Growth indices, Chemical analysis, Essential oil

INTRODUÇÃO

Lippia gracilis Schauer (Verbenaceae) is an aromatic shrub species endemic to Northeast Brazil and is popularly known in Brazil as 'alecrim da chapada' (Ragagnin et al. 2014). According to Santos et al. (2016), *L. gracilis* has emerged as one of the most important medicinal plants of the genus *Lippia*. The essential oil of this species has antimicrobial (Albuquerque et al. 2006; Bitu et al. 2014), larvicidal (Silva et al. 2008), analgesic (Mendes et al. 2010), antinociceptive (Guimarães et al. 2012), acaricidal (Cruz et al. 2013) and cytotoxic (Melo et al. 2014) properties. The antimicrobial activity is attributed to the presence of thymol and carvacrol in the essential oil extracted from the leaves of *L. gracilis* (Bitu et al. 2015).

The production potential and the synthesis of active compounds are strongly affected by environmental and crop management factors (Rosal et al. 2011; Ragagnin et al. 2014). Intensive farming and the chemical quality of medicinal, aromatic and spices plants require adequate agronomic management strategies. In this context, the global demand for organic and natural products has encouraged the adoption of organic agriculture in the production of these species (Sujatha et al. 2011).

Organic fertilization is a source of nutrients for plants and improves the physical, chemical and biological structure of the soil (Costa et al. 2013). Studies with organic fertilization in aromatic plants seek to identify the changes that these fertilizers can cause in vegetative growth and in the content, yield and chemical composition of the essential oil and in the antioxidant potential (Rosal et al. 2011).

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Research in this area has already been conducted in oregano (*Origanum vulgare* L.) by Corrêa et al. (2010), in lemon beebrush (*Aloysia triphylla* (L 'Hérit) Britton) by Brant et al. (2010), in corn mint (*Mentha arvensis* L.) by Chagas et al. (2011), in peppermint (*M. piperita* L.) by Costa et al. (2013), in *L. origanoides* HBK by Teles et al. (2014), in basil (*Ocimum basilicum* L.) by Pandey et al. (2016), in thyme (*Thymus vulgaris* L.), sage (*Salvia officinalis* L.), and marjoram (*Origanum majorana* L.) by Roby et al. (2013), and in *Morus* spp. by Kostić et al. (2013).

Phenolic compounds are produced in plants with secondary metabolism through the shikimic acid pathway. Phenolic substances are recognized for having pronounced antioxidant activity, acting as free radical scavengers and metal chelators. They act as free radicals through a donation of a hydrogen atom (Pessuto et al. 2009; Sucupira et al. 2012). According to Pascual et al. (2001), phenolic compounds of the genus *Lippia* have been less studied. The flavonoids found in these species are mostly 6-hydroxylated flavones and methoxyflavones, but some flavone sulfates have also been identified; these flavonoids have been studied and identified in only a few flavones.

Given the chemical and biological importance of the species under study, the aim of the present study was to evaluate the effects of different doses and sources of poultry manure on biomass production, photosynthetic pigment levels and volatile chemical composition of *L. gracilis*, as well as evaluate the phenolic content in ethanolic extracts.

MATERIAL AND METHODS

Cultivation and growth analysis of *Lippia gracilis*

The plant material was herborized, and a voucher specimen was deposited in the PAMG herbarium of the Agricultural Research Company of Minas Gerais (EPAMIG) under registration PAMG 57859. It was identified with the valid name *Lippia grata* Schauer; however, its synonym, *L. gracilis*, will be used in this study due to the use of that nomenclature in a greater number of published studies.

Seedlings were produced through vegetative propagation using apical cuttings (± 5 cm) collected from mother plants at the Medicinal Plants Garden of the Department of Agriculture of the Federal University of Lavras, Minas Gerais The cuttings were rooted in 128-cell polypropylene trays using the commercial substrate Tropstrato HA.

The greenhouse experiment was conducted in a completely randomized design in a 2 × 4 factorial arrangement with two types of poultry manure (quail and chicken) and four doses (0.0, 1.5, 3.0, and 6.0 kg/m^2), with four replicates, one plant per pot and three pots per plot. The doses of both manures were a) soil + sand at a ratio of 3:1 (control); b) soil + sand at a ratio of 3:1 + 1.5 kg/m^2 of manure; c) soil + sand at a ratio of 3:1 + 3.0 kg/m^2 of manure and d) soil + sand at a ratio of 3:1 + 6.0 kg/m^2 of manure. The substrates were mixed and added to 10 l plastic pots; however, transplanting of the 60-day-old seedlings occurred 15 days later to avoid phytotoxicity of the seedlings. During these 15 days, the pots were irrigated as needed.

The soil used in the experiment was from the B horizon of a dystroferric red latosol (clayey texture) and had the following chemical characteristics: pH in water = 6.0; P and K (mg/dm^3) = 1.13 and 26.0, respectively; Ca, Mg, Al and H + Al (cmol/dm^3) = 1.20, 0.20, 0.00 and 1.86, respectively; base saturation V (%) = 44.04; organic matter (dag/kg) = 1.41; and clay content (dag/kg) = 64.

The fertilizers were analyzed in the chemical analysis laboratory of the Cooperativa Regional de Cafeicultores em Guaxupé Ltda. – Cooxupé; the analyses generated the following values: a) chicken manure: pH in water = 8.0; N, P, K, Ca, Mg, and S (g/kg) = 21, 20, 7.3, 4.6, 2.6, and 3.1, respectively; and B, Cu, Fe, Mn, and Zn (mg/kg) = 17, 74, 760, 315, and 314, respectively; and b) quail manure: pH in water = 7.4; N, P, K, Ca, Mg, and S (g/kg) = 26, 17.9, 40.3, 57.2, 7.8, and 7.1, respectively; and B, Cu, Fe, Mn, and Zn (mg/kg) = 66, 71, 811, 515, and 680, respectively.

Lighting for plant growth was natural sunlight with a light/dark cycle of approximately 16/8 h, the temperature in the greenhouse was 19-26 ºC. Irrigation was performed two to three times per week, to keep the mixture moisture between 70 and 80% of field capacity. Weed control was performed manually throughout the cycle. At 120 days of cultivation, the plants were harvested for growth analysis. The roots, stems and leaves, which were dehydrated in a forced-air dryer at 40 °C until constant weight, were measured. The weight of the dry plant material was obtained to determine the leaf (LDW, g/plant), stem (SDW, g/plant), root (RDW, g/plant), aerial part (LDW+SDW) and total dry weight (TDW, g/plant).

Extraction of essential oil

The essential oil of *L. gracilis* leaves was extracted by hydrodistillation in a Clevenger apparatus for 120 min from 20 g of dry matter in 1000 ml of distilled water. The hydrolate was collected, and the essential oil was purified by liquid-liquid partitioning with dichloromethane. The organic fraction was pooled and treated with anhydrous magnesium sulfate, followed by simple filtration and evaporation of the organic solvent in a fume hood at room temperature. The purified essential oil was stored under refrigeration (4 ± 0.5 °C) until chemical analysis.

Chemical analysis of the essential oil

The chemical composition analyses were performed using a sample composed of equivolumetric aliquots of the volatile oil from the replicates of each treatment.

The quantitative analyses of the oil were performed using a gas chromatograph coupled to a flame ionization detector (CG-FIC) in an Agilent® 7890A system equipped with an HP-5 fused silica capillary column (30 m in length) (California, USA). Helium gas was used as the carrier gas at a flow rate of 1.0 ml/min; the injector and detector temperatures were set to 250 °C and 240 °C, respectively. The initial oven temperature was 50 °C, remaining isothermal for 1.5 min, followed by a temperature ramp of 4 °C/min to 200 °C and a ramp of 15 °C/min to 250 °C, remaining isothermal for 5 min. The oil was diluted in ethyl acetate (1%, v/v) and automatically injected into the chromatograph using an injection volume of 1.0 µl, in split mode, at an injection ratio of 1:50. The concentrations of the constituents were expressed as the mean percentage of relative area of the chromatographic peaks ± the standard deviation of three samples analyzed for each treatment.

The qualitative analyses of the oil were performed using a gas chromatograph coupled to a mass spectrometer (GC-MS) using Agilent® 5975C equipment operated by electronic impact ionization at 70 eV in scanning mode, with a scan range of 40 to 400 *m/z* at a speed of 1.0 scan/s. The chromatographic conditions were the same as those used in the quantitative analyses.

The chemical constituents were identified by comparing their retention indices relative to the coinjection of a standard solution of *n*-alkanes (C_8-C_{20}, Sigma-Aldrich®, St. Louis USA) and by comparing the mass spectra with those in the database of the NIST/EPA/NHI library (NIST 2008) and the literature (Adams 2007). The retention indices were calculated using the equation of Van Den Dool and Dec. Kratz (1963), and retention indices in the literature were consulted for the assignments (Adams 2007).

Quantification of photosynthetic pigments

Fully expanded leaves of *L. gracilis* located between the second and third nodes below the apex were collected at 120 days to quantify the photosynthetic pigments (mg/g of leaf fresh mass): chlorophyll *a* and *b*, total chlorophyll and carotenoids. Chlorophyll and carotenoids were determined based on the method of Lichtenthaler and Buschmann (2001) with minor modifications. A sample of leaves (0.50 g) was weighed and then ground using a mortar and pestle containing 2 ml of 80% acetone (v/v) and $CaCO_3$ in the dark and under green light. Then, the extract was filtered, and the volume was brought to 10 ml with 80% acetone.

Ultraviolet molecular absorption readings were performed in a TECAN infinite® M200 PRO reader operated with the data processing system I-control®, version 3.37. Absorbances for each sample were measured at 663 nm for chlorophyll *a,* 647 nm for chlorophyll *b* and 470 nm for carotenoids.

Measurement of phenolic compounds

Preparation of *Lippia gracilis* extracts

Leaves of *L. gracilis* from each treatment were ground in a Marconi® MAO 48 knife mill. The extract solutions were prepared by dynamic maceration at a concentration of 10% (w/v). For this purpose, approximately 20 g of the ground plant material was extracted in Erlenmeyer flasks with 200 ml of 70% ethanol using a Gio Gyrotory Shaker; the Erlenmeyer flasks were kept under constant stirring at 146 rpm for 24 h in the absence of light. Then, the extracts were vacuum filtered. In previously tared flasks, approximately 20.0 g of the liquid extracts was evaporated to dryness in a rotavapor and placed in an oven dryer at 100 ± 5 °C for desiccation until reaching constant weight. The results were expressed as the percentage of dry residue/100 g of dry leaves (m/m). Quantification of phenolic compounds of the extracts were determined using ultraviolet molecular absorption spectrophotometric techniques. The readings were obtained using a TECAN infinite® M200 PRO reader operated with I-control®, version 3.37.

Measurement of total phenols

Total phenols were quantified by the method of Singleton and Rossi (1965). A volume of 50 µl of extract was mixed with 100 µl of Folin-Ciocalteu reagent (10% w/v) and 125 µl of Na_2CO_3 (7% w/v); after two hours of rest in the dark, the absorbance was measured at 760 nm. The calibration curve ($y = 5.8937x + 0.2134$ $R^2 = 0.993$) comprised the concentration range of 0.03 to 0.41 mg/ml of ethanolic solution of gallic acid. The levels of total phenols were expressed in milligrams of gallic acid equivalent per gram of dry leaf (mg GAE/g).

Measurement of flavonoids (flavones and total flavonoids)

Flavonoids were quantified by the method of Ahn et al. (2007). A volume of 100 µl of extract was mixed with 100 µl of $AlCl_3$ solution (10% w/v). After 40 min at rest, at room temperature, the absorbance was measured at 420 nm. The calibration curve (y = 3.5028x + 0.0589, R^2 = 0.9999) comprised the concentration range of 0.01 to 0.16 mg/ml of ethanolic solution of quercetin. The levels of flavonoids were expressed in milligrams of quercetin equivalent per g of dry leaf (mg QE/g).

Measurement of total dihydroflavonoids

Total dihydroflavonoids were quantified by the modified Popova et al. (2004) method. In the first phase, 200 µl of extract (at ¼ dilution) was mixed with 400 µl of DNP methanol solution (1% w/v) and heated at 50 °C for 50 min in a water bath. After cooling to room temperature, 1400 µl of KOH (10% v/v) in methanol was added to the reagent solution (sample + DNP). Then, 50 µl of the reagent solution (sample + DNP + KOH) was diluted in 950 µl of methanol and centrifuged at 605 ×*g* for 10 min. A volume of 200 µl was transferred to microplate wells. Absorbance was measured at 486 nm. The calibration curve (y = 0.162x + 0.0651, R^2 = 0.9989) comprised the concentration range of 0.08 to 2.6 mg/ml of methanolic solution of naringenin. The dihydroflavonoid contents of the extracts were expressed in milligrams of naringenin equivalent per g of dry leaf (mg NE/g).

Statistical analysis

The data on growth, total dry weight production, chlorophyll *a* and *b* and carotenoids and phenolic compounds were subjected to analysis of variance and, when significant, subjected to the Scott-Knott test at 5% probability using SISVAR software (Ferreira 2011). Principal component analysis (PCA) was used to evaluate the influence of different doses and sources of organic fertilization on the volatile constituents and growth parameters of *L. gracilis*. PCA was performed in Statistica® version 13.4 (StatSoft, Tulsa, OK, USA).

RESULTS AND DISCUSSION

Growth analysis

The behavior of the *L. gracilis* plant cultivated

Table 1. Mean leaf (LDW), stem (SDW), root (RDW), root/aerial part ratio and total dry weight (TDW) values for *Lippia gracilis* cultivated with different doses of poultry manure.

Manures	Doses (kg/m²)			
	0	1.5	3.0	6.0
	------------------------- **LDW (g/plant)** -------------------------			
Quail	2.08 aD*	15.56 aA	13.25 aB	11.04 bC
Chicken	2.08 aC	10.58 bB	13.45 aA	13.05 aA
	------------------------ **SDW (g/plant)** ------------------------			
Quail	1.17 aD	20.00 aA	15.54 aB	11.83 bC
Chicken	1.17 aC	13.20 bB	18.77 aA	17.48 aA
	------------------------**RDW (g/plant)** ------------------------			
Quail	1.72 aC	15.13 aA	15.80 aA	5.34 bB
Chicken	1.72 aB	9.41 bA	9.00 bA	9.82 aA
	---------------------- **Ratio root/aerial part** ----------------------			
Quail	0.52 aA	0.42 aB	0.54 aA	0.23 bC
Chicken	0.52 aA	0.39 bB	0.27 bC	0.32 aB
	-------------------- **TDW (g/plant)** --------------------			
Quail	4.98 aC	50.69 aA	44.61 aA	28.22 bB
Chicken	4.98 aC	33.20 bB	41.23 aA	40.35 aA

*Means followed by the same lowercase letters in columns or uppercase letters in rows do not differ statistically by the Scott-Knott test at 5% probability in the presence of chicken and quail manure and in its absence is shown in figure 1.

Regarding the biometric growth indices, there were significant differences between the doses and sources of fertilizers used (Table 1). The growth of fertilized plants was higher than that of control plants; growth was dependent on the dose and source of manure used. Plants fertilized with quail manure at a dose of 1.5 kg/m^2 accumulated more dry weight of leaves (LDW), stems (SDW), and roots (RDW) and total (TDW) were in both 1.5 – 3.0 kg/m^2. The dose of 6 kg/m^2 of quail manure impaired the accumulation of dry weight, although there was a length of shoot (Figure 1). The total dry weight gain for plants in 1.5 to 3.0 kg/m^2 quail manure was 10 to 9 times higher than that for control plants and 6 to 8 times higher for plants in 1.5 to 6.0 kg/m^2 chicken manure for control plants. Therefore, 1.5 kg/m^2 quail manure was the best manure dose for TDW, better than the best chicken manure dose, which was 3.0 and 6.0 kg/m^2. Although the dose of 3.0 kg/m^2 does not differ statistically from the dose of 1.5 kg/m^2, it would be recommended because it is half the dose of 3.0 kg/m^2. This positive effect of quail manure is probably due to the higher concentration of nutrients in this manure than in chicken manure.

Nutrient analysis of the manures revealed that the chicken and quail had high nutrient levels, which may explain the results. The quail manure had nitrogen concentration of 26 g/kg and chicken manure at 21 g/kg. Also, phosphorus content had high concentration in quail manure 17.9 g/kg and chicken manure 20 g/kg. Chemical analysis of the manures showed that the quail manure had higher nutrient levels than in chicken manure, which may explain that lower dose of quail manure (1.5 kg/m^2) accumulated more dry weight than chicken manure. The best dry weight gain from chicken manure were 3.0 kg/m^2 and 6.0 kg/m^2. The highest potassium concentration (40.5 g/kg) was found in quail manure compared with chicken (7.3 g/kg) manure, also, the Ca, Mg, and S. Chemical analysis of the manures showed that the quail manure had higher micronutrients (B, Cu, Fe, Mn, and Zn) levels than in chicken manure.

Bibiano et al. (2019) also concluded that fertilization with poultry manure (quail), compared to cattle manure, increased the growth of *Dysphania ambrosioides*. The difference in response of each species can be explained by the influence of the genotype and the variation in nutrient content that occurs in organic fertilizers from different sources and locations (Corrêa et al. 2010). Lopes et al. (2019) reported that different doses of cattle and quail manure altered the dry weight gain, content, yield and chemical composition of the essential oil of *Cymbopogon flexuosus* (Ness ex Steudi.) Will. Watson. Comparing different sources of organic fertilizers in *Hyptis suaveolens* (L.) Poit., Maia et al. (2008) observed differences in plant height when applying mineral fertilizer, vermicompost, poultry manure and organic compost, which were superior to cattle manure and the control. They also observed lower plant height in the control treatment without fertilization.

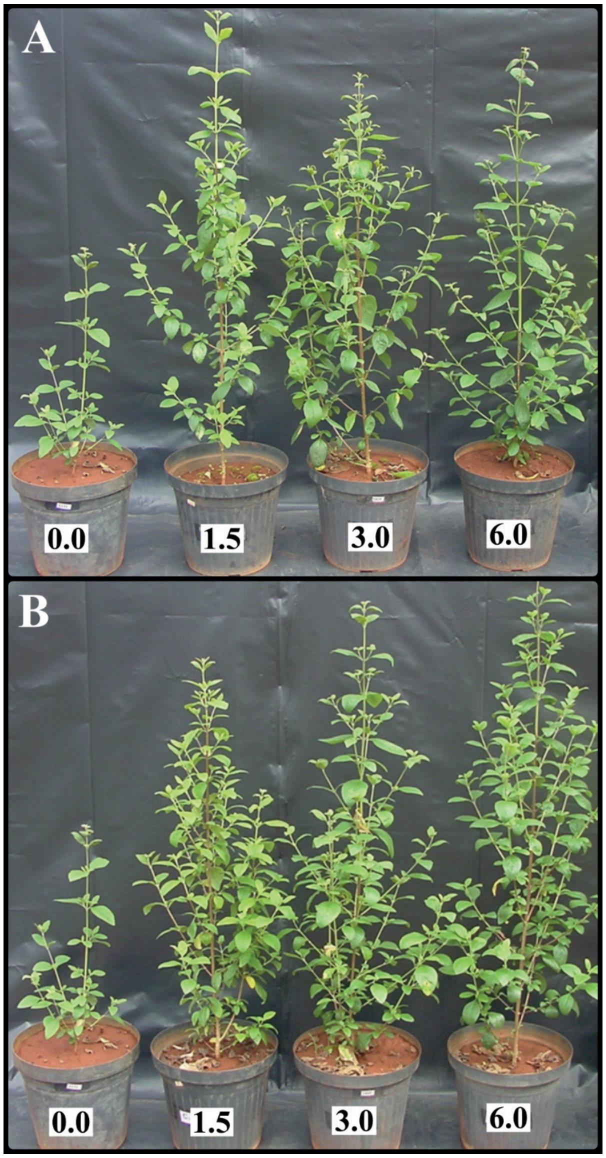

Figure 1. *Lippia gracilis* plants grown in different doses (0.0, 1.5, 3.0, and 6.0 kg/m^2) of chicken (A) and quail (B) manure. Control: without fertilization.

Regarding the root/aerial part ratio, a dose of 6.0 kg/m^2 of quail manure provided a growth index of 0.23, while at the other doses, the growth index ranged from 0.42 to 0.54 (Table 1), suggesting that plants invested more in the formation of the shoot than in the formation of the root. Regarding chicken manure, all doses showed greater investment in aerial part biomass production with respect to the control. Similarly, Bibiano et al. (2019) studied *Dysphania ambrosioides* and Costa et al. (2008) studied *Ocimum selloi* Benth. and found trends similar to those found in this study, where the root/aerial part ratio was higher in the absence of organic fertilization.

Content of photosynthetic pigments

A significant effect ($p<0.05$) of poultry manure levels was observed for total chlorophyll, chlorophyll *a* and *b*, and b/a (Table 2). For quail manure, 1.5 kg/m^2 provided the highest levels of chlorophyll *a* (0.40 mg/g), chlorophyll *b* (0.13 mg/g) and total chlorophyll (0.53 mg/g). Regarding chicken manure, an effect different from that of quail manure was observed. The highest levels of chlorophyll *a* (0.50 mg/g) and total chlorophyll (0.65 mg/g) contents were observed at a dose of 6.0 kg/m^2. For chlorophyll *b*, the highest content (0.25 mg/g) was observed at the dose of 3.0 kg/m^2. The highest b/a ratio (0.77 mg/g) was obtained at a dose of 3.0 kg/m^2 chicken manure. Regarding carotenoids showed no significant difference between the dosages and not even in relation to the control.

The highest chlorophyll concentrations using organic fertilization, either for quail or chicken, coincided with the dose where there was greater dry weight gain (growth). Plant growth is largely determined by photosynthetic capacity, where chlorophyll is an important photosynthetic pigment for the plant. Chlorophyll content in leaves varies between plant species, however, it is unclear how. Leaves with more chlorophyll are better tter able to absorb the light needed for photosynthesis, and thus, better growth can be achieved. Plants adjust their own characteristics to adapt to different environments. Thus, nutrients in the manures must play important roles in the regulation of Chlorophyll. Nitrogen (N), phosphorus (P) and magnesium (Mg) are elements required for the synthesis of chlorophyll; thus, organic fertilizers should influence Chlorophyll and growth. Quail and chicken manures from intensive farming showed to be rich in nutrients as nitrogen and phosphorus. Nitrogen (N) is an important element in plant growth and Phosphorus (P) helps transfer energy from sunlight to plants and stimulates plant growth (Hawkesford et al. 2012).

A plant with a high chlorophyll concentration is able to achieve high photosynthetic rates, and increase dry weight gain. The results of this study showed that only the utilization of organic manure, without NPK fertilizer, could maintain biomass productivity and enhance the quality of soil. Also, Hokmalipour and Darbandi (2011) working with maize concluded application of nitrogen fertilizer has positive effects on chlorophyll content and leaf

Table 2. Photosynthetic pigments contents of *Lippia gracilis* cultivated in different sources and doses (kg/m^2) of quail and chicken manure.

Manure	Doses (kg/m^2)			
	0	1.5	3.0	6.0
	-------------------------- Chlorophyll *a* (mg/g FW) -----------------			
Quail	0.25 aB*	0.40 aA	0.35aA	0.36 bA
Chicken	0.25 aC	0.22 bC	0.33 aB	0.50 aA
	-------------------------- Chlorophyll *b* (mg/g FW) ----------------			
Quail	0.08 aB	0.13 aA	0.05 bB	0.11 aA
Chicken	0.08 aC	0.05 bC	0.25 aA	0.15 aB
	----------------------------Ratio *b/a* (mg/g FW) ---------------------			
Quail	0.31 aA	0.31 aA	0.17 bB	0.30 aA
Chicken	0.31 aB	0.22 aB	0.77 aA	0.31 aB
	----------------------------Total chlorophyll (mg/g FW) -----------			
Quail	0.33 aC	0.53 aA	0.40 bB	0.47 bB
Chicken	0.33 aD	0.27 bC	0.58 aA	0.65 aA
	---------------------------- Carotenoids (mg/g FW) ------------------			
Quail	0.15 aA	0.17 aA	0.18 aA	0.16 aA
Chicken	0.15 aA	0.08 bB	0.16 aA	0.20 aA

*Means followed by the same lowercase letters in columns or uppercase letters in rows do not differ statistically from each other by the Scott-Knott test, at 5% probability.

dry weight. Both chlorophyll and carotenoids are present in the membranes of chloroplasts, more specifically in thylakoids, and are noncovalently bound to protein molecules and play important roles in the photosynthetic process.

Pandey et al. (2016), when studying basil (*O. basilicum*), found that the maximum mean values of chlorophyll *a* (0.55 mg/g) and chlorophyll *b* (0.063 mg/g) were observed for plants fertilized with chicken manure mixed with chemical fertilizers. The increase in chlorophyll may be directly related to nutrients and their balanced supply in the soil from the combined application of organic and chemical fertilizers.

The supply of nitrogen during the growth period of the leaves increases the formation of chloroplasts, thus increasing the chlorophyll content in the leaves (Singh et al. 2014). The higher chlorophyll values obtained as a function of different sources and doses of poultry fertilizer can be explained by the greater availability of nutrients such as nitrogen and magnesium in the both manure sources; such nutrients are part of the chlorophyll molecule. Correa et al. (2009), also studying doses of poultry and cattle manure, concluded that organic fertilization positively influenced the levels of chlorophyll due to higher nitrogen contents.

Chemical analysis of essential oil

The essential oil content and yield could not be determined because very rapid crystallization of the oil occurred. GC and GC/MS analyses of the essential oil of *L. gracilis* leaves found 15 chemical constituents that represented more than 93% of the total chemical composition (Table 3). Qualitative differences were evident between the control and fertilized plants. In the control, 10 chemical constituents were identified, whereas for fertilized plants, between 13 and 15 constituents were identified. Five constituents not detected in the control treatment samples were 1-octen-3-ol, trans-sabinene hydrate, thymol acetate, α-humulene and δ-cadinene. Thymol acetate and/or δ-cadinene were

Table 3. Relative percentage of essential oil compounds extracted from leaves of *Lippia gracilis* cultivated in different doses (kg/m^2) of quail and chicken manure.

		Area (%) ±SD						
		Quail				Chicken		
Compound	IR*	0.0	1.5	3.0	6.0	1.5	3.0	6.0
1-Octen-3-ol	978	nd	0.19±0.01	0.38±0.00	0.61±0.00	0.22±0.05	0.53±0.09	0.44±0.09
trans-Sabinene hydrate	1068	nd	0.27±0.01	0.31±0.00	0.31±0.00	0.29±0.02	0.29±0.03	0.35±0.06
Linalool	1100	0.23	0.40±0.02	0.43±0.00	0.42±0.00	0.40±0.03	0.43±0.04	0.44±0.08
Ipsdienol	1147	0.42	0.45±0.02	0.43±0.00	0.43±0.00	0.42±0.01	0.45±0.03	0.39±0.05
Terpinen-4-ol	1179	0.54	0.59±0.03	0.62±0.17	0.53±0.02	0.51±0.04	0.56±0.06	0.52±0.10
Thymol	1293	8.87	9.71±0.01	10.05±0.03	10.32±0.00	9.63±0.17	10.35±0.03	9.19±0.26
Carvacrol	1306	81.82	79.78±0.05	78.32±0.17	74.15±0.12	78.82±0.17	79.43±0.59	77.10±3.45
Thymol acetate	1356	nd	nd	0.16±0.00	nd	0.23±0.00	nd	0.32±0.03
Carvacrol acetate	1375	1.28	1.52±0.09	1.50±0.00	1.00±0.01	1.68±0.50	0.94±0.05	2.52±0.37
β-Caryopjyllene	1426	1.27	3.20±0.20	3.58±0.02	3.43±0.00	3.77±0.44	2.87±0.27	3.81±1.54
α-Humulene	1460	nd	0.24±0.01	0.25±0.00	0.22±0.00	0.27±0.03	0.20±0.01	0.27±0.09
2',5'-Dimethoxyacetophe-none	1485	1.23	1.30±0.07	1.23±0.00	1.12±0.00	1.16±0.44	1.12±0.13	1.28±0.09
β-Bisabolene	1512	0.29	0.34±0.02	0.34±0.00	0.34±0.00	0.44±0.06	0.31±0.01	0.39±0.14
δ-Cadinene	1528	nd	nd	0.14±0.00	0.16±0.00	0.18±0.00	0.14±0.00	0.18±0.00
Caryophyllene oxide	1590	1.50	1.20±0.02	0.89±0.07	0.85±0.00	0.91±0.16	1.19±0.07	1.33±0.08
Total area (%)		97.45	99.19	98.63	93.89	98.93	98.81	98.53
Number of compounds		10	13	15	14	15	14	15

*HP-5-MS column retention index in elution order. nd: not detected. SD: standard deviation (n=3).

responsible for qualitative differences in fertilized plants.

Regardless of the treatments, carvacrol and thymol together accounted for more than 84% of the total chemical composition of *L. gracilis* essential oil. The control treatment, despite having the lowest accumulation of total and organ-specific biomass, showed higher carvacrol synthesis (81.82%), lower thymol synthesis (8.87%) and even lower β-caryophyllene synthesis (1.27%). There was an increasing trend for thymol and *β*-caryophyllene with increasing doses of poultry manure, while carvacrol tended to decrease (Table 3). In plants fertilized with poultry manure, the mean carvacrol content was 77.93%, and the thymol content was 9.87%. These values are in agreement with Santos et al. (2014), who reported 73.9 to 77% carvacrol and 4.9 to 10.3% thymol in essential oils extracted from *L. gracilis* grown in the presence and absence of irrigation and NPK.

Pascual et al. (2001), in a review on the chemistry and pharmacology of the genus *Lippia*, found that the chemical composition of essential oils of many species of *Lippia* was investigated by gas chromatography. Based on these data, the components that were most frequently found in essential oils were limonene, *β*-caryophyllene, *p*-cymene, camphor, linalool, alpha-pinene and thymol.

The other constituents identified in the essential oil of fertilized and unfertilized *L. gracilis* plants had levels below 4.03%. These constituents include *β*-caryophyllene (1.27 to 3.81%), caryophyllene oxide (0.85 to 1.50%) and 2,5-dimethoxyacetophenone (1.12 to 1.30%), among others. Among the minor constituents, *β*-caryophyllene levels in plants fertilized with different doses of quail manure increased 2.8 times and chicken manure increased 3.0 times more compared to unfertilized plants (control).

The chemical composition results for the oil of present work suggest the presence of carvacrol chemotype. The oxygenated monoterpenes carvacrol was the primary constituents of *L. gracilis* oil with 74.15 to 81.82%. The presence of different chemotypes in *L. gracilis* is probably due to the genotype, the various seasons and collection sites, considering the variations in precipitation, temperature, soil type, light incidence, and duration of the day. Melo et al. (2014) studied three different *L. gracilis* essential oil chemotype and identified LGRA-106 - thymol (40.52%), *γ*-terpinene (8.29%), *p*-cymene (8.00%), methyl thymol (7.94%) and *β*-caryophyllene (6.45%); the LGRA-109 - carvacrol (45.84%), *p*-cymene (12.47%), *γ*-terpinene (12.81%), *β*-caryophyllene (5.38%) and methyl thymol (5.11%), and in the LGRA-201 - carvacrol (32.60%), *γ*-terpinene (25.91%), *p*-cymene (12.40%),

Table 4. Extract yield, phenolic, flavonoids and dihydroflavonoids contents from leaves of *Lippia gracilis* cultivated in different sources and doses (kg/m^2) of quail and chicken manure.

Manures	Doses (kg/m^2)			
	0	1.5	3.0	6.0
	------------------------------------ Extract yield (%) ------------------------------			
Quail	28.09 aB*	33.43 aA	29.17 bB	27.27 aC
Chicken	28.09 aB	27.80 bC	32.05 aA	27.47 aC
	----------------------------- Total phenolics (mg GAE/g) ---------------------			
Quail	19.01±0.08 aB	19.67±0.03 aA	18.02±0.25 aD	18.87±0.07 aC
Chicken	19.01±0.08 aB	19.27±0.07 aA	18.44±0.02 aC	18.78±0.02 aC
	------------------------------- Flavonoids (mg QE/g) ----------------------------			
Quail	0.95±0.06 aC	1.31±0.04 aB	1.56±0.03 bA	1.53±0.00 bA
Chicken	0.95±0.06 aC	1.19±0.01 bB	1.74±0.02 aA	1.74±0.02 aA
	----------------------------- Dihydroflavonoids (mg NE/g) -------------------			
Quail	10.79±0.3 aA	10.31±0.3 bB	6.54±0.20 bC	10.88±0.05 aA
Chicken	10.79±0.3 aC	12.17±0.4 aA	11.38±0.06 aB	11.31±0.30 aB

*Means followed by the same lowercase letters in columns or uppercase letters in rows do not differ significantly for the same class of phenolic compounds based on the Scott-Knott test, at 5% probability. Total phenolics: expressed in mg of gallic acid equivalent/g of dry leaf (mg GAE/g). Flavonoids: expressed in mg of quercetin equivalent/g of dry leaf (mg QE/g). Dihydroflavonoids: expressed in mg of naringenin equivalent/g of dry leaf (mg NE/g). The data represent the mean ± standard deviation (n=3).

β-caryophyllene (5.79%), and thymol (5.57%) were identified.

Measurement of phenolic compounds

The dry residue of the extracts obtained by dynamic maceration of dehydrated *L. gracilis* leaves in 70% ethanol ranged from 27 to 33%, denoting low yields (Table 4). Although dynamic maceration is not an exhaustive extraction process, it is usually more efficient than static maceration. Agitation exerts a strong influence on the extraction of chemical constituents from plant raw material because under rest, solvent in direct contact with plant raw material reaches a saturation concentration much faster than under agitation and extraction depends exclusively on the diffusion coefficient of the chemical constituents (Prista et al. 2011). The highest yields were observed with 1.5 kg/m² quail manure (33.43%) and 3.0 kg/m² chicken manure (32.05%) (Table 4).

There were statistically significant differences in the levels of phenolic compounds in the *L. gracilis* samples. The levels of total phenols ranged from 18.02 to 19.67 mg GAE/g, the levels of flavonoids ranged from 0.95 to 1.74 mg QE/g, and the levels of dihydroflavonoids ranged from 6.54 to 12.17 mg NE/g (Table 4). For total phenolics, there were no significant differences in levels between the types of manures. However, at the lowest fertilizer dose (1.5 kg/m²), plants fertilized with quail manure (1.31 mg QE/g) had higher levels of total flavonoids than those fertilized with chicken manure (1.19 mg QE/g). The opposite is true for the dihydroflavonoid content. Plants fertilized with 1.5 kg/m² chicken manure (12.17 mg NE/g) had a higher dihydroflavonoid content than those fertilized with quail manure (10.31 mg NE/g). The plants under these same treatments, when compared to the control, showed significantly lower levels of flavonoids than did the fertilized plants. The dihydroflavonoid content differed between the doses from quail or chicken fertilizer. The highest doses of quail manure increased the flavonoid content but reduced the total phenol content (Table 4). The variability in phenolic content is associated with the nature of the solvent, the extraction method and the part of the plant used (Miguel et al. 2014).

Principal component analysis (PCA)

PCA was used to understand the relationship among dry weight accumulation, total chlorophyll, phenolic content, flavonoids and volatile constituents and how these parameters vary according to the different doses and sources of organic fertilization. Four treatments and 11 parameters were used to evaluate the analysis of variations in PCA. The two principal components together explained 84.45% and 90.81% of the total variance for quail and chicken manure, respectively (Figure 2 and 3). The score plot revealed three distinct group, where Group 1: cultivated plants without manure, Group 2: cultivated plants with 1.5 kg/m² quail or chicken organic fertilizer and Group 3: cultivated plants with 3 or 6 kg/m² quail or chicken organic fertilizer (Figure 2 and 3). The results of Group 1 showed that *L. gracilis* grown without manures had greater synthesis of carvacrol and total phenolic compound. In Group 2 for quail manure there was greater synthesis of total chlorophyll and accumulated more TDW, SDW, LDW and RDW. However, for chicken manure was greater synthesis of β-caryophyllene, dihydroflavonoids and accumulated more RDW. And Group 3 were used 3 or 6 kg/m² of quail manure there was greater synthesis of β- caryophyllene, thymol and flavonoids and also accumulated RDW. There was also a strong correlation between the chicken manure (Group 3) and evaluated parameters dry weight production (TDW, SDW and LDW), including photosynthetic pigments, thymol and flavonoids.

PCA also confirmed that carvacrol were negatively correlated with thymol and β-caryophyllene in both manures. In addition, all growth parameters studied showed a negative correlation with the treatment without manure. In the present study, the lower dose of quail manure showed a positive and significant correlation with all studied dry weight production and for chicken manure was 3 or 6 kg/m². The PCA results corroborate and complement previous analyzes and interpretations. PCA was applied to the data of the present study to identify the interactions between the response variables and the fertilizers used.

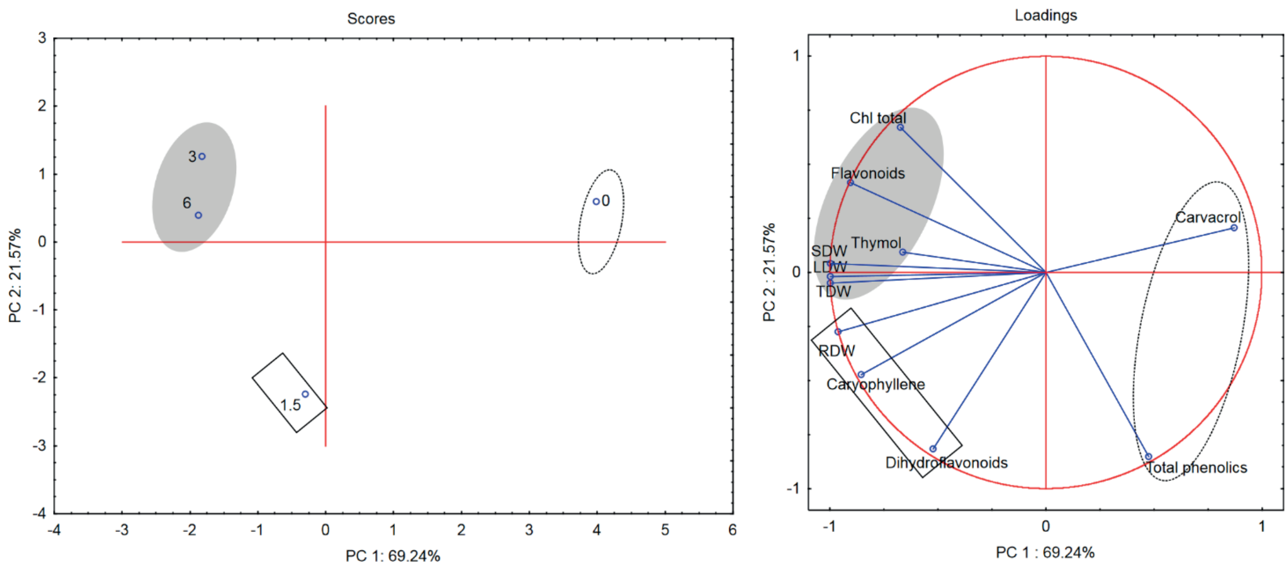

Figure 2. Score and loadings of the PCA on the matrix correlation built using data for eleven parameters from *Lippia gracilis* plants grown in chicken manure.

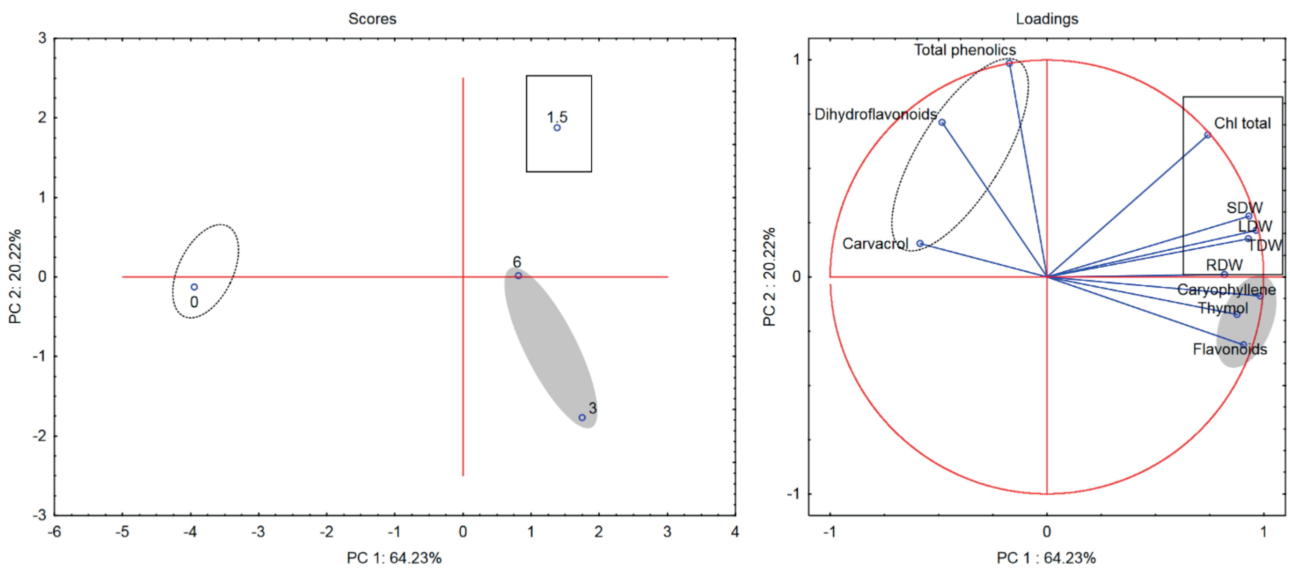

Figure 3. Score and loadings of the PCA on the matrix correlation built using data for eleven parameters from *Lippia gracilis* plants grown in quail manure.

CONCLUSIONS

The growth, photosynthetic pigment contents, chemical analyses of essential oil and phenolic compounds of *L. gracilis* were significantly affected by different sources and doses of poultry manure. The best doses were 1.5 - 3.0 kg/m^2 quail manure and 3.0 and 6.0 kg/m^2 chicken manure. However, a dose of 1.5 kg/m^2 of quail manure is recommended as it is half the dose of 3.0 kg/m^2. For chicken manure, there was no statistical difference between the doses of 3 and 6 kg/m^2 for the total dry weight, therefore the dose of 3 kg/m^2 is recommended because it is half of the dose of 6 kg/m^2. Both sources can be used successfully depending on the availability of manure where the plant is being grown. Variations in the chemical analyses, qualitative and quantitative, of the essential oil occurred depending on the fertilizer and the dose used. In the chemical analyses, the major chemical constituent identified in all treatments was carvacrol. However, the highest carvacrol synthesis was observed in the control treatment without fertilization.

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AUTHORS' CONTRIBUTIONS

All authors contributed substantially to the work reported. B.R.A., S.K.V.B., and J.E.B.P.P. conceived the study, designed the experiments; B.R.A., R.M.A.A., J.J.F.L., A.A.C., and J.E.B.P.P. performed experiments, analyzed data; S.K.V.B. did the chemical analyses. B.R.A., S.K.V.B., P.P.B., F.C.F., and J.E.B.P.P. wrote the manuscript. All authors read and approved the manuscript.

CONFLICT OF INTERESTS

The authors have no conflicts of interest to declare.

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