*Animal Journal*

***Lippia gracilis* Schauer essential oil as a growth promoter for Japanese quail**

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***Supplementary Material S1***

*Extraction of the essential oil*

To extract the essential oil, shoots of *Lippia gracillis* Schauer varieties 107 and 108 were harvested in the morning at Campus Rural (CRU) at Federal University of Sergipe (UFS), packed in plastic bags, and taken to the laboratory for processing. Subsequently, the leaves were separated from the stems and dried for five consecutive days in a forced-air oven at 55 ºC. After this step, 75 to 80 g of pre-dried leaf material were weighed and placed in a round-bottom flask to which 2 L distilled water was added for the oil extraction. The oil was extracted by the hydrodistillation method, using a Clevenger apparatus (Santos *et al*., 2004). The essential oil was then collected and packed in an amber glass jar to protect it from light and humidity. Next, the jar was stored in a refrigerator at an approximate temperature of -4 ºC (Teixeira *et al*., 2014).

The oil yield was 3% and presented myrcene (2.49%), p-cymene (12.26%), 1.8-cineole (1.41%), ϒ-terpinene (13.56%), thymol methyl ether (5.20%), thymol (3.28%), carvacrol (38.88%) and β-caryophyllene (5.05%) (Santos *et al.,*2016).

*Intestinal microbiology analysis*

The harvested material was weighed and placed in a Falcon tube containing buffered saline solution (5.61 g NaCl, 1 g KH2PO4, 2 g Na2PO4, and 0.11 g KCl in 1000 ml distilled water) and 10% glycerol (used as cryoprotectant), which was then frozen at -4 ºC. Escherichia coli (EC) Broth was used for the culture of *E. coli*; mannitol salt agar for *Staphylococcus* spp; Rappaport Vassiliadis broth for *Salmonella* spp; and BHI (Brain Heart Infusion) broth with addition of 2% dextrose for *Lactobacillus* spp. The broths also received 2.5% base agar. All media were produced following the recommendations of the manufacturers and sterilized in an autoclave at 121 ºC for 15 min. The media were prepared, placed in petri dishes, and oven-dried for 24 h.

For the analyses of all microorganisms, the samples were thawed at room temperature and serial dilutions (10–1, 10–3, 10–5, 10–7) were carried out using buffered saline solution. One milliliter of each dilution was seeded in duplicate Petri dishes to observe the growth of colonies. The inoculant was spread using Drigalski’s handle and dishes were incubated in an oven at 37 ºC for 24 h.

After the data of each replicate were obtained, the average number of colony-forming units (Log CFU/g) per treatment was calculated and turned into Log10.

$$Log CFU/g=Numberofcolony-formingunits\*Diluition$$

*Gene expression analyses*

RNA was extracted using the Trizol® reagent (Invitrogen, Carlsbad CA, USA) according to the manufacturer instructions, in the proportion of 1.0 ml for every 100 mg of tissue. The tissue was ground with a Polytron electric homogenizer (tissue + Trizol) until its complete dissociation. Next, 200 *μ*l chloroform were added to the samples, which were manually homogenized for 1 min. Samples were then centrifuged for 15 min at 12 000 g, at 4 ºC. The aqueous phase was collected and transferred to a sterile tube, followed by addition of 500 *μ*l isopropanol per tube. Tubes were then reserved at room temperature (25 ºC) for 10 min.

Subsequently, samples were centrifuged for 10 min at 12 000 g, at 4 ºC. The supernatant was discarded and the precipitate was washed with 1.0 ml 17% ethanol. The material was centrifuged once again at 7 500 g for 5 min at 4 ºC and, immediately after centrifugation, the supernatant was discarded. Lastly, the pellet was dried for 15 min and then resuspended in RNase-free ultrapure water. The total RNA concentration was measured using a spectrophotometer under the wavelength of 260 nm. GoScript™ Reverse Transcription System (Promega Corporation, USA) was used to produce cDNA, following the manufacturer instructions. Four microliters of total RNA treated with DNAse and 1 *μ*l Oligo(dT)15 Primer were added to a sterile RNA-free tube. The reaction was incubated in Thermal Cyclers (Bio-Rad Corporation, Brazil) for 5 min at 65 ºC and then for 2 min at 4 ºC. Next, 2 *µ*l GoScript™ 5X Reaction Buffer, 1.4 *µ*l MgCl2 (25 mM), 1 *μ*l PCR Nucleotide Mix, 1 *µ*l GoScript™ Reverse Transcriptase, and 5.6 *µ*l Nuclease-FreeWater were added. The solution was incubated in Thermal Cyclers (Bio-Rad Corporation, Brazil) for 30 min at 37 ºC, then 5 min at 95 ºC, and, lastly, 10 min at 4 ºC.

For the real-time PCR reactions, the fluorescent dye SYBR GREEN (SYBR® GREEN PCR Master Mix, Applied Biosystems, USA) was used. Real-time PCR analyses were carried out in a CFX96™ IVD Real-Time PCR apparatus (Bio-Rad Laboratories, USA). The amplification reaction consisted of 5 *µ*l diluted cDNA, 0.5 *µ*l of each primer (forward and reverse) at 10 *µ*M, 12.5 *µ*l SYBR® GREEN PCR Master Mix, and water up to a total volume of 25 *µ*l. To measure each gene reaction efficiency, a series of 25-*µ*l dilutions were performed similarly to the previous one, always using 5 *µ*l of cDNA pool derived from a serial dilution (80, 40, 20, and 10 ng). The primers used in the amplification reactions of sodium-glucose transporter 1 (*SGLT1*), glucose transporter 2 (*GLUT2*), glutathione peroxidase (*GPX)*, and catalase (*CAT*) were designed according to the sequences of their respective genes and deposited on the website www.ncbi.nlm.nih.gov (access codes NM\_205064.1, NM\_001163245.1, XM\_015863594.1, and NM\_204267.1) (Supplementary Table S1). The *ß-actin* gene (access codeL08165) was used as an endogenous reference gene. All analyses were performed in a volume of 25 *µ*l and in duplicates.

**Supplementary Table S1-**Primers for qRT-PCRused in the amplification reactions in the intestinal tissue of Japanese quail

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| --- | --- | --- | --- |
| Gene  | Amplicon [Bp] | AT [°C] |  Primer sequence (5’-3’) ¹ |
| *SGLT12* | 160 | 60 | GCCATGGCCAGGGCTTA  |
| CAATAACCTGATCTGTGCACCAGTA |
| *GLUT23* | 180 | 60 | CGCAGAAGGTGATAGAAGC  |
| ACACAGTGGGGTCCTCAAAG |
| *GPX74* | 140 | 60 | TTGTAAACATCAGGGGCAAA |
| TGGGCCAAGATCTTTCTGTAA |
| *CAT5* | 76 | 60 | TTGGGTTGGCTCGTTGAGG |
| CGGAGCTACAGAAGCACGAT |
| *β-actine* | 136 | 60 | ACCCCAAAGCCAACAGA |
| CCAGAGTCCATCACAATACC |

1Bp, base pair; AT, annealing temperature; 2*SGLT1*, sodium-glucose cotransporter 1; 3*GLUT2*, glucose transporter 2; 4*GPX7*, glutathione peroxidase 7; 5*CAT*, catalase.

Reference

Santos AS, Alves SM, Figueiredo FJC and Rocha-Neto OG 2004. Descrição de Sistema e de Métodos de Extração de Óleos Essenciais e Determinação de Umidade de Biomassa em Laboratório. Comunicado Técnico 99, 1-6, Embrapa, Belém, Pará, Brazil. Retrieved on 14 June 2016 from <https://www.infoteca.cnptia.embrapa.br/bitstream/doc/402448/1/com.tec.99.pdf>

Santos CP, Pinto JAO, Santos CA, Cruz EMO, Arrigoni-Blank MF, Andrade TM, Santos DA, Alves PB and Blank AF 2016. Harvest time and geographical origin affect the essential oil of *Lippia gracilis* Schauer. Industrial Crops and Products 79, 205–210.