

## ECOTOXICOLOGICAL EVALUATION OF *Lippia alba* Mill PLANT RESIDUES ON TERRESTRIAL ECOSYSTEM REPRESENTATIVES

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### ABSTRACT

*Lippia alba* is a medicinal plant commonly used in Cuba and the Caribbean for the development of herbal formulations against skin inflammatory illnesses thus generating residual biomass whose environmental impact remains unassessed. This study aimed to evaluate the ecotoxicological effects of *L. alba* residues and their aqueous extract fractions on key terrestrial ecosystem organisms. Soil microbial community activity was analyzed through CO<sub>2</sub> emissions and ammonium (NH<sub>4</sub><sup>+</sup>) quantification, as well as degradation and toxicity tests on earthworms (*Eisenia andrei*). Phytotoxicity was assessed using *Phaseolus vulgaris* seeds to determine germination rates and radicle length. Results showed that CO<sub>2</sub> emissions increased in the groups treated with *L. alba* residues, suggesting enhanced microbial respiration, whereas NH<sub>4</sub><sup>+</sup> levels remained unchanged across all treatments. Seed germination rates remained above 75% at all tested extract concentrations except at 75%. Radicle length, however, was significantly reduced at higher concentrations (75% and 100%). No toxic effects were observed in *E. andrei* (earthworms). These findings indicate that *L. alba* residues are not toxic to soil macro- or microfauna and may contribute to soil organic enrichment, although higher concentrations of aqueous extracts exhibit moderate phytotoxic effects. The study supports the potential incorporation of *L. alba* waste into sustainable soil management strategies, such as composting and vermicomposting.

**Keywords:** *Lippia alba*, plant residues, ecotoxicology, soil respiration, soil amendment, *Phaseolus vulgaris*, phytotoxicity, vermicomposting.

### INTRODUCTION

Plants have been used for medicinal purposes throughout history and have significantly contributed to the development of modern pharmacotherapy [1]. A notable example is *Lippia alba* (Mill.) N.E. Br. ex Britton & P. Wilson, an aromatic species from the Verbenaceae family, widely distributed across tropical and subtropical regions of the Americas including Cuba, Mexico, and Brazil, as well as parts of Africa and Asia [2]. Traditionally, *L. alba* has been employed for its antimicrobial, analgesic, sedative, antispasmodic, and digestive properties, attributed to its diverse secondary metabolites including monoterpenes such as carvone, citral, and linalool; sesquiterpenes including caryophyllene and germacrene; and flavonoids like quercetin and rutin [3], [4].

In Cuba, a hydroalcoholic leaf extracts of *L. alba* are commercially available as an analgesic topical preparation, recognized in the National Formulary of Phytopharmaceuticals and Apipharmaceuticals as a phytomedicine [5]. Currently, novel formulations derived from this plant, such as anti-inflammatory creams for muscular and joint pain [6], antimicrobial gels [7], and oral tablets [8], [9] are under development.

The increasing industrial use of *L. alba* in cosmetic and phytotherapeutic formulations has resulted in the generation of substantial residual biomass, primarily from the extraction of essential oils and hydroalcoholic fractions. This biomass is often discarded without valorization, potentially leading to environmental burdens if not properly managed. However, recent studies suggest that residues from medicinal plant species may retain residual bioactive properties, implying possible allelopathic or ecotoxic effects on soil organisms and cultivated plants [10], [11]. In terrestrial ecotoxicology, organisms such as *Eisenia andrei* (earthworm) and *Phaseolus vulgaris* (common bean) are widely employed as bioindicators due to their sensitivity to chemical pollutants, bioaccumulation potential, ecological roles in organic matter decomposition and nutrient cycling [12], [13]. Additionally, soil microbial respiration (measured via CO<sub>2</sub> emissions) and nitrogen mineralization (NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup>) are relevant parameters for assessing the impact of organic amendments or plant residues on soil microbial functionality [14], [15].

The incorporation of plant residues into soil may elicit contrasting effects: stimulation of microbial activity and nutrient release, or alternatively, phytotoxic effects associated with the release of phenolic and terpenoid compounds with allelopathic activity [16], [17]. In the specific case of *L. alba*, the ecological impact of its residues remains largely uncharacterized, representing a significant

knowledge gap given its expanding industrial use and the need for circular economy approaches in the medicinal plant agroindustry [18]. This study aims to evaluate the toxicological effects of *Lippia alba* plant residues and aqueous extracts on representative terrestrial organisms: the earthworm *Eisenia andrei*, the plant *Phaseolus vulgaris*, and the soil microbial community. This integrative approach seeks to determine the potential of *L. alba* residues as bioamendments, and to assess their safety or risk for soil ecosystems. The results will support the development of sustainable strategies for the reuse of waste generated during the production of phytomedicines and cosmetics from this species, through composting or vermicomposting, in alignment with ecotoxicological principles and environmentally responsible management practices.

### EXPERIMENTAL

#### Preparation of *Lippia alba* Plant Residue

The plant residue used was provided by the Department of Pharmacy of the Institute of Pharmacy and Food (IFAL), University of Havana (UH), Cuba, where the hydroalcoholic extract were prepared from *L. alba* leaves. A total of 10 kg of residual biomass were weighed, air-dried at room temperature, and subsequently ground to a homogeneous powder with a particle size of 1 mm. Eight kilograms of this material was used directly in soil assays, while another fraction underwent aqueous extraction via infusion (10% w/v, 15 minutes at 90 °C), followed by filtration and dilution to the desired concentrations for phytotoxicity assays.

#### Soil Assays

Microbial respiration, as indicated by CO<sub>2</sub> emissions, and ammonium (NH<sub>4</sub><sup>+</sup>) levels, as an indicator for nitrogen mineralization were measured in soil microfauna. In the earthworm *E. andrei*, both toxicity and physiological alterations were assessed. The interaction between these key biological components of the soil ecosystem was also analyzed.

#### Experimental Substrate

The substrate consisted of vermicompost (worm humus) obtained from the breeding and maintenance facility of the Subdirection for Toxicological and Environmental Assessments at the National Center for Toxicology (La Habana, Cuba), where a controlled feeding protocol was followed. The substrate was homogenized and adjusted to 40% of its water holding capacity using distilled water [19].

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## Experimental Design

Five experimental groups were established, each with three replicates ( $n = 3$ ). Each replicate received 300 g of substrate and 30 g of either *L. alba* plant residue or commercial peat (*Sphagnum*), depending on the treatment (Table 1). All groups were supplemented with 10 mL of 25% glucose solution to stimulate microbial activity [20], [21]. Adult *E. andrei* earthworms with visible clitellum were evenly distributed in Groups G3 and G5 using the weight-equilibration method (Bloguin) to evaluate the microbiota–macrofauna interaction during the decomposition process. To assess the toxicological effects on earthworms, the following parameters were monitored over a 28-day period: mortality, behavior, presence of lesions or morphological alterations, and final body weight variation.

**Table 1.** Distribution of experimental groups in soil microbial community trials.

Groups	Treatment
G1	Humus + glucose
G2	Humus + glucose + residue ( <i>L. alba</i> Mill.)
G3	Humus + glucose + residue +5 earthworms
G4	Humus + glucose + peat sphagnum
G5	Humus + glucose + peat sphagnum + 5 earthworms

## CO<sub>2</sub> Quantification (Microbial Respiration)

CO<sub>2</sub> emission was assessed in accordance with EPA guideline OCSPP 850.3200 (2012) [13], using an indirect measurement method based on aerobic soil microbial respiration, following the closed-system incubation technique [21], [22]. Each experimental container was hermetically sealed and incubated at  $22 \pm 2$  °C in complete darkness. Inside each container, a vessel containing 0.1 N NaOH solution was placed to absorb the CO<sub>2</sub> released. Measurements were taken at 24 hours, and on days 5 and 28 [21].

CO<sub>2</sub> was quantified by back-titration with 0.1 N HCl, and the amount of evolved CO<sub>2</sub> was calculated using the following equation:

$$R = (B - M) \times N \times E$$

### Where:

- $R$  = CO<sub>2</sub> released (mg)
- $B$  = Volume of HCl used for the blank (mL)
- $M$  = Volume of HCl used for the sample (mL)
- $N$  = Normality of the acid (eq/L)
- $E$  = Equivalent weight of CO<sub>2</sub> (22 mg·meq<sup>-1</sup>)

## Ammonium (NH<sub>4</sub><sup>+</sup>) Quantification

Ammonium (NH<sub>4</sub><sup>+</sup>) content was determined at the end of the experiment using the extraction method with 0.5 M K<sub>2</sub>SO<sub>4</sub>, followed by spectrophotometric measurement at 670 nm, as described by García Gutiérrez et al. (2014) [19]. A calibration curve was prepared using ammonium sulfate [(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>] from a stock solution at a concentration of 2 µg/mL. Aliquots of 1.0, 1.5, 2.5, 3.5, 5.0, and 5.5 mL were taken and brought to a final volume of 25 mL. The calibration curve yielded the following linear regression equation:

$$y = 0.096x + 0.005 \quad \text{with a coefficient of determination } R^2 = 0.9978.$$

## Phytotoxicity Assay in *Phaseolus vulgaris*

### Preparation of Plant Material

Phytotoxic effects were assessed by evaluating the impact on seed germination and early development of *Phaseolus vulgaris*. Seeds were selected based on uniformity in size and viability, certified by the Seed Assurance and Services Unit of the Ministry of Agriculture of Cuba. Pre-treatment included hydration in distilled water for 30 minutes, followed by disinfection with 75% ethanol for 5 minutes and three rinses with distilled water. The assay was conducted according to OECD Guideline 208 [23] and EPA (1996) protocols [24].

## Experimental Design

*P. vulgaris* was selected due to its importance as a staple food crop in several American countries such as Cuba and Chile [25], and its designation by OECD as a bioindicator species for phytotoxicity in terrestrial ecosystems [23]. Five experimental groups were established, each with six replicates ( $n = 6$ ), using aqueous extracts of *L. alba* plant residue at concentrations of 25%, 50%, 75%, and 100%, alongside a control group (distilled water). In sterile Petri dishes, five seeds were placed on filter paper moistened with 3 mL of the corresponding treatment solution. Plates were sealed with Parafilm and incubated in the dark at  $22 \pm 2$  °C for 120 hours. The parameters assessed included germination percentage, root length, and seedling morphology under stereomicroscopy [23].

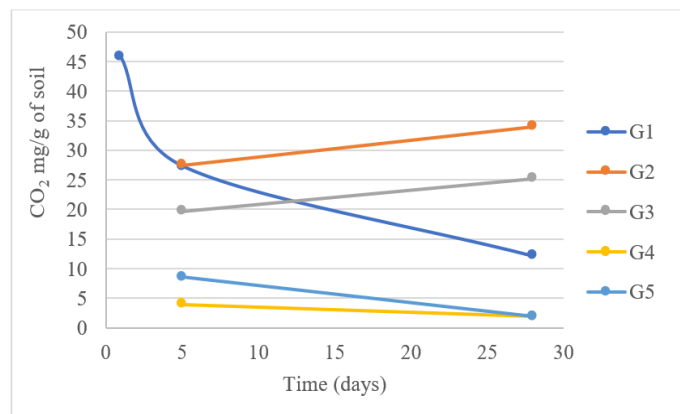
## Statistical Analysis

Data were analyzed using SPSS software version 22 (IBM, 2013). Normality was tested with the Shapiro–Wilk test, and homogeneity of variance was assessed with Levene’s test. For parametric data, one-way ANOVA was applied followed by Dunnett’s or Duncan’s post hoc tests. For non-parametric data, the Kruskal–Walli’s test was used. Statistical significance was set at  $p < 0.05$  [26].

## RESULTS AND DISCUSSION

### Effect of *Lippia alba* Residues on Soil Microbial Respiration

CO<sub>2</sub> and ammonium levels were quantified as indirect indicators of microbial community activity. These variables serve as valuable tools to evaluate the impact of *Lippia alba* residues on this critical environmental compartment. Ambient temperature ranged between 22–24 °C, remaining within the acceptable range established by EPA regulatory guidelines for this type of assay ( $22.0 \pm 2$  °C) [13]. Soil pH at the beginning and end of the experiment varied between 6.75 and 7.80, both considered suitable for the optimal functioning of soil microbial communities [27]. Soil microbial respiration is a key indicator of biological activity and organic matter decomposition efficiency [14]. CO<sub>2</sub> emission in the groups treated with *L. alba* residues (G2 and G3) was significantly higher at the end of the assay compared to the control group (G1) and the peat-amended groups (G4 and G5). Notably, group G2 (residue without earthworms) increased the CO<sub>2</sub> emission to 34.01 mg/g compared to peat group (G4) with a value of 1.97 mg/g, on day 28 ( $p < 0.01$ ), as shown in Figure 1.



**Figure 1.** CO<sub>2</sub> emissions in the time (28 days) in the different groups (G1: humus glucose, G2: humus residue, G3: humus residue earthworms, G4: humus peat sphagnum, G5: humus peat sphagnum earthworms).

The peat-supplemented groups did not show significant differences between them. Both exhibited the lowest CO<sub>2</sub> emissions, which resulted in statistically significant differences ( $p < 0.05$ ) when compared to the groups amended with *L. alba* residues, despite peat also being a source of organic matter. Peat is primarily composed of partially decomposed plant remains and is known for its high carbon content, contributing to long-term carbon storage in soils [28]. Its accumulation results from the slow decomposition and compaction of mosses and other plants in waterlogged peatland environments, which are associated with reduced decomposition rates [29] and low soil respiration. These characteristics favor organic matter stabilization and carbon accumulation over time, as observed in this assay.

Notably, the CO<sub>2</sub> emission levels in the residue-treated groups were significantly higher than those in the peat-treated groups, suggesting that *L. alba* plant material decomposes more rapidly than humified materials. This behavior is consistent with reports on other fresh plant residues, such as *Tithonia diversifolia* and *Aeschynomene fluminensis*, which also induced increased microbial respiration in amended soils [30], [31].

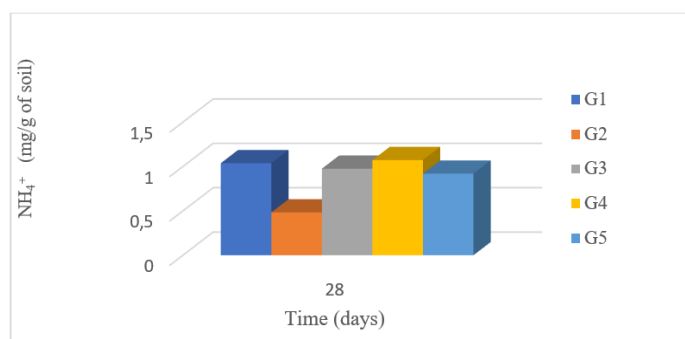
These results indicate an activation of the soil microbiota associated with the availability of easily assimilable carbon in *L. alba* residues. This phenomenon has also been described in soils amended with plant materials rich in phenolic and terpenoid compounds, such as *Tagetes* spp. and *Azadirachta indica*, where sustained increases in microbial respiration were observed [21], [31]. The elevated CO<sub>2</sub> emission rates in the groups amended with *L. alba* residues suggest their potential use as active bioamendments in composting systems, provided their allelopathic composition is well-characterized.

Upon incorporation into the substrate, *L. alba* residues promoted a sustained increase in carbon dioxide (CO<sub>2</sub>) emissions over the 28-day monitoring period, indicating an enhancement of microbial respiration. This effect is likely attributable to the availability of readily degradable organic compounds present in the residues, such as sugars, organic acids, and free phenols. According to Anderson (1982), soil respiration is a sensitive indicator of biological activity and reflects shifts in the microbial community following organic matter input [21]. Similar findings have been reported by Beesley et al. (2014), who observed elevated soil respiration rates after green compost application [32].

The presence of earthworms (*Eisenia andrei*) did not significantly affect respiration rates, which may be due to the short duration of the experiment, the early stabilization stage of the residue, or the specific type of residue used [33]. No mortality, significant weight loss, or external lesions were observed in earthworms exposed to *L. alba* residues (G3) when compared with the peat group (G5) ( $p > 0.05$ ). No behavioral changes, discoloration, or altered mobility were detected, indicating a low or negligible acute toxicity of the plant residues toward this species. These results agree with those reported by Das et al. (2022) and Noa et al. (2023), who evaluated medicinal plant residues on epigeic earthworms and found no signs of subchronic toxicity, suggesting that *L. alba* solid residues are safe for this component under controlled conditions [10], [34].

#### Ammonium (NH<sub>4</sub><sup>+</sup>) Quantification

Ammonium quantification is one of the indicators used to assess the impact of xenobiotics on soil microbial activity. **Figure 2** presents the determinations conducted at the end of the assay, which allowed evaluation of the effect of *L. alba* residues on the nitrogen mineralization process.



**Figure 2.** Ammonium quantification at the end of the test (G1: humus glucose, G2: humus residue, G3: humus residue earthworms, G4: humus peat sphagnum, G5: humus peat sphagnum earthworms)

#### Ammonium (NH<sub>4</sub><sup>+</sup>) Quantification

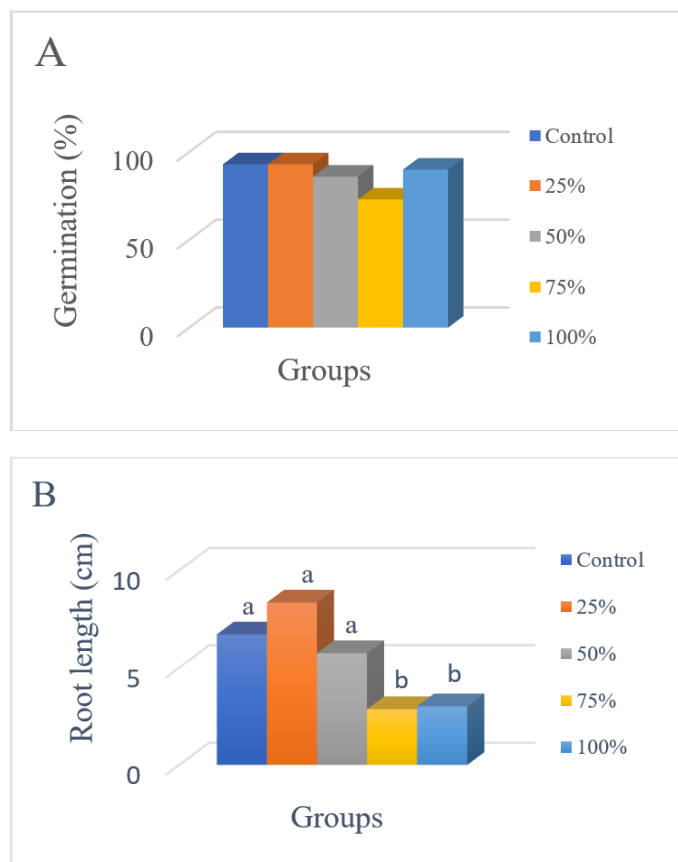
NH<sub>4</sub><sup>+</sup> levels did not show statistically significant differences between groups ( $p > 0.05$ ), regardless of the presence or absence of earthworms (**Figure 2**). Average NH<sub>4</sub><sup>+</sup> concentrations ranged from 1.8 to 2.3 mg/kg of soil, suggesting low nitrogen mineralization activity during the experimental timeframe. This contrasts with previous studies, such as that by Abbasi et al. (2007), in which the application of organic amendments increased nitrogen mineralization in agricultural soils.

A possible explanation is that *L. alba* residues may have a high C:N ratio or contain secondary metabolites that temporarily inhibit ammonification processes [11]. Moreover, the 28-day duration of the experiment may not have been sufficient to reach the active phase of nitrogen mineralization, as described in composting or vermicomposting processes, where nitrogen availability may be delayed until 50–60 days of material maturation [34], [35].

#### Effects of *Lippia alba* Extract on Phytotoxicity in *Phaseolus vulgaris* L.

##### Germination

The germination rate was  $\geq 75\%$  across all treatments, except at the 75% concentration, which showed a slight reduction (68%), without reaching statistically significant differences compared to the control ( $p > 0.05$ ), as illustrated in **Figure 3A**. This indicates that the aqueous extract does not significantly affect the germinative viability of the seeds.



**Figure 3.** Effect of the aqueous extract of *Lippia alba* residues on the germination (A) and root length (B) of *Phaseolus vulgaris* L. exposed to extract concentrations of 25%, 50%, 75%, 100%, and distilled water control

##### Root Length

Root length was assessed during the assay, and its behavior is presented in **Figure 3B**. A significant inhibition of radicle elongation was observed starting at the 50% extract concentration, with more pronounced effects in the 75% and 100% treatment groups, showing reductions of 48% and 62%, respectively, compared to the control ( $p < 0.01$ ). Morphologically, the radicles exhibited abnormal growth patterns but showed no visible necrosis. This phytotoxic effect has been reported for other plant extracts rich in flavonoids and monoterpenes, such as rutin or geraniol, which may interfere with cell division, auxin transport, and cell elongation in young roots [36], [37].

Although germination rates remained above 75% in most treatments, aqueous extract fractions at 75% and 100% induced a significant reduction in radicle length, suggesting an inhibitory effect on root development. This phenomenon may be related to the presence of phenolic compounds, flavonoids, and terpenoids in the extracts, which are known for their allelopathic potential.

Similar findings have been reported for aqueous extracts of other plant species, including *Cyperus rotundus*, *Cistus ladanifer*, and *Sargassum* spp., which reduced root growth due to interference with processes such as cell elongation, protein synthesis, and mitochondrial energy metabolism [16], [36], [38].

The presence of **rutin** as a major metabolite in *Lippia alba* extracts is consistent with the observed effects, as flavonols have been shown to act as inhibitors of mitochondrial electron transport and to alter enzymatic activity in developing plant tissues [37]. Moreover, the radicle is among the most sensitive plant structures to chemical agents released from residues, as its development relies on a fine hormonal balance and sustained availability of nutrients and water. Inhibition of root growth can negatively impact seedling establishment capacity, and consequently, their ecological and agronomic competitiveness.

Based on the results obtained, it can be concluded that the solid residues of *L. alba* do not pose an immediate ecotoxicological risk to soil organisms such as earthworms or microbial communities and may be considered a viable raw material for composting and vermicomposting processes. However, the aqueous extract of the residue, particularly at high concentrations, may exert transient phytotoxic effects, suggesting the need to control its direct application to soil or to implement proper pre-treatment (e.g., maturation) prior to agricultural use. In line with recent studies focused on the valorization of medicinal and aromatic plant (MAP) residues [18], [39], these findings reinforce the importance of incorporating ecotoxicological assessments before deploying organic reuse strategies. The present study provides a robust scientific basis for the development of composting/vermicomposting technologies specifically tailored to *Lippia alba* residues, thereby supporting the transition toward sustainable and low-impact production models.

The results obtained in this study provide relevant evidence regarding the ecotoxicological effects of *Lippia alba* (Mill.) plant residues on key components of terrestrial ecosystems and represent progress in understanding their potential impact on soil when considered for use as bio-amendments. Despite growing interest in the valorization of MAP residues, scientific literature still lacks integrative studies that simultaneously evaluate their effects on soil microbiota, edaphic macrofauna, and cultivated plants. This work represents a significant contribution in that direction.

### CONCLUDING REMARKS

Residues from *Lippia alba* serve as beneficial carbon sources for soil, showing no observable toxicity toward the earthworm *Eisenia andrei* or the soil microbiota, which suggests their ecological safety. Furthermore, the incorporation of *L. alba* residues into the substrate significantly increased CO<sub>2</sub> emissions compared to peat-treated groups, indicating microbial activation and suggesting favorable biodegradability of the residue.

On the other hand, ammonium (NH<sub>4</sub><sup>+</sup>) levels showed no significant differences between treatments, which could be attributed to the limited duration of the assay or to a low nitrogen mineralization rate from the residues. Therefore, longer-duration studies are recommended for more accurate assessment of nitrogen mineralization. The aqueous extract of the residues exhibited phytotoxic effects on root growth of *Phaseolus vulgaris* at higher concentrations (75% and 100%), evidencing the presence of secondary metabolites with allelopathic potential, although germination was not significantly affected.

Overall, the results support the viability of using *L. alba* residues as a bio-amendment in composting or vermicomposting processes and provide a scientific basis for future sustainable management strategies, aligned with the principles of circular economy and regenerative agriculture.

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