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Phytochemical Analysis, Antioxidant and Cytotoxic Activity of *Lannea egregia* Engl. & K. Krause Stem Bark Extracts

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ABSTRACT

This study investigates the phytochemical contents, antioxidants and cytotoxic activities of *Lannea egregia* Engl. & K. Krause stem bark extracts. Secondary metabolites were extracted using n-hexane, ethyl acetate, and ethanol by successive maceration. The concentrated extracts were subjected to preliminary phytochemical screening using standard procedures. The crude flavonoid was obtained from the plant material using Harborne's method and then profiled using high-performance liquid chromatography (HPLC). The antioxidant activities of the plant extract were assessed using reducing power assay and DPPH scavenging activity, while the cytotoxic activity was determined using the brine shrimp lethality assay. The crude extract of n-hexane or LEHe (0.86%), ethyl acetate or LEEa (1.42%), ethanol or LEEt (3.32%), and flavonoid or LEF (9.7+-0.01%) were obtained from the plant material, with flavonoids, anthraquinone, terpenoids, and tannins detected. The ethyl acetate extract and the crude flavonoid have the highest and comparable DPPH radical scavenging activity at IC_{50} of 22.98 ± 0.07 µg/mL and 22.48 ± 1.02 µg/mL respectively, the greatest reducing property being exhibited by the ethanol extract. The most cytotoxic activity was observed in the n-hexane extract at LC_{50} of 8.70 ± 0.58 µg/mL. HPLC detected catechin, p-coumaric acid, ferulic acid, rutin, apigenin, kaempferol and quercetin. In conclusion, *Lannea egregia* stem bark extracts possess antioxidant and cytotoxic activities and could be explored for new drugs in the management of cancer.

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INTRODUCTION

Lannea egregia Engl. and K. Krause (Anacardiaceae) is a deciduous tree. In Guinea, the Ivory Coast, and Benin, it is known as *L. barteri* (Oliv.) Engl. (Abdulrahaman et al., 2014; Rafiu, 2019), ranging in location from the savannas of Guinea to Nigeria (Jansen, 2005). It is popularly referred as "*ekudan*" in the Yoruba tribe of Nigeria and has been used in traditional remedies to cure a variety of human illnesses. A decoction is consumed to treat nausea, diarrhoea oedema, paralysis, epilepsy, and insanity (Rafiu et al, 2019). The stem bark decoction is also consumed in the Central African Republic and Nigeria as a stomachic to raise hemoglobin levels and as a component of vermifuge medication (Neuwinger 2000). Leprosy, sores, and other skin conditions can also be treated externally with the stem bark (Ogundajo et al., 2021). The macerated root is used in a poultice for the treatment of wounds, and its decoction is used to cure hernias (Neuwinger 2000). The *L. egregia* leaves are also used traditionally as an anticancer herb and to cure haemorrhoids (Soladoye et al., 2010). Antimicrobial activities of the stem bark extract and oil have also been reported (Rafiu et al*.,* 2019; Ogundajo et al., 2021).

Flavonoids are polyphenolic secondary metabolites found in flower pigments, vegetables, fruits, and some beverages, such as wine, tea, and cocoa. They have been reported to be favourable antioxidants in the management of diseases such as cancer, Alzheimer's, atherosclerosis and other non-communicable or degenerate diseases. Also, they are useful in the pharmaceutical and cosmetic industries (Panche et al., 2016).

The cytotoxic activities of plants have been studied widely to assess the toxic effect of plant extracts on living organisms. The term is often used to describe chemotherapy drugs that kill cancer cells. The cytotoxic activities will provide knowledge on the possible use of the plant extracts in the prevention and treatment of cancer, in line with its use in traditional practices (Soladoye et al*.* 2010), especially with the increasing incidence of the disease in humans.

The objective of the study is to determine and compare the phytochemical contents of *L. egregia* n-hexane (LEHe), ethyl acetate (LEEa), and ethanol (LEEt) stem bark extracts and to evaluate the antioxidant and cytotoxic activities of these crude extracts and crude

flavonoid (LEF). It also aims to determine the flavonoid profile using high-performance liquid chromatography, which has not been reported to date.

METHODS

General Instrumentation

All the solvents and chemicals used in this work were of analytical grade purchased either from Sigma-Aldrich chemical company or Fisher scientific chemical company. HPLC-grade acetonitrile and acetic acid (Sigma-Aldrich chemical company) was used for the HPLC system. *Artemia salina* (Aqua master, China) was used for the cytotoxicity assay. The ultra-violet spectroscopy data were obtained using Spectrum Lab 752s, while high-performance liquid chromatography was determined using the Agilent Technologies' HPLC 1100 series.

Preparation and Extraction of *L. egregia* Stem Bark

A fresh sample of *L. egregia* stem bark was purchased at Mushin market, Lagos state, Nigeria. The plant was authenticated at the Forest Herbarium, Ibadan (FHI 113077). Before extraction, the stem bark was washed with water and cleaned to remove sands and other debris. It was then chopped into small pieces and air dried in an open laboratory for around three weeks until dry. The dried plant material was powdered into a coarse form in a blender.

The extraction of the plant material was conducted by successive maceration in n-hexane, ethyl acetate, and ethanol. The plant material (500 g) was soaked in 1000 mL of n-Hexane for 72 h and filtered. The process was repeated twice. The ethyl acetate (1000 mL) was added to the marc, left for 72 h, and filtered, then repeated to obtain the ethyl acetate extract. Finally, 1000 mL of ethanol was added to the marc and then macerated for 72 h. This was repeated to obtain the ethanol extract. The extracts were then concentrated to dryness in vacuo at 30°C using a rotary evaporator.

Phytochemical Screening of *L. egregia* Stem Bark Extracts

A preliminary qualitative test to determine secondary metabolites was performed on the different extracts of *L. egregia* using the standard protocols described by Harborne (1998) and Sofowora (2008). The plant extracts were tested for the presence of alkaloids, flavonoids, tannins, saponins, anthraquinones, terpenoids, cardiac glycosides, steroids and reducing sugar.

Extraction of Flavonoids from *L. egregia* Stem Bark

Flavonoids were extracted from the plant material using Harborne's method (Harborne, 1998). Aqueous methanol (80%, 50 cm^3) was added to 10 g of the coarse plant sample in a 250 cm^3 beaker, covered, and allowed to stand for 24 h at room temperature. The extract was separated from the residue using Whatman filter paper number 42 (125 mm). The residue was further re-extracted with the same volume of methanol (three times). The extract was pooled together and then transferred into a crucible and evaporated to dryness over a water bath. The content in the crucible was cooled in an oven at 40°C and weighed until a constant weight was obtained. The experiment was conducted three times, and the percentage of crude flavonoid obtained was calculated.

High-Performance Liquid Chromatography (HPLC) Method for the Estimation of Flavonoid Content

High-performance liquid chromatography was used to measure the levels of catechin, p-coumaric acid, ferulic acid, rutin, apigenin, kaempferol and quercetin in LEF. Methanol (100%) was used to dissolve 1.0 mg of LEF in 1.0 mL. The final product was filtered through a Millipore membrane filter measuring 0.45 m. The LEF was subjected to HPLC analysis using Agilent Technologies' HPLC 1100 series. The extract was separated on a Zorbax Eclipse XDB RP C8 (150 4.6 mm, 5 m) column at a flow rate of 0.5 mL/min while the temperature was set at 40°C. Acetonitrile and 0.2 percent acetic acid were used to elute the mobile phase. The injection volume was 10 µL, and detection was monitored at 280 nm. Sample identification was achieved by contrasting the retention times and spectra of the chemicals in the sample with those of reference standards.

Antioxidant Assay

The antioxidant activity of the extracts was measured using the reducing power assay and 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay, as described by Omotayo et al. (2021), with some modifications. The assay concentrations for all the crude extracts and flavonoid for the reducing power and DPPH assays were 10 µg/mL, 20 µg/mL, 40 µg/mL, 80 µg/mL and 160 µg/mL. The reducing activities of all the extracts were illustrated by the increase in absorbance in line with the increased concentration of the samples. The DPPH scavenging activity was expressed as the percentage inhibition extrapolated using the formula:

$$Scavenging\ activity\ (\%) = \frac{\text{Control absorbance} - \text{Sample absorbance}}{\text{Control absorbance}} \times 100$$

The absorbance of the stock DPPH was the control. The IC_{50} was deduced from the graph of the concentration of extracts against the corresponding percentage scavenging activity.

Cytotoxicity Assay (Brine Shrimp Lethality Assay)

Sample preparation

Samples were prepared by dissolving 10 mg of each crude extract in 10 mL of 5% DMSO to give the stock solution. Five different concentrations, 10 µg/mL, 20 µg/

Table 1. Percentage yield of the *L. egregia* extracts and crude flavonoid

Extract	Extract Colour	Yield (%)
LEHe	Reddish brown solid	0.86
LEEa	Dark brown solid	1.42
LEEt	Dark brown solid	3.32
LEF*	Brown solid	9.7 ± 0.01

*% mean of the three experiments ± SD; LEHe: *L. egregia* crude n-hexane extract; LEEa: *L. egregia* crude ethyl acetate extract; LEEt: *L. egregia* crude ethanol extract; LEF: *L. egregia* crude flavonoid

Table 2. Phytochemical contents of the *L. egregia* extracts

	***L. egregia* extracts**		
Phytochemical	**LEHe**	**LEEa**	**LEEt**
Alkaloids	+	+	-
Flavonoids	+	+	+
Tannins	+	+	+
Saponins	+	-	+
Anthraquinones	+	+	+
Terpenoids	+	+	+
Cardiac glycosides	-	-	-
Steroids	+	-	+
Reducing sugars	+	-	+

Key: + = detected; - = not detected; LEHe: *L. egregia* crude n-hexane extract; LEEa: *L. egregia* crude ethyl acetate extract; LEEt: *L. egregia* crude ethanol extract.

mL, 40 µg/mL, 80 µg/mL, and 160 µg/mL, were prepared in triplicate by serial dilutions from the stock solution of the crude plant extract and the crude flavonoid. Each concentration was made up to 10 mL with seawater and a control was prepared using only DMSO.

Hatching the shrimp

Artemia salina leach (brine shrimp eggs) was purchased and used as the test organism. The brine shrimp eggs were hatched in a shallow rectangular dish (150 mm x 5 mm) filled with seawater, as described by Vanhaecke et al. (1981). The shrimp eggs (ca 50 mg) were sprinkled into the rectangular dish, which was covered and well illuminated externally. After 24 h the phototropic nauplii were collected using Pasteur pipettes from the illuminated dish (Vanhaecke et al., 1981). Ten brine shrimps (nauplii) larvae were transferred to each of the prepared vials of different concentrations using a dropper with a long tip, and the total volume in the vials was adjusted to 10 mL with seawater. To facilitate the easy transfer, the nauplii were counted under a magnifying lens (3x) in the stem of the dropper against an illuminated background. The vials were maintained under illumination. The shrimps that survived were counted after 24 h. The total death and percentage mortality at each dose level and control were determined (Vanhaecke et al., 1981). The LC_{50} was determined from the graph of logarithm to base ten of concentration against percentage mortality.

Statistical analysis

All the bioassays were conducted three times and data were reported as mean ± standard deviation of three values. Results were analyzed using one-way analysis of variance (ANOVA). Student T-test was used to compare the mean and all data were considered statistically significant at $p<0.05$. All the graphs were drawn using microsoft excel 2020.

RESULTS AND DISCUSSION

***L. egregia* Stem Bark Extracts**

The colour of the LEHe, LEEa, LEEt and LEF crude extracts and the percentage yields were summarized in Table 1. The mean percentage yield of the crude flavonoid was 9.70 ± 0.01.

Phytochemical Screening

Phytochemical screenings of the extracts were performed using standard methods, with the results summarized in Table 2. Flavonoids, tannins, anthraquinones, and terpenoids were detected in all the extracts, while alkaloids were detected in the n-hexane and ethyl acetate extracts. Saponins, steroids and reducing sugar were also detected in the n-hexane and ethanol extracts.

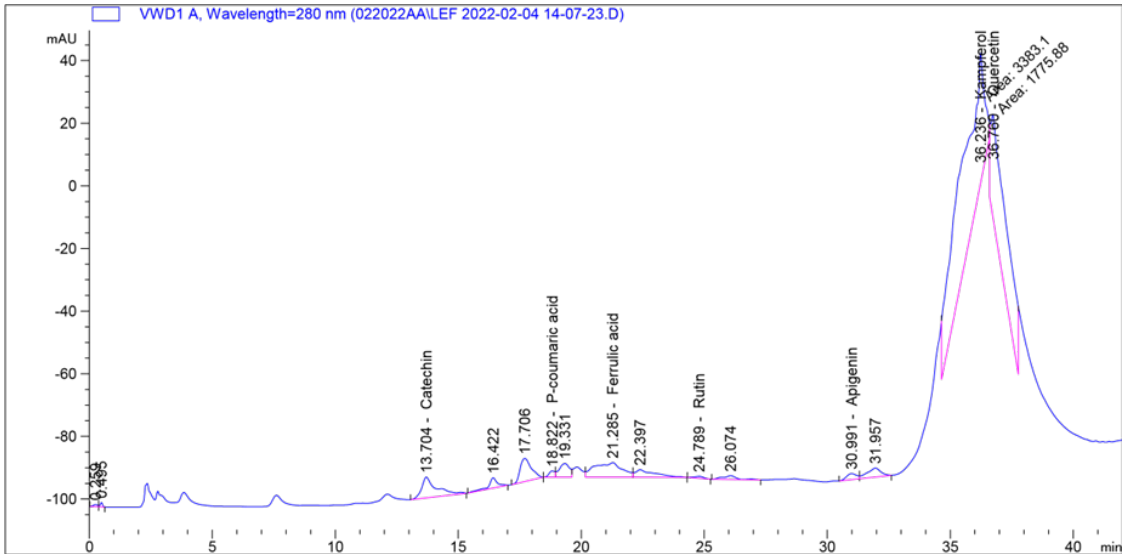

Figure 1. HPLC chromatogram of the crude flavonoid of *L. egregia* stem bark

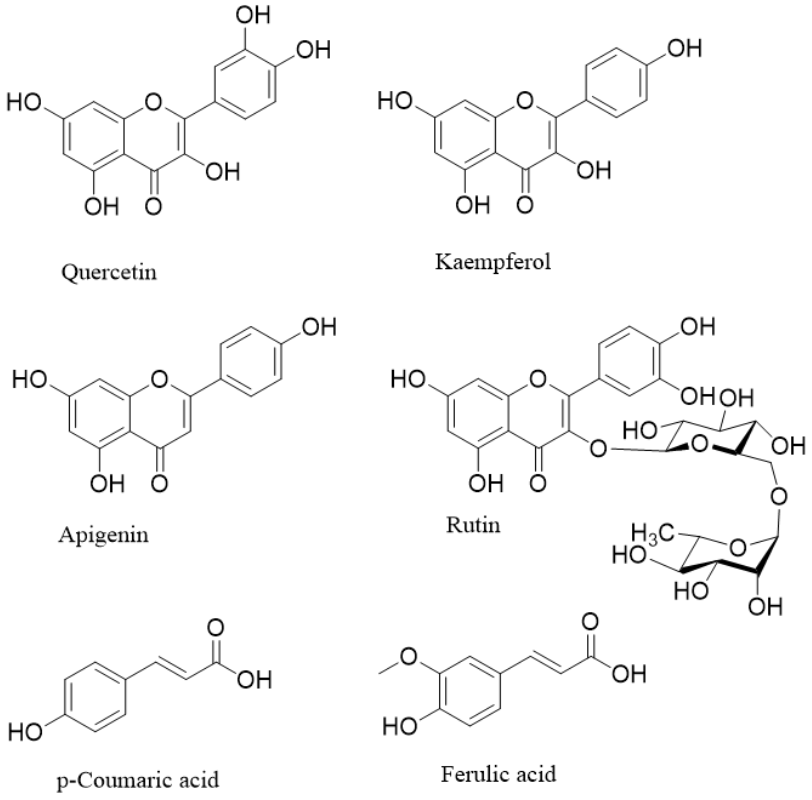

Figure 2. The chemical structures of compounds identified from the crude flavonoid of *L. egregia* stem bark

High-Performance Liquid Chromatography Profile of Crude Flavonoid

The high-performance liquid chromatography profile of crude flavonoid detected the presence of catechin, p-coumaric acid, ferulic acid, rutin, apigenin, kaempferol and quercetin, along with several other undetected flavonoids (Figures 1 and 2).

Antioxidant Activity

The antioxidant activities of the plant extracts were assessed using reducing power assay and DPPH scavenging activity. The results indicate a significant dose-dependent reducing property and DPPH scavenging activities by all the crude extracts.

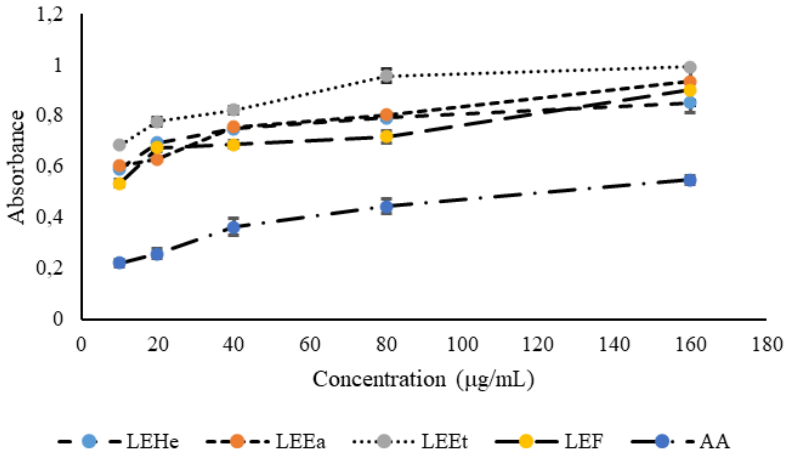

Figure 3. Reducing power of the various extracts of *L. egregia* stem bark

Values are represented as mean ± SD of the three experiments. LEH, LEEa and LEF are not significantly different ($p>0.05$); AA is significantly different from others ($p<0.05$).
LEHe: *L. egregia* crude n-hexane extract; LEEa: *L. egregia* crude ethyl acetate extract; LEEt: *L. egregia* crude ethanol extract; AA: Ascorbic acid.

Table 3. Flavonoids identified from LEF

Retention time (min)	Flavonoid	Amount (ppm)
13.704	Catechin	6.99283
18.822	p-Coumaric acid	1.11333×10^{-1}
21.285	Ferulic acid	3.96432
22.397	Unknown	117.89650
24.789	Rutin	7.89171×10^{-2}
30. 991	Apigenin	3.62114
31.957	Unknown	98.66263
36.236	Kaempferol	197.35148
36.760	Quercetin	96.44073

Reducing power assay is one of the tools used for assessing the antioxidant capacity of natural products based on their ability to reduce ferric ions to ferrous ions. The increase in absorbance value with an increase in concentration shows that the plant extract had good reducing properties (Xiao et al., 2020). In our study, all the extracts indicate significantly higher reducing activity than ascorbic acid, a standard antioxidant compound, with the highest reducing activity recorded in the LEEt (Figure 3). The extracts LEH, LEEa and LEF showed a comparable but not significant ($p>0.05$) reducing activities.

The synthetic-free radical, DPPH (1, 1-diphenyl-2-picrylhydrazyl), is used by researchers across the world to screen for plants with *in-vitro* antioxidant activity (Omotayo, 2021). The compounds or extracts with antioxidant activities show reduced absorbance values when compared to the methanolic or ethanolic solutions of DPPH (Omotayo, 2021). The results obtained in this study suggest that all the extracts studied possess antioxidant activities; however, the highest DPPH scavenging activity was recorded in both LEEa and LEF extracts, as demonstrated by their low IC_{50} values. The antioxidant activities of LEEa and LEF are not significantly different ($p>0.05$), showing comparable IC_{50} values of 22.98 ± 0.07 µg/mL and 22.48 ± 1.02 µg/mL respectively (Figure 4).

Phenolic compounds are natural antioxidant compounds comprising phenolic acids and flavonoids. These compounds act as radical terminators by donating electrons or hydrogen to radicals and breaking or altering the initiation of lipid oxidation (Mutha et al., 2021). From our results, the flavonoids detected from the LEF were catechin, p-coumaric acid, rutin, apigenin, quercetin and kaempferol together with two unknown flavonoids (Figures 1-2, Table 3). The most abundant flavonoid in *L. egregia* was kaempferol (197.35 ppm), while the least abundant was rutin (7.89×10^{-2} ppm) (Figures 1-2, Table 3).

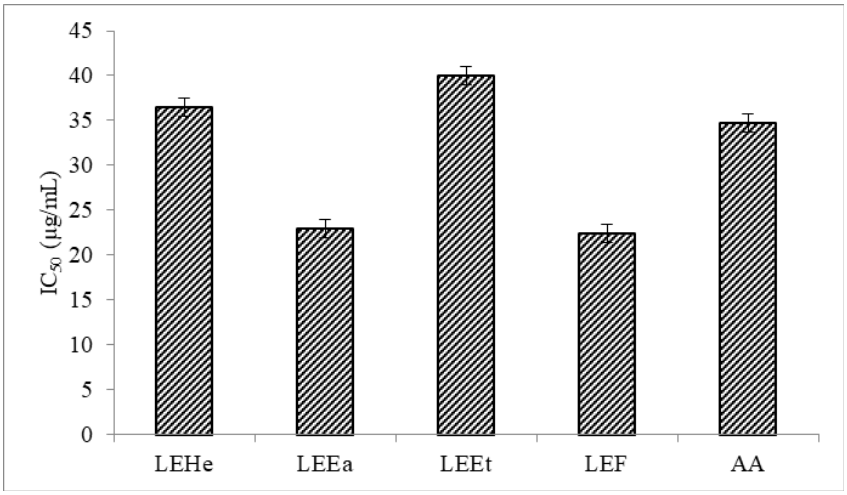

Figure 4. IC_{50} of *L. egregia* extracts and the ascorbic acid for the DPPH scavenging activity

Values are represented as mean ± SD of three experiments. LEEa and LEF are not significantly different (p>0.05).
LEHe: *L. egregia* crude n-hexane extract; LEEa: *L. egregia* crude ethyl acetate extract; LEEt: *L. egregia* crude ethanol extract; AA: Ascorbic acid

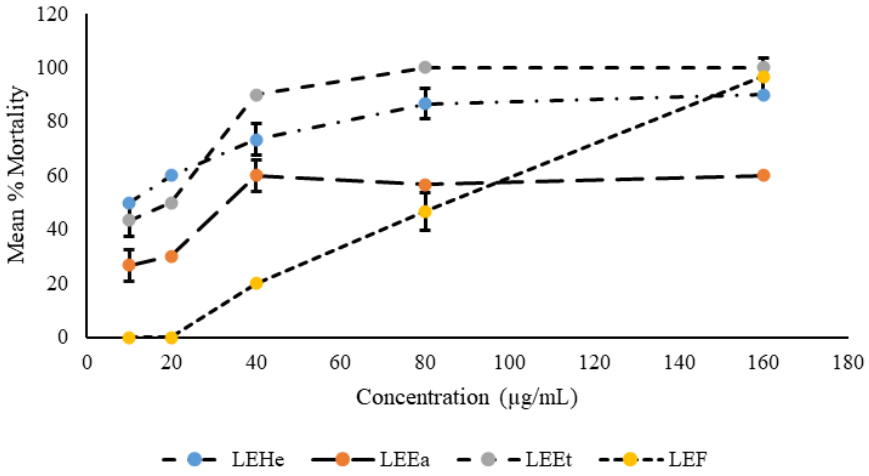

Figure 5. Mean percentage mortality of *L. egregia* crude extracts and flavonoids

Values are represented as mean ± SD of the three experiments. LEHe and LEEt are not insignificantly different (p>0.05); LEHe and LEF are significantly different(p<0.05); LEEa, LEF and LEEt are significantly different (p<0.05).
LEHe: *L. egregia* crude n-hexane extract; LEEa: *L. egregia* crude ethyl acetate extract; LEEt: *L. egregia* crude ethanol extract; AA: Ascorbic acid.

Flavonoid compounds and phenolics have been reported to have great benefits for the health of the heart, brain and blood (Bae et al., 2020; Ganeshpurkar & Saluja, 2017). Catechins are polyphenolic compounds that are reported to be beneficial for the human heart and brain and have anti-cancer properties (Bae et al., 2020). p-Coumaric acid has been reported to possess anti-tumor, anti-microbial, anti-inflammatory, and antioxidant properties and is used in the cosmetic industry as a component of products because of its anti-melanogenic effects (Boo, 2020; Taofiq et al., 2017). Rutin is reported to have anti-inflammatory properties to assist in blood circulation, prevent blood clotting, manage cholesterol, assist the human body to utilizing vitamin C, and helping in the production of collagen (Ganeshpurkar & Saluja, 2017). In addition, apigenin is associated with muscle relaxant properties and also linked to the management of Alzheimer's disease (Salehi et al., 2019). Quercetin and kaempferol have been reported to exhibit anti-inflammatory, antioxidant and cardioprotective properties (Espley et al., 2014; Dabeek & Marra, 2019). The antioxidant activities of the *L. egregia* stem bark extracts might be associated with these flavonoids and phenolic compound contents.

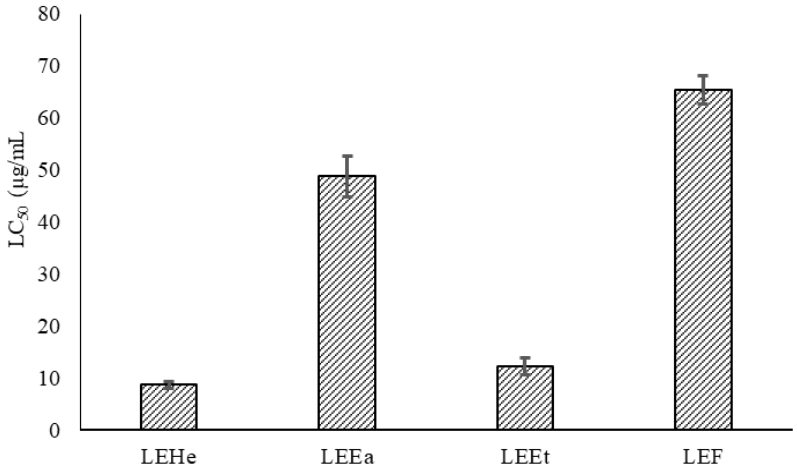

Figure 6. LC_{50} values of *L. egregia* stem bark extracts

Values are represented as mean ± SD of the three experiments.
LEHe: *L. egregia* crude n-hexane extract; LEEa: *L. egregia* crude ethyl acetate extract; LEEt: *L. egregia* crude ethanol extract; LEF: *L. egregia* crude flavonoid

Cytotoxic Activity

The brine shrimp lethality bioassay is a simple, inexpensive and rapid cytotoxicity assay of plants' bioactive compounds based on their ability to kill *Artemia salina* (brine shrimp). It is widely used to evaluate the toxicity of substances such as heavy metals, pesticides and medicines, especially natural products serving as a preliminary toxicity screening for the future experiments on a mammalian animal models (Sarah et al., 2017). From this study, the mean percentage mortality of brine shrimp increased as the concentration of *L. egregia* extracts increased, suggesting a cytotoxic activity (Figure 5). Sasikumar and Ghosh (2019) reported that high potential cytotoxic activity in the brine shrimp lethality assay is considered effective against tumor cell lines when LC_{50} is lower than 100 µg/mL. In this study, all the crude extracts showed substantial but different ($p<0.05$) cytotoxic effects when compared with each other. The highest cytotoxic activity was recorded in the n-hexane extract, with an LC_{50} of 8.70 ± 0.58 µg/mL (Figure 6). The cytotoxic activity of the extracts is in the order LEHe>LEEt>LEEA>LEF. The trend in cytotoxic activity also suggests that the cytotoxic agent in *L. egregia* might be due to the presence of flavonoids or other phytochemicals.

CONCLUSION

In conclusion, *L. egregia* stem bark extracts display antioxidant and cytotoxic activities and could be a source of new anticancer drugs. The results suggest that the cytotoxic activities shown by *L. egregia* stem bark extracts might be resulted by flavonoids or other phytochemicals. Hence, there is a need for further studies to determine other cytotoxic agents present in the plant apart from flavonoids.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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