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Table of Content

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Full Length Research Paper

Fungistatic activity of essential oils for the control of bipolaris leaf spot in maize

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Research institutions have emphasized alternative disease control agents because they represent a great option with beneficial effects on human and animal health and ecosystem balance. Despite the studies already done on botanical fungicides, their effective use requires knowledge about the applicability of natural products to different production systems. The main objective of this study is to evaluate the efficacy of essential oils of *Ocimum pupuraceus* L., *Cymbopogon nardus* (L.) Rendle, *Cymbopogon citratus* (DC.) Stapf, and *Lippia sidoides* Cham. on the inhibition of mycelial growth and germination of conidia of *B. maydis*. The other objectives of this study were to perform gas chromatographic and phytotoxicity analyses, and test the control of bipolaris leaf spot using essential oils of *L. sidoides* applied as a preventive and curative agent. Among the treatments studied, the essential oil of *L. sidoides* is effective in inhibiting mycelial growth and conidial germination at the concentrations of 5 and 1%, respectively. The main constituent of the oil is thymol (92.68%). The concentration range 0.75-3% of *L. sidoides* essential oil is phytotoxic to maize plants. Lower values of the area under the progress curve of bipolaris leaf spot are observed at concentrations 0.1-0.5%, when the essential oil was applied as a preventive agent prior to the colonization of plant tissues by the pathogen. The application of the oil as a curative to plants with the disease also shows efficacy at the concentration 0.1%, reducing the severity by more than 54%. These results demonstrate the potential effects of *L. sidoides* essential oil on preventive and curative control of bipolaris leaf spot in maize and mycelial growth.

Key words: Medicinal plants, alternative control, phytopathogens, *Zea mays*, *Bipolaris maydis*, *Lippia sidoides*.

INTRODUCTION

Brazil is classified as the third largest producer and the second largest exporter of Maize (*Zea mays* L.) in the world. Brazil is relevantly involved with the agricultural scenario (Peixoto, 2014; USDA, 2016; FAO, 2018);

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however, in Brazil, several factors have hindered maize production, among them, diseases. Diseases significantly affect the plant's productive potential (Conab, 2016), and may reduce the productive potential in whole or in part (Manfroi et al., 2016).

Maize diseases can be grouped according to plant organ and the resulting symptoms. They can be divided into leaf diseases, stem and root rot, and spike and grain rot (Ferrari and Possamai, 2015). In disease monitoring, mappings conducted by the agency Embrapa Milho and Sorgo, a high severity of bipolaris leaf spot has been detected in some Brazilian states, such as Rondônia, Mato Grosso, Goiás and Tocantins (Costa et al., 2014).

Because most cultivars are susceptible to bipolaris leaf spot, fungicides have been widely used, especially in tropical Brazilian areas, where the disease meets favorable conditions for its development (hot and humid climate). The indiscriminate use of agrochemicals has raised the resistance to phytopathogens and negatively affected the environment and human health through contamination of food, soil microbiota, water and the ecosystem. Seeking to reduce the problems generated by agrochemicals, natural products have been considered an alternative for disease control (Benvenuti, 2012; Ootani et al., 2013). Currently, research institutions have been emphasizing alternative disease control agents as they are an option with beneficial effects on human and animal health. They also promote ecosystem equilibrium. There are numerous alternatives to conventional chemical control of phytopathogens as a way to reduce the damages associated with their use.

One alternative is to use agents capable of inducing resistance in treated plants, thus activating their own natural defense mechanisms (Walters et al., 2013). Several authors have demonstrated the efficacy of some essential oils applied at certain concentrations for disease control (Diniz et al., 2008; Dalcin et al., 2017).

However, the issue is complex since leaf spot bipolaris is a disease that causes great damage to the corn crop due to the partial defoliation of the plants, and the current control of this disease is still made mainly by the use of fungicides and, thus, requires collective efforts by conducting studies for the purpose of creating knowledge and applicability of natural products to different production systems and patosystems.

Thus, the objective of this work is to evaluate the efficacy of essential oils for the control of leaf spot bipolaris in maize.

MATERIAL AND METHODS

Plant species

The plants used for the extraction of essential oil were *Ocimum pupuraceus* L. (dark opal basil) - Lamiaceae, *Cymbopogon nardus* (L.) Rendle (citronella) – Poaceae, *Cymbopogon citratus* (DC.) Stapf. (lemon grass) - Poaceae, and *Lippia sidoides* Cham. (rosemary-pepper) – verbenaceae.

The extraction of essential oils was carried out using hydro-distillation in a modified Clevenger equipment (Guimarães et al., 2008). 200 g of leaves of each test substance, dehydrated and chopped, were placed in a round bottom flask and covered with distilled water. The extraction time was two hours of boiling. Subsequently, the supernatant was collected using a micropipette, and stored in amber bottles covered with aluminum foil (protection from light), and then stored in a refrigerator at 4°C until the bioassays were conducted.

Isolation of *Bipolaris maydis*

The phytopathogen was obtained from maize plants presenting typical symptoms of bipolaris leaf spot. The isolation was performed on Petri dishes containing PDA culture medium (potato, dextrose, agar). The fungus was identified using optical microscopy according to the characterization of its vegetative and reproductive structures, which was made by consulting specialized literature (Barnett and Hunter, 1972; Ellis, 1971).

Inhibition of mycelial growth

The fungitoxic activity was evaluated *in vitro* using increasing concentrations of essential oils (1, 2, 3, 4 and 5%). The experiment was completely randomized in a factorial design of four replications and five evaluation periods (2, 4, 6, 8 and 10 days of incubation).

In order to evaluate the effects of essential oils on the mycelial growth of *B. maydis*, the methodology described by Seixa et al. (2008) and Ferreira et al. (2018) was used.

Chromatographic analyses of essential oils

Qualitative and quantitative analyses of the essential oils were performed using gas chromatography together with GC-MS mass spectrometry. The chromatograph used was the Shimadzu GC-210, equipped with a QP2010 Plus mass selective detector. The equipment was operated with the following setup: RTX-5MS fused silica capillary column (30 m x 0.25 mm x 0.25 µm film thickness), temperature programming in the column: 60-240°C (3°C/min), injector temperature: 220°C, carrier gas: Helium, and splitless injection with injected volume of 1 µl of a 1:1000 solution in hexane. For the mass spectrometer (MS), the following setup was used: Impact energy of 70 eV, and temperature of the ion source and the interface: 200°C. The spectra obtained were compared with the library database Nist and Wiley 229, and the retention index calculated for each constituent was compared with tabulated levels according to Adams (2007). The quantification of the contents of the compounds was expressed as percentage.

Phytotoxicity of essential oil in maize plants

The phytotoxicity test was performed with the most promising essential oil for inhibition of mycelial growth *in vitro*. The experimental design was completely randomized with four replications. The treatments consisted of one control (water) and nine oil concentrations. Maize seeds were sown in plastic pots containing soil manure, soil and commercial Plantmax® substrate at a ratio of 1:2:1. Each vase was sown with four seeds of the cultivar Tractor® because it is susceptible to the disease and widely cultivated in the region. Irrigation was performed daily by hand. Twenty days after sowing, different concentrations (0.01, 0.1, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5 and 3.0%) of the essential oil were applied. The solutions were prepared as described above for the *in vitro* assay. The application on the leaves was carried out using a spray

Table 1. Effects of the essential oil of *Lippia sidoides* at increasing concentrations on mycelial growth (mm) of *B. maydis*.

Concentration	Evaluation time (days)					Inhibition*	Regression equation	R^2
(%)	1	2	3	4	5	(%)		
T (water)	15.06±1.52	20.81±0.34	26.48±0.87	33.85±0.68	39.28±0.58	0	y = 6.148x + 8.652	0.99
1	7.41±1.01	11±1.78	15.87±1.73	19.83±2.08	24.95±0.20	36	y = 4.391x + 2.639	0.99
2	-	4.91±0.20	12.07±2.08	21.08±3.79	25.44±4.10	35	y = 6.705x − 7.415	0.98
3	-	5.1±0.24	7.51±0.46	15.56±1.86	20.17±1.09	48	y = 5.08x − 5.572	0.98
4	-	3.79±0.51	7.34±0.84	15.24±0.53	19.82±1.11	49	y = 5.109x − 6.089	0.98
5	-	-	-	-	-	100	-	-

(-): no growth, (*): evaluation considered until the tenth day of incubation (mean ± standard deviation).

(capacity of 500 ml) until the point of flowing from leaves. 24 h after the application, the evaluations were carried out using a scale adapted from Freitas et al. (2009) and Cogliatti et al. (2011), where; 0% = absence of phytotoxicity, 1-25% = mild leaf necrosis or mild plant chlorosis, 26-50% = moderate leaf necrosis or moderate plant chlorosis, 51-75% = high leaf necrosis or high plant chlorosis, 76-100% = wilting and plant dryness.

Inhibition of *Bipolaris maydis* conidial germination

This assay was conducted in a completely randomized design with three replicates. An 1 mL aliquot of the conidial suspension of *B. maydis* (10^4 conidia mL^{-1}) and another 1 mL conidia suspension at different concentrations (0.0, 0.05, 0.1, 0.25, 0.5 and 1%) of the essential oil containing tween 80 (2%) was applied to each container (small glass) (Balbi-Peña et al., 2006). They were incubated in a humid chamber with a 14 h photoperiod. 300 conidia were counted per treatment by observing germinated and not germinated conidia using an optical microscope (Aguiar et al., 2014). After this period, the percentage of germination of conidia inhibition was calculated according to an adapted methodology (Aguiar et al., 2014; Balbi-Peña et al., 2006).

Preventive and curative control of bipolaris leaf spot using the essential oil

From the data obtained in the *in vitro* and phytotoxicity bioassays, the tests of preventive and curative control of *B. maydis* leaf spot at different essential oil concentrations (0.01, 0.05, 0.1, 0.25 and 0.5%) and the control using only distilled water were conducted. The experiment was completely randomized with three replications. The solutions were prepared as described for the *in vitro* assay.

For the preventive control test, three completely healthy maize plants were used containing five leaves each, to which the essential oil solution was previously applied at the concentrations already described. The application was carried out using a spray until the point of flowing from leaves. After one hour of oil application, the maize plants were inoculated with 5 mL of 1 x 10^4 mL^{-1} of solution of *B. maydis* conidia. Then, the plants were transferred to the humid chamber for 48 h to provide adequate conditions for pathogen development.

After 48 h, five evaluations of disease severity were performed at a two-day interval adopting the score developed by Santos et al. (2005) as follow; 0: Healthy plant, 1: Less than 1% of diseased leaf area, 3: 1-5% of diseased leaf area, 5: 6-25% of diseased leaf area, 7: 26-50% of diseased leaf area, and 9: more than 50% of diseased leaf area. In the curative assay, maize plants were initially inoculated with 5 mL of 1 x 10^4 mL^{-1} of solution of *B. maydis*

conidia. Then, the plants were transferred to the humid chamber for 48 h to provide adequate conditions for pathogen development. After the appearance of the first lesions characteristic of the disease, the different concentrations of essential oil were applied. Five evaluations of disease severity were carried out after the application of the essential oil at intervals of two days using the grading scale previously described.

Statistical analysis

The results were expressed as mean ± standard deviation for the *in vitro* mycorrhizal growth inhibition and conidial germination assays. The *in vivo* curative and preventive control was subjected to linear regression. The area under the disease progression curve (AUDPC) was calculated according to Schneider et al. (1976). Regression equations were adjusted using the Excel® software.

RESULTS

Inhibition of mycelial growth

The essential oils of *O. pupuraceus*, *C. nardus* and *C. citratus* showed no inhibitory effects on the mycelial growth of the pathogen *in vitro*. Only the essential oil of *L. sidoides* had a fungitoxic effect at the concentrations tested as shown in Table 1 and Figure 1. There was inhibition of mycelial growth at the concentrations 2-4% of essential oil until two days of incubation in relation to the control (water). Thus, these same concentrations initially inhibited the mycelial growth of the pathogen, and maintained a decelerated growth in relation to the control (water) until the tenth day of evaluation. The 5% concentration of the oil completely inhibited the mycelial growth of *B. maydis* at the evaluated times.

Chemical constituents of *Lippia sidoides* essential oil

The chromatographic analysis of *L. sidoides* essential oil revealed, in quality and quantity, its chemical constituents. Thymol (92.6%) was the main component, followed by caryophyllene (2.2%) and ρ-cymene (1.1%), among other constituents that form this compound as indicated in
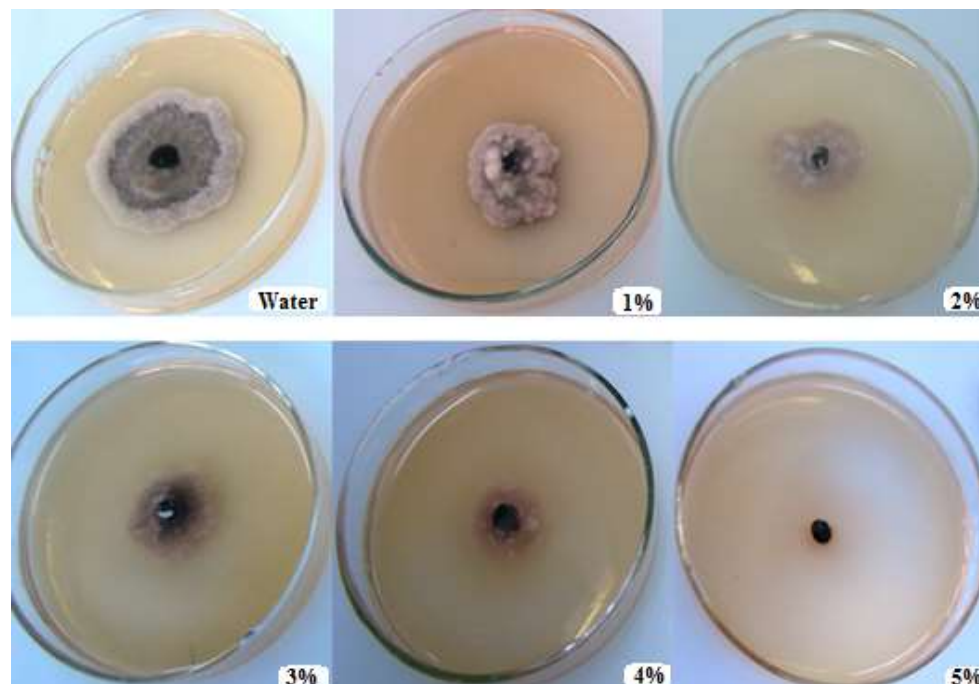
Figure 1. Inhibition of mycelial growth of *Bipolaris maydis* subjected to different concentrations of the essential oil of *Lippia sidoides* six days after the incubation.

Table 2. Chemical constituents of *Lippia sidoides* essential oil identified by GC/MS and their respective contents expressed as percentage.

[a]NC	Compound	[b]RT	[c]CRI	(%)
1	α-thujeno	5.915	927	0.051
2	α-terpinene	8.680	1018	0.091
3	ρ-cimene	8.944	1025	1.162
4	γ-terpinene	10.176	1058	0.250
5	cis-sabinene hidrate	10.656	1071	0.102
6	4-terpineol	15.19	1182	0.453
7	Thymol methyl ether	17.264	1230	0.430
8	Thymol	20.075	1294	92.684
9	(E)-caryophyllene	25.369	1419	2.235
10	α-humulene	26.849	1456	0.134
11	caryophyllene	31.878	1582	0.617
	Total (%)	-	-	98.179

[a]NC = Number of compounds; [b]RT = Retention time; [c]CRI = Calculated retention index.

Table 2.

Phytotoxicity of *Lippia sidoides* essential oil to maize plants

Among the oils tested *in vitro*, only *L. sidoides* was efficient to inhibit mycelial growth of *B. maydis*, and thus was selected for the phytotoxicity test. This essential oil causes phytotoxicity from the concentration 0.75%. It causes burning or necrosis in 60% of the leaf area, besides yellowing or plant chlorosis. The concentration 3.0% causes a high degree of phytotoxicity, and irreversible lesions were verified in all plants, with burning in 96.6% of the leaf area. Because the concentration 0.5% did not cause any phytotoxicity to maize plants, it was chosen as the maximum dosage to be used in the preventive and curative control of bipolaris leaf spot.
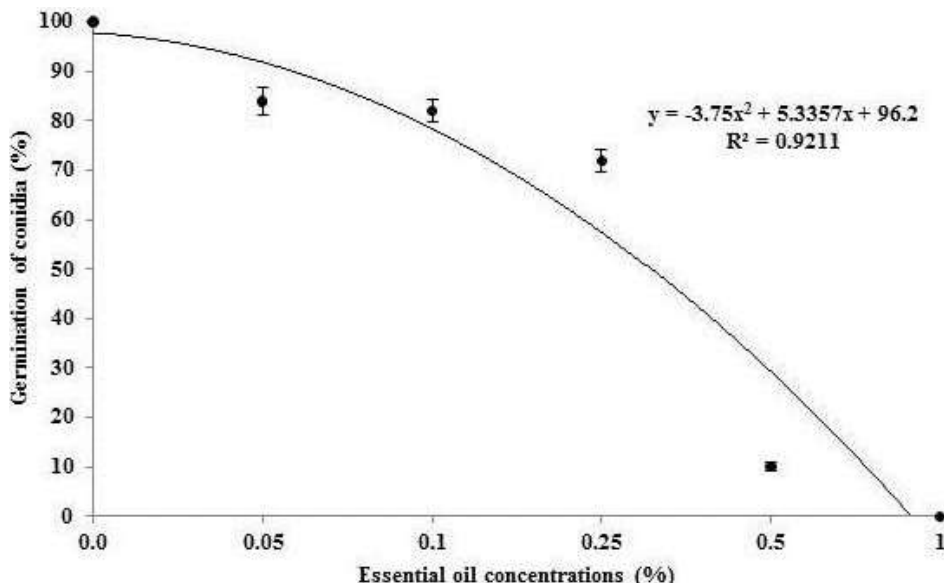
Figure 2. Inhibition of germination of conidia of *Bipolaris maydis* in function of increasing concentrations of *Lippia sidoides* essential oil (mean ± standard deviation).
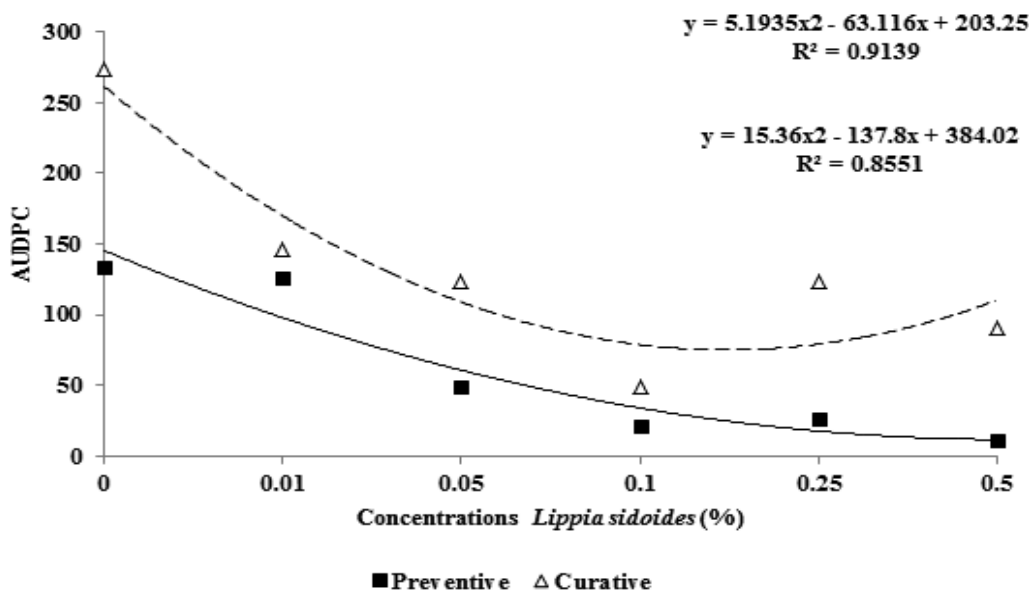
Figure 3. Area under the disease progression curve (AUDPC) of bipolaris leaf spot in maize leaves in function of increasing concentrations of essential oil of *Lippia sidoides* applied in a preventive and curative manner.

Inhibition of *Bipolaris maydis* conidial germination

Inhibition of conidial germination was proportional to the increase in concentrations as presented in Figure 2.

Considering the absolute control with 100% germination of conidia after 14 h of incubation, and comparing it with the germination of conidia subjected to the different concentrations of the essential oil of *L. sidoides*, the concentrations 0.05, 0.01 and 0.25% decreased the germination of *B. maydis* by 16, 18 and 28%, respectively as depicted in Figure 3. The concentration 0.5% inhibited 90% of conidial germination. At the concentration 1% of the essential oil of *L. sidoides*, there was total inhibition of conidia (100%), evidencing a great potential of pathogen inhibition by the oil. In spite of the efficiency and due to the phytotoxicity verified up to the oil dose 1%, doses at

the concentrations up to 0.5% were tested for the *in vivo* control of the disease.

Preventive and curative control of bipolaris leaf spot in maize

Concerning the preventive effects of *L. sidoides* essential oil on the progress of bipolaris leaf spot as indicated in Figure 3, the severity of the disease decreased from the concentration 0.05. A good control level was observed at the concentrations 0.1-0.25 when compared to the absolute control. At the highest concentration tested (0.5%), there was a decrease in disease severity up to 91% when compared to the absolute control.

Concerning the curative application on the presence of the disease, although it did not have the same effectiveness as the preventive control, there was a greater control level at the concentration 0.1%, with an 82% reduction in disease severity. This same concentration also demonstrated an efficacy in preventive application when there was an 84% reduction of leaf spot. The essential oil of *L. sidoides* presents a great potential as an alternative preventive and curative control that is, before or after the occurrence of pathogen infection in maize leaves.

DISCUSSION

In relation to the benefits of using the essential oil of *L. sidoides* in maize plants, emphasizing the control of bipolaris leaf spot, we proved that there is a technical feasibility. However, its active principles are chemical compounds that may cause phytotoxicity or irreversible damage to plant leaves. Thus, recommendation of use should only be made after tests to verify the sensitivity of the plant and the maximum oil concentration, taking into account the cultivar. The definition of doses represents an economically interesting practice for researchers and for future use by farmers.

The effects of *L. sidoides* essential oil on bipolaris leaf spot result from the effects of both the main compound (thymol) and the interaction of minor compounds. Moreira et al. (2011) isolated two antifungal proteins from flowers of *L. solides* that were able to inhibit the development of *Botrytis cinera*. Ferreira et al. (2018), testing the fungitoxic activity of the essential oil of *L. sidoides* on *Curvularia lunata*, found a total pathogen inhibition at the concentration 50 mg/mL. Oliveira et al. (2008) evaluated the effects of essential oils of plants of the genus Lippia on contaminating fungi during plant propagation. The authors showed that the essential oil of *L. sidoides* was effective in inhibiting the mycelial growth of all evaluated fungi (*Aspergillus niger*, *Penicillium* sp., *Fusarium* sp., and *Fusarium oxysporum*).

Studies have shown the potential of essential oils for the control of phytopathogens by both direct effects on pathogen structures and induction of phytoalexins (Schwan-Estrada and Stangarlin, 2001). Guimarães et al. (2011), evaluating the fungitoxic activity of the essential oil of *C. citratus* and citral, verified that both the oil and its components showed activity against phytopathogens. *Alternaria alternata* and *Bipolaris* sp. were the most sensitive, and *Fusarium oxyporium* was the most resistant to the action of these compounds. Seixas et al. (2011) studied the control of *Fusarium subglutinans* using essential oil of *C. nardus* L. and the compound citronellal, and found a greater inhibitory effect by citronellal.

In this work, the other oils tested did not present any inhibitory effect on *B. maydis.* These results should not be extended to other phytopathogens, which may have a different sensitivity or a greater capacity to metabolize chemical constituents. Different from the results of this work, Gonçalves et al. (2015) analyzed the chemical characterization of *L. sidoides* oil and verified the presence of carvacrol (33.2%) and 1,8-cineol (24.4%) as the main components. However, Ferreira et al. (2018) found results similar to those of this study. The authors demonstrated that primary constituents of *L. sidoides* oil included thymol (92.6%), (E) - cariophilene (2.2%), and ρ-cymene (1.1%). It is worth mentioning that the composition of essential oils may vary according to the location and time of collection, plant age, used substrates, fertilization etc. These requirements may increase or decrease the metabolites present in plants (Pina et al., 2018; Ribeiro et al., 2018).

The concentrations tested for inhibition of conidial germination of *B. maydis* were efficient; a 100% inhibition occurred at the dose 1%. Ferreira et al. (2018) explained that it is necessary to understand the mechanisms of action of essential oils against pathogenic fungi. According to Solórzano-Santos et al., (2012), the potentiality of essential oils can be attributed to their hydrophobicity, which allows them to partition with cell membrane lipids and mitochondria of bacteria, causing disturbance in cellular structures, increasing membrane permeability, which leads to the leakage of molecules essential to survival and consequently kills the bacteria (Miranda et al., 2016).

For fungi, according to Juven et al. (1994), there is a release of cell contents through changes in the permeability of fungal membranes. Therefore, the presence of the -OH group in the molecular structure, a major compound of the essential oil of *L. sidoides*, has the ability to bind to amine and hydroxylamine groups of proteins present in fungal cell membranes.

The effects of the essential oil on bipolaris leaf spot in maize plants is promising, yet preventive applications are better than curative applications. In the scientific literature, there are few reports on the effects of essential oils for the control of bipolaris leaf spot. Oil solutions of *L. sidoides* are preventively applied, forming a protective layer on maize leaves and inhibiting the germination of

the conidial germinative tube. Ferreira et al. (2018) observed the same effect regarding the preventive effect of the treatment with essential oil of *Lippia*. The authors showed that the compounds of the substance might have a greater contact with *C. lunata* conidia on the imminence of germ-tube formation and the subsequent development of hyphae. Veloso (2016) concluded that the essential oil of *Morinda citrifolia* has fungitoxic properties, inhibiting the mycelial growth of *B. maydis* and *Exserohilum turcicum*, as well as the decreasing the severity of the disease.

Conclusions

In view of the results, the inhibitory effects of the oil on mycelial growth, as well as on the germination of conidia of *B. maydis*, are confirmed. It is possible to state that the essential oil of *L. sidoides* is a promising antifungal agent due to its mixture of active components for a preventive and curative control of the progress of bipolaris leaf spot.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Journal of Medicinal Plants Research

Full Length Research Paper

Evaluation of active phytochemical constituents linked to the analgesic and anti-inflammatory property of *Cassia singueana* Del. root bark

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***Cassia singueana* is used in ethnomedicine for the management of pain and other related ailments. This study aimed to investigate the phytochemical constituents of the plant's root bark linked to the therapeutics claims of its analgesic and anti-inflammatory activities. The plant material was obtained and identified, and its phytochemical fingerprint profile was established using GC-MS technique. The plant's root bark was dried and ground into powder. The powdered material was extracted using 70% ethanol to obtain the crude extract (CECs). Another portion of the material (200 g) was extracted sequentially using soxhlet apparatus to obtain hexane (HFCs), chloroform (CFCs) and methanol MECs) fractions, respectively. The acute toxicity test (2000 mg/kg) and preliminary analgesic and anti-inflammatory activity (100 mg/kg, p.o.) of the extract and fractions were performed in mice using acetic acid-induced writhing and formalin respectively. The most active hexane fraction (HFCs) was subjected to HPLC analysis to investigate the active phytochemical constituents. They were then evaluated for analgesic and anti-inflammatory efficacy at 25, 100 and 400 mg/kg, p.o. using formalin (50 µl) induced pain in rats. Results showed that the root bark of *C. singueana* exhibited analgesic and anti-inflammatory activity against the pain induced model. HPLC analysis revealed that the phytochemical constituents linked to these activities are gallic acid, caffeic acid, luteolin and ferulic acid.**

Key words: *Cassia singueana*, analgesic, anti-inflammatory, gallic acid, caffeic acid, luteolin, ferulic acid.

INTRODUCTION

Cassia singueana Delile -- in Caill. Voy. Meroe, Bot. IV. 27 (Cent. Pl. Afr. 28). (IK), family: Caesalpiniceae (IPNI, 2019) is a woody annual herb or shrubs with small yellow flowers that can grow up to 1.2 and 1.5 m high. It is

widespread in India and tropical Africa, and is used traditionally for treatment of pain and body ache associated with acute malaria attack (Adzu et al., 2003). The plant is also used against stomach spasm, and biological studies showed that it exhibited antipyretic, antiplasmodial activities and anti-ulcer activities (Adzu et al., 2003; Ibrahim et al., 2013). Such medicinal plants had served as agent for scientific exploration, and yielded products that are being applied in healthcare. Most compounds of these plants originally used in ethnomedicines provided direct source of phytomedicines or served as research tools and starting points for the synthesis of bioactive analogues (De Smet, 1998). Despite some advantages in the medical use of plant extracts over isolated entities, there is a need to identify the chemical constituents linked to the observed beneficial effects. The objective of this study was to evaluate the phytochemical component of *C. singueana* root bark linked to its therapeutic benefit in treating pain and related ailments. The plant material was sequentially extracted and fractions obtained were evaluated using analgesic and anti-inflammation activity models in mice and rats. The phytochemical fingerprint of the plant as well as its safety profiles were also evaluated.

MATERIALS AND METHODS

Preparation of test agents

The plant material was collected within the premises of the Adamawa State University, Mubi (10°16′N 13°16′E), Nigeria in December, 2017. The rootbark was cleaned, washed and dried under shade. The dried material was ground into powder using pestle and mortar, stored in air-tight containers, and 200 g of the powdered material was sequentially extracted using soxhlet extractor (Gallemkamp, England) with hexane, chloroform and methanol to obtain HFCs, CFCs, MFCs, respectively. Another portion of the powdered material (200 g) was macerated in 70% ethanol (Malone, 1983) for 7 days with occasional shaking to obtain the crude extract (CECs). The mixture was then filtered, and the solvents were evaporated in vacuum at 40°C using a rotary evaporator. The resultant sample in each case was weighed, transferred to sample bottle tubes and stored at 4°C.

Animals

Swiss albino mice (20-28 g) and Wistar rats (180-260 g) of both sexes obtained from the Animal Facility Centre, NIPRD, were used for the study. The animals were kept in plastic cages at room temperature and moisture, under naturally illuminated environment. They were fed with standard feeds and have access to tap water *ad libitum*. The animals were used in accordance with standard procedures of NIPRD Animal Care and Ethics (NIPRD/SOPs/05:3:05).

Hydro-distillation

Fresh sample of *C. singueana* root bark was used. The root bark (300 g) was chopped into pieces, immersed in water, and then boiled using a Clevenger type apparatus continuously for 2 h. The vapors of the volatile components were carried by the steam to a condenser as droplets onto 1 mL HPLC grade hexane layer at the front of the receiver. This was then separated by decantation.

Chemical fingerprinting using gas chromatography-mass spectrometric (GC-MS) analysis

The recovered essential oil was analyzed using GC-MS (Shimadzu QP-2010 GC) equipped with QP-2010 mass selective detector and Shimadzu GCMS solution data system. The sample was diluted in hexane (1/100, v/v) of which 1.0 µl was injected into the instrument using auto sampler. The analysis was performed in triplicate. The retention indices of all the volatile constituents were calculated using a homologous series of n-alkane under the same conditions of analysis. Individual constituents were identified by comparing their retention time with literature compounds (Adams, 2017).

High performance liquid chromatography (HPLC) analysis

The bioactive constituents of the CECs and HFCs were analysed by HPLC (Shimadzu Corporation, Kyoto Japan) technique using chromatographic conditions earlier described (Adzu et al., 2014). Flavonoids and phenolic acid standards that include apigenin, rutin, quercetin, caffeic acid, ferulic acid and morin were employed for the identification of the phytoconstituents of the extracts by comparing their retention time under similar experimental conditions (Krishna Murthy and Manohar, 2014).

Acute toxicity testing

The Limit Test (OECD, 2008) was adapted for the study with little modification. Three male and female mice each were treated with 2000 mg/kg of the extract. One male and female mouse served as control and received 10 ml/kg of water. All the mice were closely observed for signs of toxicity at 15 min, 30 min, 1, 2, and 4 h, and then once daily for 14 days.

Acetic-acid-induced writhing response

The writhing test was used. Mice were separated into six groups of five mice each. Group 1 served as control and was administered vehicle. Groups 2 to 5 received CECs, HFCs, CFCs and MFCs, respectively at 100 mg/kg, p.o., while Group 6 was administered acetylsalicylic acid (ASA, 100 mg/kg, p.o.) Thirty minutes later, writhe was induced by 0.7% acetic acid (10 ml/kg, i.p.). A latency period of 5 min was given to each mouse, and the writhing's were cumulatively counted for 15 min.

Anti-inflammatory activity

Thirty mice were weighed and randomized into six groups (*n* = 5). Group 1 was given 10 ml/kg of distilled water (negative control), Groups 2 to 5 were treated with CECs, HFCs, CFCs and MFCs (100 mg/kg, p.o.) and Group 6 was treated with 100 mg/kg, p.o. of ASA. Inflammation was induced by injecting 50 µl of 2.5% solution of formalin into the sub-plantar surface of the right hind paw 30 min after treatment. The mice paw volume was measured with a Letica (Spain) digital plethysmometer (LE 7500). Readings were taken before, 5 min later, and at intervals of 20 min for a total of 120 min.

Formalin test

Rats were grouped into six (*n* = 5) and pretreated orally with water (10 ml/kg), HFCs (25, 100 and 400 mg/kg), acetylsalicylic acid

Table 1. The GC-MS chemical fingerprint profile of *Cassia singueana.* Twenty two peaks were detected as follows.

Peak	MF	Chemical name	MW	RT	Area %
1	CH_2NHCH_2	Aziridine	43.069	3.529	0.35
2	$C_3H_4O_2$	Pyruvaldehyde	72.63	3.638	0.46
3	C_5H_8O	4-penten-2-one	84.118	6.629	0.51
4	C_4H_9NO	Nitroisopropane	87.122	6.780	0.75
5	$C_5H_8O_2$	Vinyl propionate	100.117	6.909	2.38
6	$C_9H_{11}NO$	4- Ethylphenylformamide	147.193	12.348	0.38
7	$C_6H_5NO_2$	Nicotinic acid, 2-tetrahydrofurylmethyl	124.117	12.546	1.91
8	$C_6H_{12}N_2O_2$	Acetylmethylamide	144.174	14.991	0.46
9	$C_{16}H_{32}O_2$	Palmitic acid	256.43	17.353	9.91
10	$C_{11}H_{22}O_3$	Methyl 2-hydroxydecanoate	202.29	18.926	1.65
11	$C_6H_{12}O$	Cyclobutylethanone	100.161	19.135	0.59
12	$C_5H_9O_3$	Ethyl alpha-hydroxyisobutyrate	118.13	19.273	1.29
13	$C_8H_{16}O$	3-Methyl-4-heptanone	128.215	19.388	0.84
14	$C_{15}H_{26}O$	1-Tridecyn-4-ol	222.372	19.747	1.42
15	$C_{13}H_{28}$	5-Methyl-5-propylnonane	184.367	20.093	1.63
16	$C_{21}H_{44}$	Henicosane	296.582	20.446	16.72
17	$C_{18}H_{35}NO$	9-Octadecenamide	281.484	20.568	8.93
18	$C_{10}H_{20}O$	3,5-Dimethyl-4-octanone	156.269	20.747	4.86
19	$C_{10}H_{20}O$	1,2-Dihydrolinalool	156.269	20.992	28.34
20	C_9H_{20}	2,4,4-Trimethylhexane	128.26	21.419	7.25
21	$C_8H_{14}O_2$	Allyl isovalerate	142.198	22.145	3.64
22	$C_8H_{14}O_2$	Allyl pentanoate	142.198	22.267	5.69

MF; Molecular formula; MW: Molecular weight (g/mol.); RT: Retention time (min).

(ASA,100 mg/kg) for anti-inflammation or morphine (Mor, 10 mg/kg) for pain relief evaluation. One hour after, 50 µl solution of 2.5% formalin was injected into the sub-plantar surface of each rat left hind paw. Activities against severity of pain and the anti-inflammatory processes were measured as previously described (Adzu et al., 2014).

Statistical analysis

The data were expressed as a mean ± standard error of mean (S.E.M.). The statistical analyses were obtained by the one-way analysis of variance (ANOVA), followed by the Dunnett's test using Graph Pad Prism Version 5.01 for Windows, Graph Pad Prism Software (San Diego California). $P < 0.05$ was considered significant.

RESULTS

GC-MS analysis

Table 1 shows the GC-MS fingerprint profile of *Cassia singueana* with detection of twenty two peaks.

HPLC analysis of CECs

As shown in Figure 1, nine peaks with retention times (in min) of 3.286, 4.018, 4.663, 5.669, 6.543, 7.560, 8.362, 22.761 and 25.564 were identified.

HPLC analysis of HFCs

The chromatogram (Figure 2) showed four peaks of major constituents with retention times (in min) of 3.288, 3.992, 4.703 and 7.600.

Acute toxicity test

Within 2 h of administration, the predominant sign of toxicity observed were reduced motility and sedation. Afterwards, other signs observed were mouth scratching, paw licking and some non-specific signs. No treatment-associated mortality was recorded in all mice throughout the 14 days duration of the study.
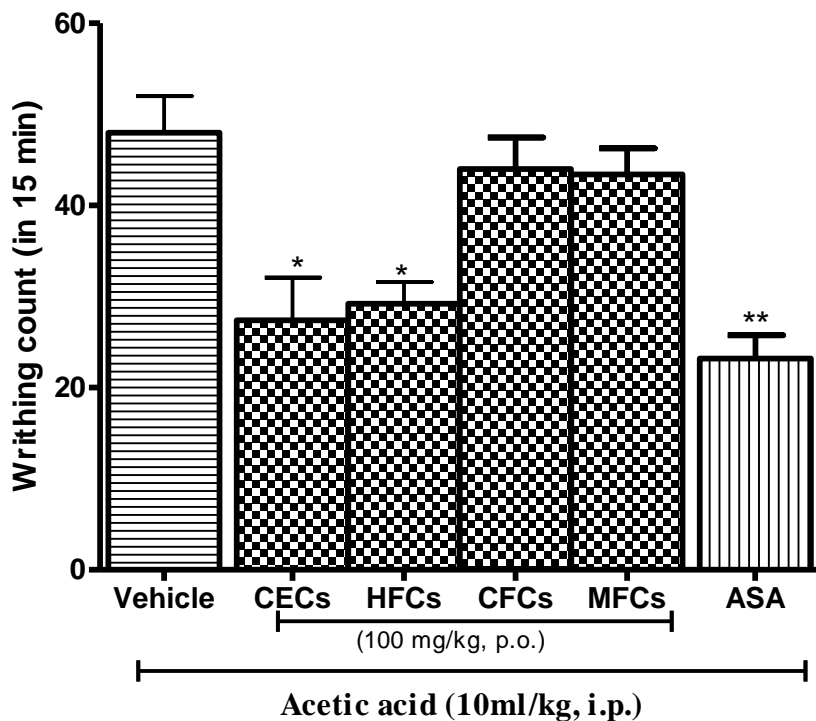
Acetic acid-induced writhing

Injection of acetic acid induced writhes (abdominal constriction) in the control. Treatment with the crude extract and fraction of *C. singueana* reduced the mean
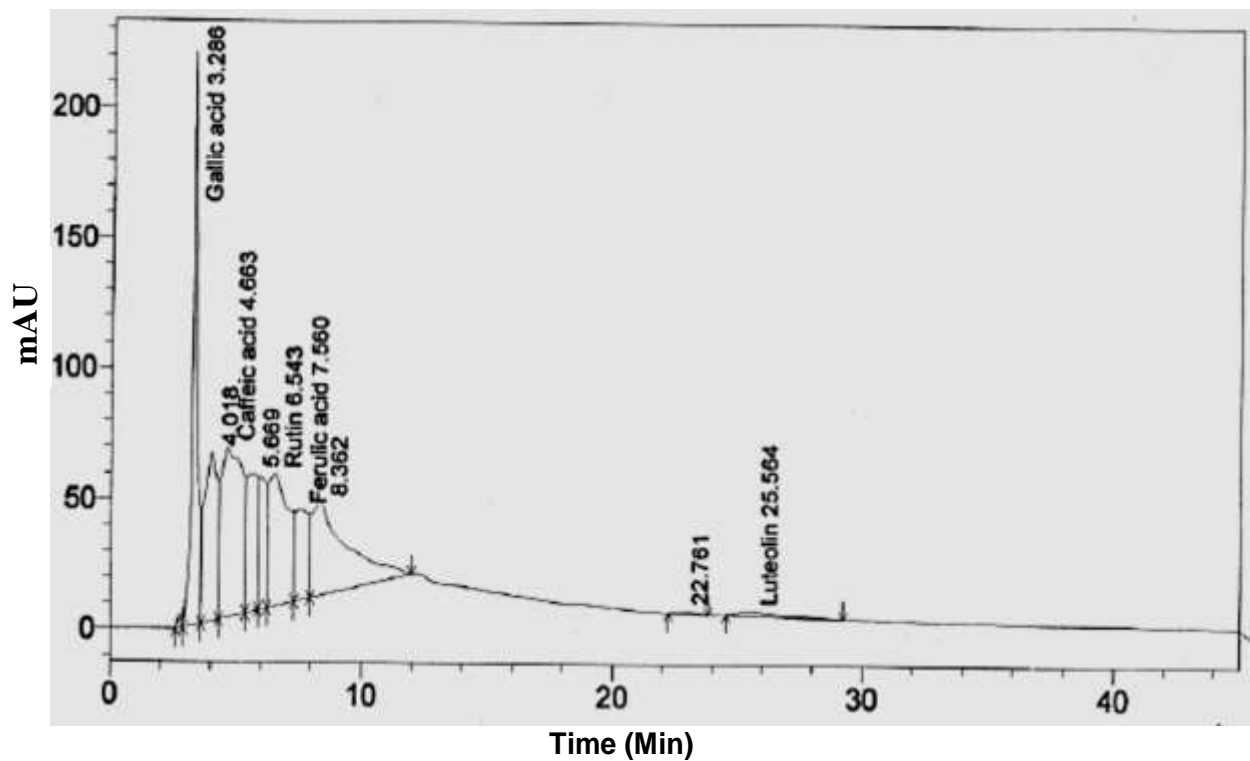
Figure 1. HPLC profile of crude ethanolic extract of *Cassia singueana* (CECs) that include: gallic acid (16.21%), caffeic acid (20.76%), rutin, ferulic acid (6.94%), and luteolin (1.03%).
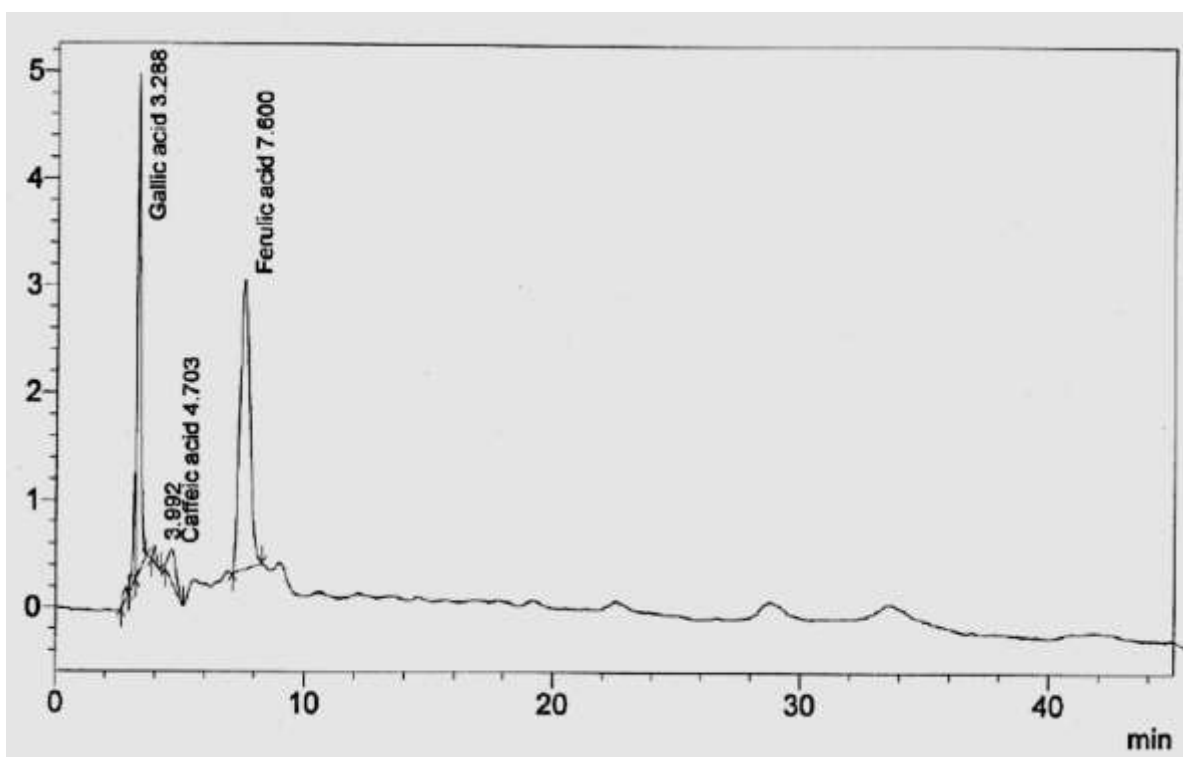
Figure 2. HPLC fingerprints of hexane fraction of *C. singueana* (HFCs) showing a total of 4 peaks that Include: gallic acid (34.37%), caffeic acid (5.61%) and ferulic acid (58.93%).

Figure 3. Effect of oral administration of vehicle (10 ml/kg), crude extract (CECs); hexane fraction (HFCs), chloroform fraction (CFCs) and methanol fraction (MFCs) of *C. singueana* rootbark (100 mg/kg), and aspirin (ASA, 100 mg/kg) on acetic acid induced writhing in mice. Each column represents the mean ± S.E.M. of 5 mice. One-way ANOVA followed by Student-Newman-Keuls test. *p < 0.05; and ** p < 0.01 vs. control.

count of writhing from 12.5 to 24%, with ASA causing 51.67% protection of the induced abdominal writhing when compared with the control group (Figure 3).

Anti-inflammatory activity

Injection of formalin into the sub-plantar surface of the mice right hind caused acute inflammatory process in the control group (Figure 4). Treatment with the crude extract and fraction of *C. singueana* reduced this processes.

Formalin test (analgesic effect)

Injection of formalin into the sub-plantar surface of the mice right hind caused high pain score in the control group; whereas treatment with HFCs attenuated this score (Figure 5).

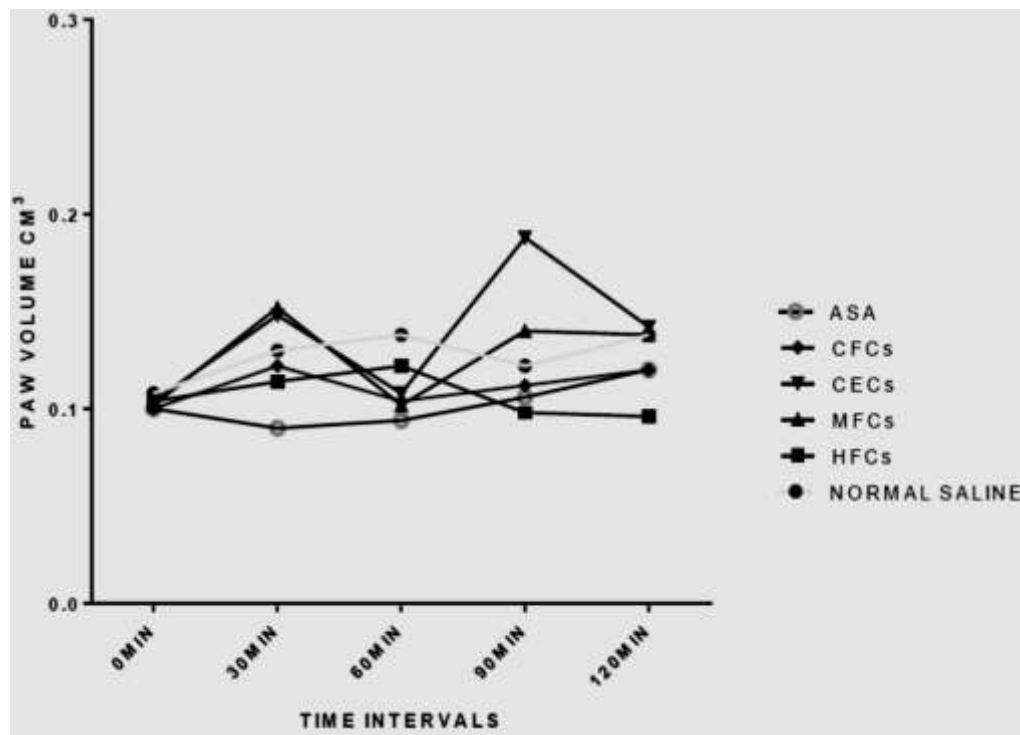### Formalin Test (anti-inflammatory activity)

Injection of formalin into the sub-plantar surface of the mice right hind caused acute inflammatory process in the

control group. Treatment with the crude extract and HFCs of *C. singueana* reduced this processes (Figure 6).

DISCUSSION

The phytochemical fingerprints of fresh *C. singueana* root bark investigated using GC-MS technique revealed various constituents of its essential oil. Such fingerprint profiling are used for quality control; and is important for validity and subsequent stability checks, even if they are not directly related to activity (Bilia, 2014).

The HPLC profile of the crude ethanolic extract of *C. singueana* (CECs) and the most active hexane fraction (HFCs) revealed the presence of gallic acid, caffeic acid, rutin, ferulic acid and luteolin. Gallic acid (3,4,5-trihydroxybenzoic acid) has been reported to possess antioxidant, anti-inflammatory, analgesic, neuroprotective, anticancer, and anti-diabetic properties (Yang et al., 2016). Caffeic acid (3,4-dihydroxy-cinnamic acid) is known to have antioxidant as well as anti-inflammatory, anticancer, and antiviral activities (Magnani et al., 2014). Ferulic acid (3-(4-hydroxy-3-methoxy-phenyl) prop-2-enoic acid) is a well-known antioxidant against free radicals that causes oxidative damage of cell membranes

Figure 4. Anti-inflammation activity of crude extracts (CECs) and hexane (HFCs), chloroform (CFCs) and methanol (MFCs) fractions of *C. singueana* and ASA on formalin induced oedema in mice.
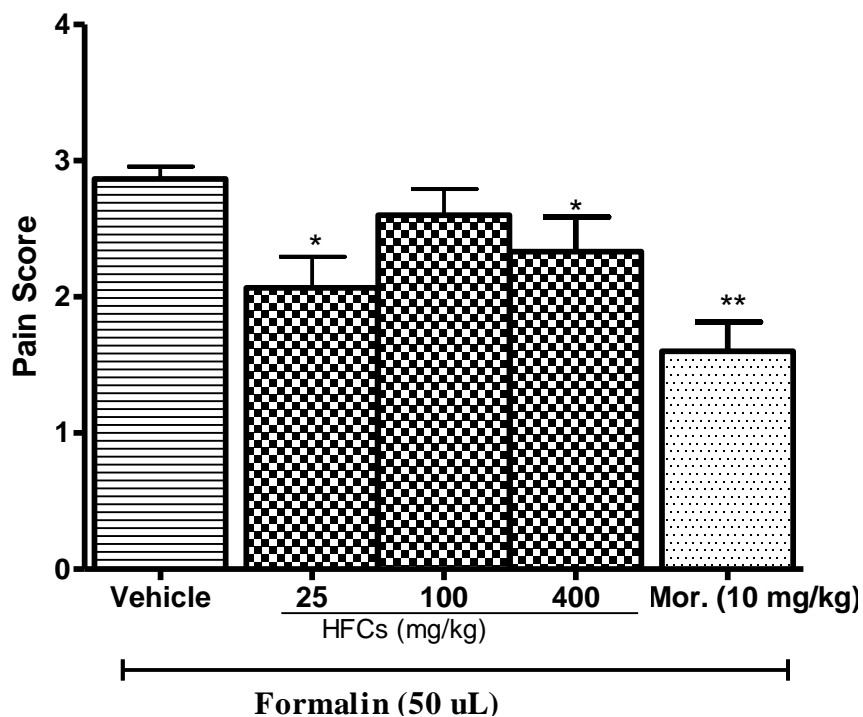
Figure 5. Effect of oral administration of vehicle (10 ml/kg), HFCs (25, 100 and 400 mg/kg), and morphine (Mor, 10 mg/kg) on first phase (0 – 10 min) of formalin (50 µl) induced pain in rats. Each column represents the mean ± S.E.M. of 5 animals. One-way ANOVA followed by Student-Newman-Keuls test. *p < 0.05; and ** p < 0.01 vs. control.
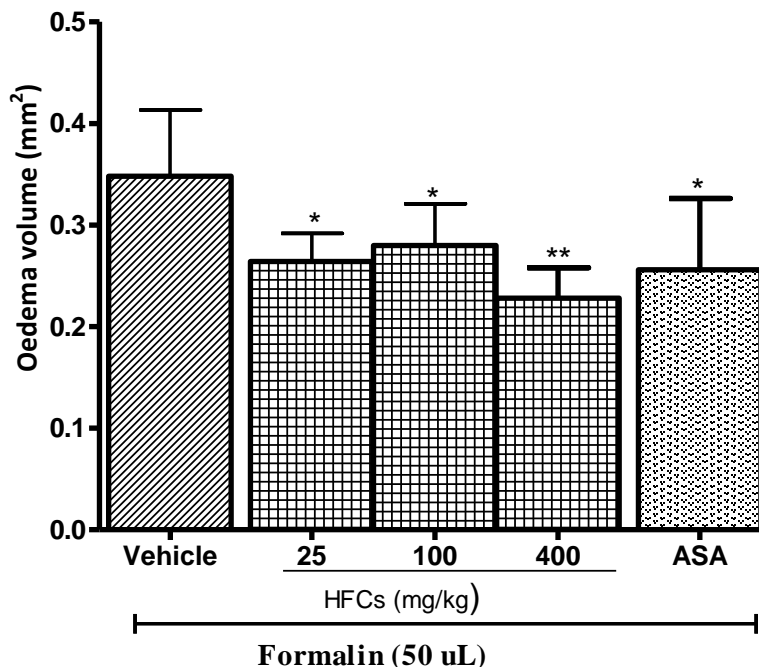
Figure 6. Effect of oral administration of vehicle (10 ml/kg), hexane fraction (HFCs) of *C. singueana* rootbark (25, 100 and 400 mg/kg), and aspirin (ASA, 100 mg/kg) on formalin (50 µl) induced inflammation in rats. Each column represents the mean ± S.E.M. of 5 animals. One-way ANOVA followed by Student-Newman-Keuls test.

(Zduǹska et al., 2018), thereby making it useful in managing inflammatory conditions. Luteolin (3′,4′,5,7-tetrahydroxyflavone) uses are primarily relating to its ability to manage inflammatory conditions including cancer (Lin et al., 2008).

CECs and HFCs were safe in mice. They also demonstrated anti-inflammatory and analgesic activities against the experimentally induced laboratory models. Acetic acid-induced writhes is used in detecting analgesic effect of medicinal agents and is considered as a model of prostaglandin synthesis sensitive response. The most active HFC_S exhibited potent analgesic activity on both phases of the formalin test. This test which is biphasic is recommended for studying the mechanisms of analgesic agents. The first phase (0 -15 min) involves stimulation of nociception in the paw mediated centrally, while the second phase (15 – 60 min) is linked to the activation of local inflammatory processes that stimulates pain sensation similar to clinical pain (Meunier et al., 1998). HFCs suppressed both phases of pain suggesting evidence of dual activity involving both centrally mediated and peripherally localized pain relief mechanisms.

Conclusion

The active constituents responsible for the analgesic and anti-inflammatory activities of *C. singueana* rootbark are gallic acid, caffeic acid, luteolin and ferulic, of which ferullic acid is made up 59% of the most active HCFs. This is unique because it linked the analgesic and anti-inflammatory activities of the plant material to these compounds acting alone or synergistically. This might stimulate more interest in these compounds for their development as pain relief agents.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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