Assessment of the analgesic potential of ethylacetate leaf fraction of *Sida linifolia* L. (Malvaceae)

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**ABSTRACT**

*Sida linifolia* L. is a valuable plant of West Tropical Africa with several folklore claims and growing evidence of its bioactivity, including its pain-relieving potential; however, scientific validation of these claims is still limited. This study investigated the phytochemical composition and analgesic potential of the ethyl acetate fraction of the hydro-alcoholic extract of *S. linifolia* leaves (SEAL). Gas chromatography-flame ionization detector (GC-FID) and high-performance liquid chromatography (HPLC) techniques were used to determine the phytochemistry of SEAL. Formalin and acetic acid models were employed to determine the analgesic properties of SEAL. The result of GC-FID analysis revealed varying concentrations of lunamarine, naringenin, ephedrine, catechins, cardiac glycosides, flavonates, kaempferol, flavones, and naringenin were detected in SEAL via HPLC analysis. In addition, varying concentrations of polyphenolics, such as phenylacetic acid, caffeic acid, ellagic acid, and naringenin were detected in SEAL. The LD<sub>50</sub> study showed that SEAL was safe up to 5000 mg/kg body weight per oral (p.o.) in Swiss mice. Pretreatment with oral doses (200, 400, and 600 mg/kg bw) of SEAL significantly (p < 0.05) inhibited all phases of formalin-induced hind paw licking and acetic acid-induced writhing syndrome in mice compared to positive control and was on par with aspirin (100 mg/kg bw p.o.). The observed bioactivity of SEAL could be anchored to its phytoconstituents. Therefore, the plant leaf fraction represents a good source of bioactive compounds with immense potential for exploration in therapeutic research.

1. Introduction

In recent times, where herbal medicines are rapidly gaining popularity among individuals and the pharmaceutical industry, the elucidation of the phytochemical profile of medicinal plants is critical for the synthesis of novel, potent, and less toxic therapeutic principles as an alternative to synthetic drugs which are not without adverse effects (Welz et al., 2018). The African tropical forest, by its extraordinary biodiversity and endemism, is well-known for being an ideal environment for discovering novel pharmaceuticals (Cao & Kingston, 2009). As a result, many of its indigenous communities rely on medicinal plants as food or disease panacea for humans and livestock due to their rich phytoactive composition (Aziz et al., 2018). Moreover, in the face of increased abiotic stresses, such as excessive UV radiation and drought, and biotic stressors,
such as pathogens and herbivores, plants have evolved to synthesize
a variety of secondary metabolites that aid their survival. Fortunately, exploring these bioactive compounds is relevant to the medical and pharmaceutical industries (Aondoaver et al., 2023; Yeshi et al., 2022).

Furthermore, the phytocomposition of plants represents a rich repository of biologically active compounds with several documented bioactivities. From time immemorial, plants have provided a source for developing food, drugs, and other modern-day pharmaceuticals due to their rich phytochemical composition (Anyamele et al., 2023; Madueke et al., 2020b). The consumption of African medicinal plants as food or medicine stems from the rich profile of bioactive secondary metabolites naturally situated in their roots, leaves, stem bark, flowers, and seeds (Altemimi et al., 2017; Mahomoodally, 2013). Moreover, due to several adverse effects associated with synthetic drugs (in this context, NSAIDs), there is an increased demand for alternative remedies, preferably from plants, to manage pains and inflammation associated with several disease conditions (Madni et al., 2022; Madueke & Anosike, 2017).

Sida linifolia L., a valuable plant of West Tropical Africa (particularly in Nigeria and Sierra Leone), is a virgin medicinal weed with folkloric applications across Africa (Akubue et al., 1983). It is found ubiquitously in dry forest areas and belongs to the Sida genus (which comprises about 200 species of weeds) and the Malvaceae family (Saensouka et al., 2016). The phytochemical compositions and pharmacological activities of some weeds belonging to the Sida genus and the Malvaceae family, such as S. hombibifolia, S. cordifolia, S. acuta, S. corymbosa, and S. tiagi, have been extensively documented (Dinda et al., 2015); however, reports on the pharmacological properties of S. linifolia are still limited. The folklore uses and growing evidence of the anti-inflammatory and pain-associating properties of S. linifolia leaves warranted the present study. Generally, African herbalists employ the alcoholic decoction of the leaves to treat malaria, fever, painful whitlows, and other inflammatory diseases (Nieuwinger, 2000; Saensouka et al., 2016). In line with this, few studies have reported potent anti-inflammatory and antinociceptive activities of the polar leaf extracts and fraction of the plant leaves (Ezeako et al., 2023; Nwanwko et al., 2023b; Nwankwo et al., 2023c); however, reports on the analgesic potential of the non-polar fraction are still limited. Similarly, a more recent study demonstrated appreciable antioxidant and anti-inflammatory actions of the ethyl acetate leaf fraction of S. linifolia (Nwanwko et al., 2023a); however, not much was reported regarding the phytochemistry. In addition, the anti-nociceptive mechanism of the leaf fraction was not reported (Nwanwko et al., 2023a). Moreover, several investigators have reported rich phytochemistry and potent pharmacological activities of non-polar fractions of closely related medicinal plants (Eseyin et al., 2010; Mah et al., 2017; Rodrigues & de Oliveira, 2020; Shah et al., 2017). For instance, Shah and Khan (2014) anchored the anti-diabetic action of the ethyl acetate fraction of crude methanol leaf extract of S. cordata in alloxan-induced diabetic rats to its rich composition in polyphenols. In their report, the non-polar aspect of the plant extract exhibited appreciable bioactivities compared to respective standards. In another study, Shah et al. (2017) reported that the ethyl acetate leaf fraction of S. cordata showed excellent renal protective effect in CCl4-induced nephrotoxic rats and linked this bioactivity to its rich composition in antioxidant compounds detected using HPLC-DAD analysis. Similarly, Mgbelema et al. (2015) reported that n-hexane and ethyl acetate fractions of S. acuta improved liver function indices in thioacetamide-challenged hepatotoxic rats. In the same vein, Ekramul Islam et al. (2003) reported that ethyl acetate and chloroform leaf extracts of S. rhombifolia exhibited higher cytotoxicity and antibacterial activity compared to other polar extracts and related this to the rich mixture of phytoactive compounds present in the non-polar fractions. On this premise, this study investigated the phytochemical profile and anti-nociceptive mechanisms of the ethyl acetate fraction of S. linifolia leaves to further validate the folklore claims of the plant leaves.

2. Materials and methods

2.1. Reagents and chemicals

The reagents and chemicals used for the study were of analytical grade. Some equipment and reagents were acquired from the Department of Biochemistry, University of Nigeria, Nsukka. Other chemicals used in the study were purchased from scientific shops in Nsukka and Enugu in Enugu State, Nigeria.

2.2. Procurement and identification of plant leaves

Fresh leaves of S. linifolia were harvested from grazing land in Nsukka town, Enugu State, Nigeria (latitude 6.8429° N and longitude 7.3733° E) in May 2021. After that, a botanist at the Bio-resources Development and Conservation Program Research Centre, Nsukka, Enugu-Nigeria, identified and authenticated the plant. A deposit of the plant was kept at the herbarium for future retrieval (with voucher no: BDCP20210724).

2.3. Extraction and fractionation of plant material

The extraction procedure followed the methods outlined by Parvin et al. (2015), with minor modifications, while the fractionation process followed the procedures described by (Hwang et al., 2009). In this case, the harvested plant leaves were washed carefully in a tap, free of debris, and shed-dried for 5 days. After that, the plant leaves were pulverized with a high-speed electric grinder (High-Speed, China). Then, a weighted quantity (2500 g) of the powdered plant leaves was soaked in 98% ethanol (8.0 l) for 24 h. The mixture's vessel was sealed with cotton wool and kept in a rotary incubator (Kottermann, Germany) operated at 200 × g. After that, filter paper (Whatman No. 1) was employed to filter the mixture, and the resultant filtrate was evaporated at 45 °C and reduced pressure using a rotary evaporator. The crude leaf extract obtained was loaded into a fractionating column and partitioned (3 times) with ethyl acetate. The eluent was evaporated in a rotary evaporator operated at similar conditions (reduced pressure and 45 °C). The concentrated fraction was transferred into a properly labeled sterile screw-capped vessel and left to stand at 4 °C in a refrigerator until needed. The study was meant to reproduce the alcoholic decoction of the plant leaves in the form used in African folklore and to investigate the bioactivity of the non-polar aspect of the crude extract. The ethyl acetate leaf fraction (SEAL) was adopted for the study in line with previous submissions (Eseyin et al., 2010; Mah et al., 2017; Shah et al., 2017).

2.4. Preliminary phytochemical screening

Preliminary phytochemical screening of the secondary metabolites composition of the leaf fraction was performed according to standard procedures (Harborne, 1998; Trease & Evans, 1989). Qualitative phytochemical screening was performed to evaluate the relative abundance of different classes of phytochemicals present in the non-polar leaf fraction.
2.5. Gas chromatography-flame ionization detector (GC-FID) profiling

Profiling of the phytochemical composition of the plant leaf fraction was further carried out using the GC-FID technique.

2.5.1. Sample preparation

The study adopted the protocol described by Newman and Cragg (2010) and Kelly and Nelson (2014). A volume (15 ml) of ethanol mixed with 10 ml of potassium hydroxide (50% (m/v)) was dispensed into a test tube containing a known mass (1 g) of the study material. The resultant solution was incubated in a water bath at 60 °C for 60 min. Subsequently, the mixture was collected through a separatory funnel. After washing consecutively with 20 ml of ethanol, a volume of cold water (10 ml), 10 ml of hot water, and a volume of hexane (3 ml), the entire tube content was dispensed into the funnel. Thereafter, a volume (10 ml) of 10% ethanol (v/v) was used to wash the mixture thrice and later subjected to dehydration using anhydrous sodium sulfate. The concentrate was then transferred into 1000 µl of pyridine solution, and a volume of 200 µl was aspirated for analysis.

2.5.2. Gas chromatography-flame ionization detector (GC-FID) quantification

The phytochemistry of the study material was determined using a BUCK M-910 Gas Chromatography system equipped with RESTEK 15-meter MXT-11 (15 m × 250 µm × 0.15 µm) column and a flame ionization detector. The carrier gas (helium, 5.0 pas) was maintained at a 40 ml/min constant flow rate. The injector was operated at a splitless injection of 2 µl, at a temperature of 280 °C, and at a linear speed of 30 cm/s. An initial oven temperature of 200 °C was maintained. After that, the temperature was uniformly increased from 3 °C min⁻¹ to 330 °C; this temperature was kept constant for 5 min. The flame ionization detector was operated at 320 °C. Standards solutions (1000 ppm) of various phytochemicals (epicatechin, flavones, steroids, tannins, catechins, cyanogenic glycosides, rutin, sapogenins, kaempferol, cardiac glycosides, oxalate, lunamarin, flavonones, phytate, flavan-3-ols, spartein, resveratrol, ephedrine, naringin, anthocyanin, and proanthocyanidins) were prepared. Thereafter, a known concentration (50 ppm) of the various standard solutions was used to register the reference peak height, peak area, and retention time in the system. After that, the concentration of the various phyto compounds in the analyte was evaluated by taking the ratio of the area and mass of internal standards and the peak area of the identified compounds. The identified compound concentrations were presented in ppm (which equals µg/ml and µg/g).

2.6. High-performance liquid chromatography (HPLC) determination of polyphenolic compounds

2.6.1. Sample and standards preparations

This study was carried out following the procedure reported by Mizzi et al. (2020). Standard solutions of 18 phenolic compounds, namely luteolin, coumaric acid, apigenin, phenylacetic acid, naringenin, p-vanillic acids, syringic acid, chrysirin, sinapic acid, 4-methoxycinnamic acid, gallic acid, quercetin, chlorogenic acid, ellagic acid, ferulic acid, 3,4-dimethoxybenzoic acid, caffeic acid, and benzoic acid (Sigma-A, Germany) were prepared. Stock solutions (100 mg/l) were prepared using ultra gradient grade HPLC methanol (Carlo-Erba, Milan, Italy), and standard curves were plotted with various concentrations. Standard phenolic solutions were mixed in different concentration ratios, and spectrophotometrical readings were taken within the UV-Vis region (180 and 480 nm) using a UV-2600 spectrophotometer (Shimadzu, Tokyo, Japan) to arrive at an appropriate wavelength for the HPLC-DAD determinations.

2.6.2. High-performance liquid chromatography quantification

Quantification of phenolic compounds in the test sample was carried out with an HPLC system (Waters-2695 Alliance, Milford, Connecticut, U.S.A.) coupled with a UV-Vis Diode array detector (DAD). A 4.6 mm width, 5 µm particle size, and 250 mm length Waters SunfireTM HPLC system, equipped with a C18 reverse-phase chromatographic column, were employed in the separation process. Injection of standard phenolic solutions and the test sample at equal volumes into the system separately was done with an auto injector. The resultant gradient after a series of prior studies using HPLC grade (≥ 99.9 %) acetonitrile (Honeywell) as the mobile phase A while phosphoric acid made by introducing 85% orthophosphoric acid (Sigma-Aldrich, Germany) in drops to HPLC grade water to obtain a pH of 2, served as the mobile phase B. A 0.5 ml/min constant flow rate, 5 °C operating temperature, and total runtime of 6 min were employed in the analysis. The specific wavelengths (210, 280, and 360 nm) used in the study were obtained from prior studies of the UV-Vis spectra of the different phenolic standards using HPLC-DAD.

2.7. Animals

In this study, 10-11 weeks old male and female albino mice weighing 28-32 g were used. Animals were collected from the animals of the animal holding unit, Department of Zoology and Environmental Biology, University of Nigeria, Nsukka. The animals were kept in a well-hygience cage at an adequate temperature (24 ± 1 °C), relative humidity (55.5%), light/dark period (12/12 h), and fed quality rat feed. The animals were acclimatized to their new surroundings for 14 days before the commencement of the experiment.

2.8. Ethical clearance

The experiments were carried out following international ethical guidelines described in the revised National Institute of Health handbook on the use of laboratory animals (NIH, 1985). The animals utilized in the study were treated decently. Ethical clearance documents were received from the Department of Biochemistry’s Research and Ethics unit at the University of Nigeria, Nsukka (No. UNN/BCH/9014). The study did not include any human subjects.

2.9. Lethal dose (LD50) toxicity study

The procedure outlined by Lorke (1983) was employed to determine the median lethal dose (LD50) of SEAL. Six groups of mice (three mice in a group) were given the test sample through oral intubation at different doses (10, 100, 1000, 1600, 2900, and 5000 mg/kg bw) after being deprived of rat chews for 12 h before the start of the experiment. Mice were monitored for behavioral abnormalities (dullness, in-coordination, and anxiety) or death 24 hours after treatment. Furthermore, the treated animals were observed for 7 days for signs of delayed toxicity. Following that, three sub-maximal doses, 200, 400, and 600 mg/kg bw, denoting low, mid, and high doses, respectively, which have been established to be safe for oral administration in mice (Konaté et al., 2012) were adopted for subsequent biochemical tests.
The anti-nociceptive actions of SEAL were determined using two pain models (formalin and acetic acid models). In each case, mice were randomly assigned into five groups of eight animals each (n = 8). Twenty-four-hour fasted mice in the various groups were administered 10 mg/kg bw per oral distilled water, 200, 400, and 600 mg/kg bw (p.o.) SEAL and 100 mg/kg bw (p.o) aspirin, respectively. One hour post-administration of the various treatment regimens, animals were challenged with phlogistic agents, respectively.

2.10.1. Formalin-induced arthritis model

The method outlined by Akindele and Adeyemi (2007) was employed in this study. A known volume (0.02 ml) of freshly prepared formalin solution (1% v/v) was delivered intraperitoneally into the right hind paws of mice 30 min after the respective treatments were administered to the various groups. After induction, time (in sec) spent by each mouse responding to the effect of the phlogistic agent by licking and biting the affected paw (indicative of pain) was recorded at the various pain phases; phase I (0-5 min) and phase II (15-30 min).

2.10.2. Acetic acid-induced writhing model

The study was done according to the method documented by Mbagwu et al. (2007). Post-administration of various treatments in the respective groups, animals were allowed to stay for 30 min; after which, 10 ml/kg bw acetic acid (0.6% v/v) was infused into the hind paws of mice intraperitoneally. Thirty minutes post-acetic acid infusion, the number of writhes produced in each mouse was recorded every 5 min for 30 min. The ability (%) of SEAL to inhibit writhing syndrome caused by acetic acid injection in mice was estimated with the formula as follows:

\[
\text{Writhing Inhibition} \% = 1 - \frac{\text{Mean writhes number (test)}}{\text{Mean writhes number (control)}} \times 100
\]

2.11. Statistical analysis

Data was statistically analyzed with one-way and two-way ANOVA using Statistical Product and Service Solutions (SPSS) version 23. The analyzed data were presented as mean ± standard deviation (SD). Significant differences in mean across different groups were analyzed using Duncan post hoc. The degree of significance of the study was established at p < 0.05.

3. Results and discussion

3.1. Percentage yield

Maceration of 2500 g of the plant leaves resulted in a yield of 70.65 g of crude extract (which accounts for about 2.83% of the starting material). Furthermore, fractionation of a known amount (70.65 g) of the crude extract in a fractionating column with ethyl acetate, yielded 21.29 g ethyl acetate leaf fraction (SEAL). This amount (21.29 g) accounts for about 30.14% of the weight of crude extract (70.65 g) introduced into the fractionating column. The SEAL was used for the study. Our choice of fraction corresponds with Mah et al. (2017), which reported rich phytochemistry and pharmacological properties of the ethyl acetate fraction of S. rhombifolia. Our result showed a higher yield (30.14%) for ethyl acetate fraction than that of Shah et al. (2017), which realized a 15% ethyl acetate fraction from crude methanol extract. The variation in yield could be due to several factors. For instance, reports have shown that variations in the solubility of phytochemicals, such as phenolics in various solvents of varying polarities, significantly influence their extraction yield (Rababah et al., 2010; Zlotek et al., 2016). It has been established in the literature that extraction and purification of phytochemicals are generally affected by several factors, including the type of material, polarity, concentration of solvent, texture of the pulverized material, time of harvest, storage conditions, drying temperature, and extraction duration (Arana et al., 2022; Ekpo et al., 2020). Despite the common starting material, reports have demonstrated that the solvent used in extraction influences the phytochemical profile and bioactivities of the respective extracts/fractions (Nakamura et al., 2017; Ngo et al., 2017). Depending on their structural chemistry, different phytochemicals are extracted in solvents of varying polarity because no single solvent can reliably extract all of the phytochemicals present in a given plant material (Iloki-Assanga et al., 2015; Lapornik et al., 2005). Therefore, to allow for the extraction of a broader range of phytochemicals with varying polarities, the serial exhaustive extraction procedure involving sequential extraction with solvents of increasing polarity from non-polar (such as n-hexane and ethyl acetate) to more polar (aqueous) solvent is usually employed (Abdel-Aal et al., 2015; Das et al., 2010). Moreover, reports have shown that solvent polarity significantly affects plant extracts’ yield and phenolic composition (Barchan et al., 2014; Ghassemzadeh et al., 2015). Nonetheless, specific mixes of phytoactive compounds exist in the non-polar phase after fractionation. Nawaz et al. (2020) reported higher total phenolic and total flavonoid content in the non-polar fractions of Phaseolus vulgaris seeds indicating that some phenolic compounds could be non-polar. In line with this, several studies have reported excellent bioactivities of non-polar fractions of medicinal weeds of the Sida genus (Mgbemena et al., 2015; Rachel et al., 2008; Shah et al., 2017) and anchored these activities to their rich phytochemistry.

3.2. Qualitative phytochemical composition of SEAL

Presented in Table 1 is the result of the preliminary phytochemical analysis of SEAL. The result showed that flavonoids, phenols, alkaloids, and steroids were moderately abundant in SEAL, while tannins, cyanogenic compounds, saponins, terpenoids, and glycosides were present in minute abundance in the plant leaf fraction. Studies have linked the health benefits of medicinal plants to their rich composition of bioactive ingredients naturally present in their leaves, roots, stems, seeds, or flowers as secondary metabolites capable of influencing the body’s physiology (Chidi et al., 2021; Okey et al., 2020; Sokmen et al., 2004). Among these phytochemicals, polyphenolics (e.g., flavonoids, tannins), alkaloids, glycosides, terpenoids, and steroids are well documented for their potent pharmacological activities (Kurukov, 2012; Madueke et al., 2020a; Moriasi et al., 2020; Moriasi et al., 2021). Based on the result, flavonoids were relatively higher in abundance in the leaf fraction. Reports have shown that flavonoids and other phenols with adjacent phenol substituents exhibit anti-inflammatory and anti-thrombotic actions via their ability to exert inhibitory actions on serine proteases at a micromolar scale as well as to attenuate superoxide anions (Robak & Gryglewski, 1996; Tungmunnithum et al., 2018; Xue et al., 2017). Similarly, a recent submission by Jakimiuk et al. (2021) expounded on the potential of flavonoids to act as human neutrophil elastase inhibitors. In line with this, Kumawat et al. (2012), Bahar et al. (2013), and Khan et al. (2014) demonstrated potent anti-thrombolytic properties of S. acuta, S. rhombifolia, and S. tiagii, respectively, and anchored these activities to their flavonoids composition. The plant extract’s rich phenolic
(particularly flavonoids) composition may lend credence to its local use to treat whitlow and malaria.

Table 1. Result of the phytochemical screening of SEAL

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>Relative abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonoids</td>
<td>++</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
</tr>
<tr>
<td>Phenols</td>
<td>++</td>
</tr>
<tr>
<td>Cyanogenic compounds</td>
<td>+</td>
</tr>
<tr>
<td>Glycosides</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>+</td>
</tr>
<tr>
<td>Steroids</td>
<td>++</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>++</td>
</tr>
</tbody>
</table>

+++ highly abundant, ++: moderately abundant, +: minutely abundant

3.3. Phytochemicals detected in SEAL using GC-FID analysis

The result of the GC-FID study of the phytochemical composition of SEAL is presented in Table 2. From the result, varying concentrations of lunamarine (14.7703 ppm), naringenin (13.9470 ppm), ephedrine (13.6169 ppm), catechins (12.5734 ppm), cardiac glycosides (10.9097 ppm), flavonanes (9.3867 ppm), kaempferol (9.0277 ppm), naringin (8.0388 ppm), flavones (7.5346 ppm), phytate (6.7060 ppm), rutin (5.4329 ppm), steroids (5.0650 ppm), tannins (4.6313 ppm), oxalate (3.8593 ppm), flavan-3-ols (3.8542 ppm), sapogenins (3.7255 ppm), cyanogenic glycosides (3.3538 ppm), anthocyanin (2.65171 ppm), and proanthocyanidins (2.4496 ppm) were present in SEAL. The GC-FID chromatogram (Figure 1) showed different bands of various peak areas indicative of the various phytoconstituents. For instance, flavone present in SEAL is a flavonoid with a double bond between the C2 and C3 in the flavonoid skeleton and an unsubstituted C3 and oxidized C4. This compound exhibits potent antioxidant and anti-inflammatory properties in vitro and in vivo (Chagas et al., 2022). Flavanone detected in SEAL displays a wide range of bioactivities, including its potential to attenuate free radicals, inhibit inflammatory cascades, reduce blood pressure, as well as halt microbial activities that have been documented (Testai & Calderone, 2017). The presence of naringin in SEAL indicates its antioxidant and anti-inflammatory potential. Naringin and its aglycone naringenin are flavonoids with several reported pharmacological profiles, such as anti-inflammatory and antioxidant actions (Salehi et al., 2019). A study has suggested that naringin supplementation could treat metabolic syndrome-related diseases, including diabetes, obesity, and hypertension (Alam et al., 2014). Naringenin has also been shown to attenuate free radicals, inhibit protein carbonylation, and lipid peroxidation, inordinate inflammatory responses, and improve sugar metabolism (Alam et al., 2014). Flavan-3-ols detected in the leaf fraction are known to exhibit antioxidant and anti-inflammatory potentials and exhibit cardioprotective, antitumor, antitumor, and antiviral potentials (Ullah et al., 2020). The result also showed the presence of catechin in the plant fraction. Several in vivo and in vitro studies have shown that catechin possesses excellent free radical scavenging potentials and interacts with transcription factors and enzymes to elicit anti-inflammatory and antioxidant activities (Fan et al., 2017; Kim & Heo, 2022; Li et al., 2022). The polyphenolic catechin possesses the potential to modulate oxidative stress and inflammation-associated molecules, including mitogen-activated protein kinases (MAPKs), transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2), nuclear factor-kappa B (NF-kB), and activators of transcription 1/3 (STAT1/3) pathways The presence of some potent types of flavonols, such as kaempferol and rutin, in the leaf fraction also suggests its radical scavenging and anti-inflammatory potential. Reports have shown that kaempferol could supplement the human antioxidant defense, protect against cancer growth, angiogenesis, and metastasis, and promote apoptosis of infected cells (Chen & Chen, 2013). On the other hand, rutin detected in the leaf fraction possesses antioxidant, vasoprotective, neuroprotective, cytotoxic, cardioprotective, and anti-carcinogenic potentials (Ganeshpurkar & Saluja, 2017). Sapogenins (triterpenes), also detected in the fraction, are reported to possess antibacterial (Na et al., 2016) and anti-inflammatory activities (Yang et al., 2021) and are potent against several types of cancer via their ability to modulate intracellular Ca2+ levels (Sun et al., 2020).

The presence of tannins in the leaf fraction, although in relatively small preponderance suggests its anti-inflammatory properties. Reports have shown that tannins possess inhibitory potential against cyclooxygenases and abrogate the endemic action of irritants, such as carrageenan and egg albumin (Attiq et al., 2018; Wagner, 1989). Tannins also enhance blood clotting, lower blood pressure, and modulate immune responses. However, the type and dosage of tannins are critical to these effects (Chung et al., 1998). Proanthocyanidins, detected in SEAL, are a class of condensed tannins commonly found in plants with notable pharmacologic activities (Rauf et al., 2019). Proanthocyanidins are powerful antioxidant phytochemicals capable of scavenging free radicals and exerting anti-inflammatory and antitumor actions (Dai et al., 2014; Wang et al., 2020). Anthocyanins detected at high levels in SEAL exhibit anti-inflammatory, anticancer, anti-diabetic, antimicrobial, cardioprotective, and anti-obesity potentials (Naseri et al., 2018; Oliveira et al., 2020; Sivamaruthi et al., 2020).

Ephedrine, a naturally occurring alkaloid detected in SEAL, possesses excellent anti-inflammatory properties (Fiebich et al., 2012; Parsaemehr & Sargyean, 2013). The anti-hypotensive effect of ephedrine has been reported (Dabisch et al., 2003). Studies have shown that ephedrine functions both as a direct and indirect sympathomimetic. It is an agonist of both α- and β-adrenergic receptors; however, its action indirectly results in the secretion of norepinephrine (NE) from sympathetic neurons, as well as the inhibition of NE reuptake and displacement of additional NE from storage vesicles. Similarly, a previous study demonstrated the anti-inflammatory potential of pseudoephedrine/ephedrine against TNF-α-mediated acute lipopolysaccharide/D-galactosamine-induced liver toxicity (Wu et al., 2014). The ethanolic fraction of S. linfola did not show the presence of ephedrine in GC-FID analysis as previously reported (Nwankwo et al., 2023b). This could be due to the non-polar nature of ephedrine structural chemistry. The presence of lunamarine in SEAL also implies its immunomodulatory properties. Studies have reported that lunamarine (punarnavine), a quinoline alkaloid, possesses anticancer and immunomodulatory potentials (Manu & Kuttan, 2009a, 2009b).

The results also revealed the presence of steroids and cardiac glycosides in SEAL. Steroids are well-known inflammatory inhibitors that interact with receptors in the nuclear membrane via trans-activation or trans-repression, resulting in the modulation of inflammatory gene expression (Timmermans et al., 2019; Vandeveer et al., 2013). Cardiac glycosides are steroidal glycosides capable of resuscitating failed hearts by improving cardiac output via the renin-angiotensin axis, making the myocardium more efficient in sustaining the circulatory system demands (Kelly, 1990; Khudmiri, 2014; Patel, 2016). Although acute toxicity is associated with a high intake of cyanogenic glycosides, which manifests in growth retardation and damage to the central nervous system (CNS), nevertheless, a recent study by Figurová et al. (2021) reported concentration-dependent anti-inflammatory action of a cyanogenic glycoside. While oxalate increases the risk of
hyperoxaluria (Siener et al., 2013) and kidney stone disease (Holmes & Assimos, 2004), phytate binds and renders essential minerals inaccessible and unavailable (Akter et al., 2020; Gibson et al., 2018; Gupta et al., 2015). On the contrary, recent submissions have posited that phytate in modest amounts may exert antioxidant and anticancer effects and could prevent kidney stone formation caused by calcium oxalate (Pires et al., 2023; Pujol et al., 2023; Shamsuddin, 2002).

Table 2. Phytochemical profile of SEAL

<table>
<thead>
<tr>
<th>Peak No</th>
<th>Compound</th>
<th>Retention time (min)</th>
<th>Peak area</th>
<th>Peak height</th>
<th>Conc. (ppm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lunamarine</td>
<td>3.190</td>
<td>7628.0534</td>
<td>596.491</td>
<td>14.7703</td>
</tr>
<tr>
<td>2</td>
<td>Naringenin</td>
<td>1.280</td>
<td>8428.6252</td>
<td>644.168</td>
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<tr>
<td>3</td>
<td>Ephedrine</td>
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<td>4428.2046</td>
<td>349.075</td>
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<tr>
<td>4</td>
<td>Cardiac glycoside</td>
<td>7.090</td>
<td>7704.1709</td>
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<td>10.9087</td>
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<td>5</td>
<td>Flavonones</td>
<td>9.113</td>
<td>15862.0896</td>
<td>1220.495</td>
<td>9.3867</td>
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<tr>
<td>6</td>
<td>Kaempferol</td>
<td>25.080</td>
<td>10027.6642</td>
<td>781.331</td>
<td>9.0277</td>
</tr>
<tr>
<td>7</td>
<td>Naringin</td>
<td>17.033</td>
<td>17990.4274</td>
<td>843.163</td>
<td>8.0388</td>
</tr>
<tr>
<td>8</td>
<td>Flavone</td>
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<td>10118.3940</td>
<td>786.357</td>
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<tr>
<td>9</td>
<td>Phytate</td>
<td>30.986</td>
<td>6172.2460</td>
<td>483.357</td>
<td>6.7060</td>
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<td>10</td>
<td>Rutin</td>
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<td>7295.8673</td>
<td>570.542</td>
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<td>Steroids</td>
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</tr>
<tr>
<td>14</td>
<td>Flavo-3-ol</td>
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<td>5224.3806</td>
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</tr>
<tr>
<td>15</td>
<td>Sapogenin</td>
<td>1.640</td>
<td>4719.6824</td>
<td>336.190</td>
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</tr>
<tr>
<td>16</td>
<td>Cyanogenic glycosides</td>
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<td>279.848</td>
<td>3.3538</td>
</tr>
<tr>
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<td>Anthocyanin</td>
<td>0.246</td>
<td>9957.768</td>
<td>782.312</td>
<td>2.65171</td>
</tr>
<tr>
<td>18</td>
<td>Proanthocyanidins</td>
<td>43.876</td>
<td>5092.5632</td>
<td>401.247</td>
<td>2.4496</td>
</tr>
</tbody>
</table>

*Conc.: Concentration (1 ppm = 10⁻¹ mg/100 g = 1 mg/l = 1 µg/ml = 1 µg/g)

![Figure 1. Gas chromatography-flame ionization detector (GC-FID) chromatogram of SEAL](image)

3.4. Polyphenolic compounds detected in SEAL using HPLC technique

Table 3 shows the identified polyphenolic compounds in SEAL using the HPLC technique. From the result, phenylacetic acid (96.5956 ppm), caffeic acid (3.1631 ppm), ellagic acid (0.2076 ppm), and naringenin (0.0337 ppm) were presented in the leaf fraction at different concentrations. The HPLC chromatogram (shown in Figure 2) showed four peaks representing phenylacetic acid (which retained at 2.515 min), caffeic acid (which retained at 1.182 min), ellagic acid (which retained at 1.682 min), and naringenin (which retained at 4.973 min). The presence of these compounds in SEAL suggests its potent pharmacological potential. Studies have documented phenylacetic acid’s excellent antioxidant, anti-inflammatory, and antineoplastic potential (Spiegel et al., 2020; Wellendorph et al., 2009). Moreover, Diclofenac, a potent NSAID used to treat pain and inflammatory diseases, is a phenylacetic acid derivative (Wellendorph et al., 2009). Caffeic acid is a bioactive phenolic compound with several reported pharmacological activities, including its ability to attenuate free radicals, inhibit inflammation, and impede the proliferation of tumor cells. The antioxidant potential of caffeic acid is anchored to its structural chemistry, such as its free phenolic hydroxyl, the position and
number of hydroxyls in the catechol group, and its pie bond on the carbonic acid (Espindola et al., 2019). Ellagic acid’s potent free radical scavenging activities have been reported (Priyadarshini et al., 2002). The anti-inflammatory activity of ellagic acid has been documented (BenSaad et al., 2017). In addition, its antitumor, antiangiogenic, and antimetastatic activities have also been reported (Ceci et al., 2018). In line with our findings, Mah et al. (2017) highlighted the rich phenolic composition of ethyl acetate leaf fraction of S. rhombifolia and its excellent antioxidant potential in 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric ion reducing antioxidant power (FRAP), and ferrous ion chelating (Fe(III)) assays. The rich phenolic composition of SEAL implies its possible antioxidant potential, which could partly explain the medicinal potential of the plant leaves in African folklore. In the same vein, several studies have documented that polyphenolic compounds such as tannins and flavonoids, synthesized in plants as defensive mechanisms against assaults from microbial infestation and UV radiation, possess several health benefits, including anti-inflammatory and antioxidant potentials (Robak & Gryglewski, 1996; Tungmunthum et al., 2018). The antioxidant potentials of most phenolics stem from their rich phytochemistry composition of hydroxyl, electron-donating, or withdrawing substituents available in their ring structure (Zlotek et al., 2016). In line with this, Altemimi et al. (2017) submitted that the antioxidant properties of plant materials correlate with their rich composition of flavonoids, phenolics, saponins, and alkaloids. Therefore, the rich antioxidant phytochemical composition of SEAL gives credence to the folklore application of the plant leaves for managing inflammation-related diseases. Furthermore, various bioactive compounds in the SEAL could act additively or synergistically to exhibit diverse pharmacological activities, which justifies the folkloric applications of plant leaves in managing many diseases. This concurs with the proposition of Zhang et al. (2019) that phytochemicals in plant extracts may follow synergistic or additive mechanisms in eliciting pharmacological effects. Yao et al. (2010) opined that antioxidant phytochemicals in plant materials, such as polyphenolics, synergize action toward antioxidant activities. Similarly, Zhang et al. (2019) posited that the varieties of phytocompounds in a plant extract correlate positively with its biological properties, such as antioxidant, anti-inflammatory, anti-diabetic, cytotoxic, antimicrobial, and neuromodulatory properties. Plants and herbs produce a diverse mix of phytoactive compounds capable of attenuating oxidative stress, which is the hallmark of most degenerative diseases (Suffredini et al., 2004). The rich phytochemistry of SEAL suggests the participation of the non-polar aspect of the plant extract in exerting its bioactivities. Our data agrees with Mah et al. (2017), which showed rich phytochemistry and potent anti-inflammatory, cytotoxic, and anti-cholinergic actions of the ethyl acetate leaf fraction of S. rhombifolia. Similarly, Mgbenema et al. (2015) demonstrated the potential of n-hexane and ethyl acetate fractions of S. acuta to protect against thioacetamide-induced liver damage in rats. Likewise, Ekramul Islam et al. (2003) reported potent cytotoxicity and moderate antibacterial activity of ethyl acetate extract of S. rhombifolia leaves. Another study by Shah and Khan (2014) highlighted the antidiabetic potential of S. cordata in alloxan-induced diabetic rats. Similarly, Shah et al. (2017) reported a rich phytochemical composition and excellent nephroprotective effect of ethyl acetate leaf fraction of S. cordata in rats. In a comparative study, Rachel et al. (2008) reported the potent inhibitory action of ethyl acetate leaf extract of S. acuta against fungi (Aspergillus niger and Candida albicans), gram-negative bacteria (Proteus vulgaris and Escherichia coli), gram-positive bacteria (Bacillus subtilis and Staphylococcus aureus), and clinical isolates of S. aureus and Enterococcus faecalis. Similarly, Sholichah (2017) reported that the antioxidant activity of the ethyl acetate leaf fraction exhibited more potential compared to that of the ethanolic leaf extract. Their report was anchored to the phytochemical mix and rich composition of polyphenolics, flavonoids, and alkaloids in the ethyl acetate fraction of Ocimum basilicum leaves compared to the crude ethanolic leaf extract.

Table 3. High-performance liquid chromatography (HPLC) profile of SEAL

<table>
<thead>
<tr>
<th>Peak No</th>
<th>Peak ID</th>
<th>Retention time (min)</th>
<th>Peak height</th>
<th>Peak area</th>
<th>Conc. (ppm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Phenylacetic acid</td>
<td>2.555</td>
<td>44987.012</td>
<td>11770.000</td>
<td>96.596</td>
</tr>
<tr>
<td>2</td>
<td>Caffeic acid</td>
<td>1.182</td>
<td>1293.333</td>
<td>36927.199</td>
<td>3.1631</td>
</tr>
<tr>
<td>3</td>
<td>Ellagic acid</td>
<td>1.682</td>
<td>214.939</td>
<td>2423.200</td>
<td>0.2076</td>
</tr>
<tr>
<td>4</td>
<td>Naringenin</td>
<td>4.973</td>
<td>27.679</td>
<td>831.700</td>
<td>0.0337</td>
</tr>
</tbody>
</table>

*Conc.: Concentration (1 ppm = 10-4 mg/ml/100 g = 1 µg/l = 1 µg/ml = 1 µg/g)

3.5. Acute toxicity

The result of the acute toxicity study showed that oral administration of SEAL was safe up to 5000 mg/kg bw (p.o.) and did not pose any apparent adverse effect in mice.

3.6. Analgesic properties of SEAL

3.6.1. Inhibitory action of SEAL on acetic acid-induced pain model

The ability of SEAL to inhibit writhing syndrome induced by acetic acid was tested. The result (as presented in Figure 3) showed that SEAL efficiently and dose-dependently suppressed acetic acid-induced writhing in mice. Mice in the positive control group produced 68.75 ± 1.11 writhes in 30 minutes after induction. In contrast, writhes count (28.01 ± 0.89, 33.40 ± 2.51, and 36.05 ± 1.36) observed in animals pretreated with various dosages (200, 400, and 600 mg/kg bw) of SEAL, respectively, reduced (p < 0.05) after 30 min of induction and was on par with the number of writhes (26.00 ± 1.08) observed in acetic acid-challenged mice pretreated with aspirin. The peak inhibitory action of SEAL on writhing syndrome (60.00%) occurred at the minimum dose (200 mg/kg bw) and was on par (p > 0.05) with the % writhing inhibition (62.18%) observed in the standard control. Conversely, Nwankwo et al. (2023b) recorded peak inhibitory action (66.54%) of ethanolic leaf fraction of S. linifolia at 600 mg/kg bw (p.o.), and this was superior to that of SEAL. This could be due to their differences in phytochemical profile. Furthermore, the possible mechanism of the analgesic action of SEAL was investigated using different models. Writhing, a term that denotes the nociceptive response of hind limb extension, stretching, trunk twisting, or contraction of the animal’s abdomen such that it touches the floor, is used to indicate pain. The irritating, acetic acid, is usually injected intraperitoneally to elicit discomfort and writhing in laboratory animals (Koster, 1959). The writhing model can screen for antinociceptive properties of pharmacological agents (Okokon et al., 2012). The pain-assuaging potential of the test compounds is deduced from a decrease in writhes count throughout the study. In animals, intraperitoneal injection of acetic acid induces pain and writhing by increasing the permeability of tiny capillaries and elevating the levels of prostaglandin F2 (PGF2) and PGE2 in peritoneal fluid (Bentley et al., 1983). Although this model could be used to assess the analgesic
attributes of pharmaceutical drugs, it is insufficient to determine precisely the involvement of a peripheral or central mechanism of action of anti-nociception since it can stimulate a broad range of nociceptors that respond to narcotics, morphine, NSAIDs, and other CNS-potent medications, which indirectly results in prostanoids production (Chan et al., 1995). Investigators usually combine pain models such as formalin with the writhing model to discriminate between central and peripheral pain (Okokon et al., 2012). Our data (Figure 3) showed that pretreatment with SEAL exerted dose-dependent inhibition of writhing syndrome in mice and was comparable to the NSAID. Our findings suggest the involvement of a peripheral mechanism of pain inhibition by SEAL. Our data could be explained further by the model’s possible activation of peripheral receptors, specifically local peritoneal receptors lining the surface of cells populating the peritoneal cavity (Liu et al., 2021). Furthermore, whereas aspirin was considerably ($p > 0.05$) efficacious in this model, the antinociceptive action of SEAL at a lower dosage (200 mg/kg bw) was not significantly ($p > 0.05$) different from that of 100 mg/kg bw aspirin. Our findings are consistent with those of da Rosa et al. (2019) and Shaheen et al. (2017), who used acetic acid-challenged mice to demonstrate the antinociceptive effects of leaf extracts of $S$. tuberculatae and $S$. rhombifolia, respectively.

3.6.2. Inhibitory effect of SEAL on formalin-induced pain model

The inhibitory action of SEAL on paw biting and licking in formalin-challenged mice is shown in Figure 4. From the result, the infusion of freshly prepared formalin solution into mice’s left hind paws resulted in biphasic nociceptive responses, which included licking and biting of the affected paw for an extended period. The positive control mice spent 313.20 ± 15.02 sec, being the first phase (0 - 5 min) and 307.2 ± 15.07 sec, being the second phase (15 - 30 min) biting and licking the affected paw in response to the irritant. However, mice in groups administered SEAL before induction spent less time ($p < 0.05$) responding to the irritant when compared to the positive control. Animals in groups pretreated varying concentrations (200, 400, and 600 mg/kg bw p.o.) of SEAL before induction spent 231.60 ± 8.04, 212.4 ± 3.05, and 205.2 ± 5.10 sec, respectively, responding to the irritant, and were on par with the time (181.8 ± 9.50 sec) spent by formalin-challenged mice pretreated with 100 mg/kg bw (p.o.) aspirin in response to the irritant. Furthermore, in the first phase of the model, administration of 600 mg/kg bw (p.o.) SEAL produced appreciable inhibitory action (34.48%) on formalin irritation; however, 100 mg/kg bw (p.o.) aspirin exhibited superior ($p < 0.05$) inhibition (41.95%).
Furthermore, in the second phase, mice in groups that received 200, 400, and 600 mg/kg bw (p.o.) SEAL before induction, spent a shorter time (131.00 ± 7.10, 135.01 ± 6.60, and 127.01 ± 3.50 sec) responding to the irritant, and these were on par with the irritation response time (109.8 ± 4.02 sec) spent by aspirin pretreated mice challenged with formalin. The highest inhibitory action (58.79%) on formalin irritation exhibited by SEAL occurred at its highest concentration (600 mg/kg bw). However, aspirin demonstrated superior inhibitory action (64.26%). Infusion of formalin in animals has been established to magnify significantly the spontaneous response of C fiber afferents, culminating in distinctive quantifiable pain behaviors, such as licking and biting the affected paw. The formalin model is deemed better than other pain models because it can simulate tonic and acute pain in a relatively short period (∼1 h) (Zakaria et al., 2008). It has been proposed that the formalin model produces specific biphasic nociceptive responses in mice (Hunskaar et al., 1985). The formalin direct interaction with nociceptors characterizes the first phase (15-30 min), while the second phase (15-30 min) is characterized by inflammatory reaction cascades (Hunskaar et al., 1985; Tjølsen et al., 1992; Zakaria et al., 2008). Pain remedies that work centrally, such as morphine, act by inhibiting both stages of the model; however, anti-pain agents that act peripherally, such as aspirin, primarily suppress the later stage of the model. The formalin test (Figure 4) indicated that SEAL efficiently (p < 0.05) inhibited both stages of nociception, implying a peripheral anti-nociception pathway while also implying the participation of a central anti-pain mechanism. Our data agrees with the reports of Bonjardim et al. (2011) which showed that S. cordifolia leaf extract suppressed orofacial pain response in mice.

![Figure 4](image-url)  
**Figure 4.** Inhibitory action of SEAL on paw biting and licking induced with formalin

Data are presented as mean ± SD (n = 8). Asterisks (‘*, ’**, and ’***’) on a column indicate the degree of significant difference (p < 0.05) of the column compared to the control.

4. Conclusions

Natural products derived from medicinal plants, whether as a pure compound or standardized extract, offer limitless prospects for new therapeutic leads due to the unrivaled abundance of chemical variety. Due to an increased desire for chemical diversities in screening programs seeking potent drug-like compounds from natural sources, worldwide interest in botanicals and herbs with folklore relevance has grown. Our data revealed that SEAL comprises phytoactive compounds with known bioactivities. The SEAL exhibited peripheral and CNS-mediated antinociceptive actions, validating its extensive folkloric use in treating inflammatory diseases. Based on our findings, the potenti biological activities of the plant decoctions/extract may involve the active participation of bioactive compounds in the plant extract’s non-polar fraction. Hence, further research to purify, isolate, and characterize the individual bioactive principles of the leaf fraction will be relevant to the medical and pharmaceutical industries.

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Conflict of interest

The authors confirm that there are no known conflicts of interest.

Statement of ethics

Ethical clearance was obtained from the Research and Ethics unit of the Department of Biochemistry, University of Nigeria, Nsukka (No: UNN/CH/9102). No human subjects were used in the research.

Availability of data and materials

The datasets of this study is available upon request.

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Supplementary File

None.

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