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Phytochemical Composition, Antioxidant, and Antinociceptive Activities of *Spilanthes Oleracea* Methanol Leaf Extract

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ABSTRACT

BACKGROUND

The pharmacological effects of medicinal plants are linked to their phytochemical contents. *Spilanthes oleracea* is a popular medicinal plant commonly used for the treatment of pain, including toothaches. This study is aimed at identifying the phytochemicals, antioxidant and antinociceptive activities of the crude methanol extract of S. *oleracea* and validating its use for traditional treatment of pain.

METHODS

Dry *S. oleracea* leaf samples were pulverized and their bioactive components extracted using analytical grade methanol (Fisher Scientific, Fair Lawn, New Jersey). Crude extract was screened for presence of phytochemicals and analyzed for antioxidant and anti-nociceptive activities, using the Reducing Power assay and Mouse Writhing model, respectively.

RESULTS

Results obtained revealed a total phenol concentration of 17.73 mgGAE/g ± 0.02 mgGAE/g of extract; total flavonoid concentration of 43.52 mgQE/g ± 0.01 mgQE/g of extract and total tannin concentration of 7.0 mgTA/g ± 0.05 mgTA/g of extract, while the reducing power of 200 mg/ml of extract compared favorably with that of 1 mg/ml of standard ascorbic acid. Also, 200 mg/kg of extract reduced the average number of acetic acid-induced writhing reactions in mice from 18.5 ± 1.6 in the normal control to 9.0 ± 0.4, as against 100 mg/kg of standard aspirin which reduced the average number of writhing reactions from 18.5 ± 1.6 to 11.3 ± 0.7.

CONCLUSION

The methanol extract of *S. oleracea* was found to contain important phytochemicals with significant antioxidant and anti-nociceptive activities that could be responsible for its traditional uses in the treatment of pain.

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KEYWORDS

Spilanthes oleracea; Antinociceptive; Phytochemical; Antioxidant

INTRODUCTION

Phytochemicals are a group of biologically active compounds that occur naturally in plants, where they influence their color and aroma and protect them against diseases and environmental hazards [1]. They are generally referred to as '*plant secondary metabolites*' and include polyphenols, flavonoids, steroidal saponins, tannins and alkaloids [2], all of which play important roles in the prevention of chronic diseases when consumed by man. A large number of phytochemicals occur as components of food, beverages and herbs, where they are often called '*nutraceuticals'* in reference to their capacity to prevent and treat complex human diseases such as cancers, diabetes mellitus, cardiovascular diseases, nervous disorders and Alzheimer's diseases [3].

Animal studies have also revealed several biological activities of phytochemicals, including antioxidant, anti-microbial and anti-nociceptive activities, with wide applications in pharmaceutical products [4]. Presently, over 200 known species of plants are considered to be medicinal, with their phytochemical contents mediating their pharmacological activities. Approximately 25% of all known medicines are also of plant origin [5].

The plant *Spilanthes oleracea* is a popular plant across the world, with its extracts being applied for treatment of various diseases in different countries. Different parts of the plant are also widely applied for treatment of toothaches in many countries, with leaves and flower heads observed to be very effective against caries-induced dental pain [6]. Apart from pain arising from dental caries, extracts from different species of *Spilanthes*, including *S. oleracea* are also used for the management of specific conditions in different countries. For example, *S. oleracea* from Nigeria and Sri Lanka have been observed to be effective against sialagogue [7, 8], while *species* from India are effective against mouth ulcers, boils and wounds [9]. In Uganda, extracts of *S. oleracea* are commonly used for the induction of labor during childbirth [10]. The variation in the pharmacological uses of medicinal plants in different parts of the world is influenced by their phytochemical content and biochemical compounds identified. Biosynthesis of phytochemicals is in turn, influenced by environmental factors such as extremes of temperature, salinity, drought, radiation, quality of soil and other stressful conditions [11].

Identification of the phytochemical constituents of medicinal plants is therefore considered as an important step towards establishing a relationship between bioactive compounds of indigenous medicinal plants and their observed pharmacological effects [12]. Considering its importance in the traditional management of pain, the present study aims to examine the phytochemical composition, antioxidant and anti-nociceptive activities of *S. oleracea* leaf methanol extract.

MATERIALS AND METHOD

Collection, Identification and Preparation of Plant Materials

Whole plants of *Spilanthes oleracea* were collected from their natural habitat in a small swampy garden around the Otite axis of Sapele, Delta State, Nigeria. Oral and written permission for collection of *S. oleracea* samples were obtained from local owners of the garden. Plants were collected during the rainy season and authenticated by Dr. E. I. Aigbokhan of the Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin. Following proper authentication, *S. oleacea* specimen was assigned voucher number UBH-S501 and fresh samples deposited at the Herbarium of the University of Benin.

Plants were washed once under a running tap, after which all leaves were detached, dried initially in the sun for 24 hours and further air-dried for 52 hours at temperatures below 40°C. Dried *Spilanthes oleracea* leaves were pulverized using a mortar and pestle, after which powdered plant materials were stored in sealed glass containers until when needed.

Preparation of Methanol Extract of S. oleracea Leaf

Pulverized *S. oleracea* leaf (200 g) was introduced into a glass beaker. A measuring cylinder was used to measure 1 liter of analytical grade methanol (Fisher Scientific, Fair Lawn, New Jersey) purchased from MOSDELIC Nigeria Limited, University of Benin, Benin City Nigeria and introduced into the glass beaker containing 200 g of pulverized *S. oleracea* leaf. Samples were left to stand for 48 hours, with frequent vigorous shaking. After 48 hours, all solvents were decanted into fresh clean glass containers and filtered through fresh cotton, followed by filtration through filter papers. Filtrates were collected in clean glass beakers, after which solvents were evaporated at 38°C in a rotary evaporator. Following solvent evaporation, residual masses of extracts were collected, weighed and percentage yield of extracts calculated (Table 1), using the formula [13]:

Percentage Yield = Mass of extract/Mass of pulverized samples × 100

Concentrated extracts were transferred into sterile sample bottles, sealed tightly, labeled and stored in the refrigerator at 4°C until when needed.

Sample	Weight of Pulverized Plant Sample (g)	Weight of Crude Extract Obtained (g)	Yield of Crude Extract (%)
Methanol Extract of *S. oleracea* (Leaf)	200	13.4	**6.7**

Table 1: Percentage yield of methanol extracts of *S. oleracea* leaf.

Qualitative Phytochemical Screening of Methanol Leaf Extract

A sensitive weighing balance was used to weigh 2 g of crude plant extract and placed in a glass beaker. This was followed by addition of 15 ml of analytical grade methanol and mixture shaken vigorously, until extract was completely dissolved.

Solutions of crude extract was filtered using Wart's filter papers and glass funnels in order to remove any impurities or debris present. Filtrates of crude extract was collected in clean dry glass beakers and qualitatively screened for presence of alkaloids, saponins, terpenoids, steroidal saponins, tannins, phenols and flavonoids, using methods previously described by different authors [14-17].

Quantitative Phytochemical Screening of Methanol Leaf Extract

Determination of total phenol

Total phenol in crude methanol extract of S. oleracea was estimated using methods previously described [18]. Specifically, 1 mg/ml of crude extract was prepared by dissolving 0.02 g of extract in 20 ml of distilled water. Serial dilutions of 0.01 mg/ml, 0.025 mg/ml, 0.05 mg/ml, 0.075 mg/ml, 0.1 mg/ml and 0.125 mg/ml of gallic acid were prepared in methanol. A 10-fold dilution of Folin Ciocalteau's reagent was also prepared by adding 1 ml Folin Ciocalteau's reagent to 9 ml of distilled water (1:10 v/v). Next, 0.5 ml each of filtrate of crude extract was added to 2.5 ml of 10-fold dilution of Folin Ciocalteau's reagent and 2 ml of 7.5 % Na_2CO_3 in test tubes and mixed vigorously. Similarly, standard solution of gallic acid was prepared by dissolving 0.02 g of gallic acid in 20 ml of methanol and serial dilutions prepared as previously described. Mixtures were incubated at room temperature for 30 minutes, after which absorbance were measured at 760 nm. All experiments were performed in triplicates and total phenolic content in crude extract estimated as gallic acid equivalent.

Determination of total flavonoid

Total flavonoid in crude methanol extract of S. oleracea was estimated using methods previously described [19]. A weighing balance was used to weigh 0.02 g of crude extract and dissolved in 20 ml of distilled water to give a concentration of 1 mg/ml solution, from which 0.5 ml of prepared extract was added to 1.5 ml of methanol and 0.1 ml of 10% Al_2Cl_3, followed by 0.1 ml of potassium acetate and 2.5 ml of distilled water. Mixture was properly shaken and incubated for 30 minutes at room temperature, after which the absorbance was measured at 415 nm using a spectrophotometer. Results were expressed in milligram quercetin equivalents/gram of extract (mg QE/g Extract). The standard curve was prepared with quercetin in six different concentrations of 10 mg/ml, 25 mg/ml, 50 mg/ml, 75 mg/ml, 100 mg/ml and 150 mg/ml.

Determination of total tannin

The total tannin in crude extract was also estimated using methods previously described [20]. A 1 mg/ml concentration of crude extract was prepared by dissolving 0.02 g of extract in 20 ml of distilled water, after which 0.5 ml of prepared extract was added to 1.25 ml of Folin Dennis reagent and 2.5 ml of 10% Na_2CO_3. Mixture was vigorously mixed and incubated at room temperature for 30 minutes and absorbance measured at 760 nm, using a spectrophotometer. Results were expressed in milligrams tannic acid equivalents/gram of extract (mg TA/g Extract). Standard curve was also prepared with tannic acid in six different concentrations of 10 mg/ml, 25 mg/ml, 50 mg/ml, 75 mg/ml, 100 mg/ml and 150 mg/ml.

Reducing Power of Methanol Extract of S. oleracea Leaf

The reducing power of the methanol plant extract was evaluated according to methods previously described [21]. Different concentrations of aqueous solutions of crude extract ranging between 0.1 mg/ml - 1.0 mg/ml were prepared and 1 ml of each of prepared extract added to 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. Following proper mixing using a vortex, solutions of extract were incubated at 50°C for 20 minutes, after which 2.5 ml of 10% trichloroacetic acid was added to each solution of extract in order to stop the reaction. Next was addition of 0.5 ml of 0.1 % Fe_2Cl_3 to each solution of extract and measurement of absorbance at 700 nm.

Ascorbic acid (1 mg/ml) was used as positive control and higher absorbance values indicated higher reducing powers. The reducing power of the crude extract was calculated using the equation:

% Reducing power = Change in absorbance of sample/Change in absorbance of control × 100

Peripheral Anti-nociceptive Activity of Crude Plant Extract - Mouse Writhing Experiments

The peripheral anti-nociceptive activity of the crude plant extract was evaluated, using methods previously described [22], with slight modification.

Preparation of animals

A total of 15 adult (12 weeks - 14 weeks old) female Swiss albino mice weighing between 20 g and 29 g were purchased from the animal house of the Department of pharmacology, University of Benin, Benin City Nigeria. Animals were kept under standard conditions with 12 hours light and 12 hours dark cycle and provided with standard diet (pelletized finisher from top feeds Nigeria limited) and water, ad libitum.

All animals were kept in the laboratory for 12 days prior to experiments, in order to allow adequate acclimatization and fasted overnight before the experiments, in order to avoid any possible food-extract/drug interactions. Strict adherence to ethical principles in accordance with guidelines provided by the ethics committee on animal experiments, University of Benin, was ensured.

Preparation of Stock Solution of Extract and Standard

Stock solution of 20 mg/ml of extract of *S. oleracea* leaf was prepared by dissolving 0.1 g of sample in 5 ml of distilled water.

Grouping of Animals and Administration of Extract and Controls

Following adequate acclimatization, all mice were weighed in a Mettler Toledo PM4800 Delta Range weighing balance, divided into three groups of five animals per group and marked as appropriate. The first group of five mice (group 1) was administered 200 mg/kg body weight of crude extract orally, while groups 2 and 3 animals were orally administered 100 mg/kg body weight of Aspirin and 0.2 ml of 0.9 % Normal Saline, respectively. All animals were left for one hour.

After one hour, all animals were administered 0.2 ml of 0.6 % acetic acid intraperitoneally and observed for writhing reactions at time intervals of 0 minutes - 5 minutes, 5 minutes - 10 minutes, 10 minutes - 15 minutes, 15 minutes - 20 minutes, 20 minutes - 25 minutes and 25 minutes - 30 minutes. The average number of writhing reactions for individual animals at the end of 30 minutes was calculated, after which the average number of writhing reactions for each group of animals was also estimated and recorded. The group with the lowest average number of writhing reactions was regarded as possessing the most potent anti-nociceptive activity against Acetic acid-induced nociception and vice versa.

Statistical Analysis

Values obtained were analyzed using Graph pad prism, version 8. Data was presented as mean ± SEM and statistical significance between treated and control groups were calculated using one way ANOVA, followed by Turkey's multiple comparisons test, where $P \leq 0.05$ was considered statistically significant.

RESULTS

Figure 1 - Figure 3.

Table 2 and Table 3.

Figure 1: Samples of whole plants of S. oleracea obtained from a swampy area around the Otite axis of Sapele, Delta State, Nigeria.

	Alkaloids	Flavonoids	Phenols	Saponins	Tannins	Steroidal Saponins	Terpenoids
Methanol Extract of *S. oleracea* (leaf)	+++	++	+++	++	++	++	+++

KEY: +++: Abundantly Present; ++: Moderately Present; +: Minimally Present

Table 2: Qualitative phytochemical screening of methanol extracts of *S. oleracea* leaf.

Plant Extract	Total Phenol (mg GAE/g of Extract)	Total Flavonoid (mg QE/g of Extract)	Total Tannin (mg TA/g of Extract)
Methanol Extract of *S. oleracea* (Leaf)	**17.73 ± 0.02**	**43.52 ± 0.01**	**7.0 ± 0.05**

Values are expressed as percentage dry weights (% dw) of mean ± SEM, n = 3/group.

Table 3: Total phenol, flavonoid and tannin contents of methanol extracts of *S. oleracea*.

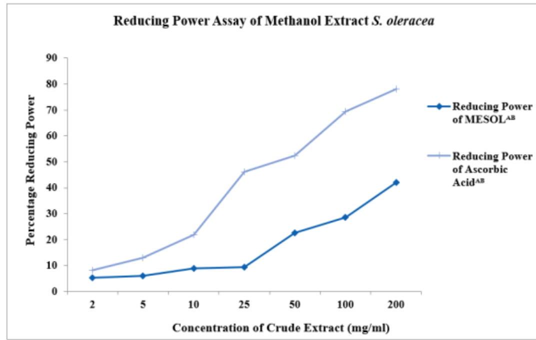

Figure 2: Reducing power of crude methanol extract of *S. oleracea*.

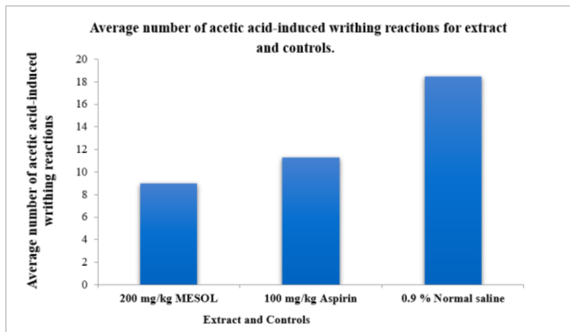

Figure 3: The methanol extract of S. oleracea leaf reduced the average number of acetic acid-induced writhing reaction in mice from 18.5 ± 1.6 (0.9% Normal saline) to 9.0 ± 0.4 (200 mg/kg MESOL), as against 100 mg/kg of standard aspirin which reduced the average number of writhing reactions from 18.5 ± 1.6 (0.9% Normal saline) to 11.3 ± 0.7 (100 mg/kg Aspirin).

DISCUSSION

Medicinal plants have been widely used for treatment of many disease conditions since time immemorial, with minimal side effects [23]. The pharmacological effects of medicinal plants are mediated by phytochemicals which are chemical substances with strong protective and disease-preventive properties [24]. Among the numerous medicinal plants known to possess strong pharmacological effects, *Spilanthes oleracea* is a popular member of the Asteraceae family found abundantly in tropical and sub-tropical regions of India, South America, Brazil and Africa. Many studies on *S. oleracea* plant have reported antifungal, antipyretic, anticonvulsant, antioxidant, antimicrobial, anti-inflammatory, anti-nociceptive, immune-stimulatory, vaso-relaxant, diuretic, analgesic and anesthetic effects [25]. Consequently, the plant is used for treatment of many diseases in different countries, with several biochemical compounds mediating observed pharmacological effects.

Phytochemical analysis of *S. oleracea* species has revealed presence of alkaloids, tannins, steroids, carotenoids, carbohydrates and alkamides [26]. In consonance with previous observations, results of the present study revealed an abundance of alkaloids, phenols and terpenoids in the methanol extract of *S. oleracea* leaf. Other phytochemicals observed in moderate concentrations in the methanol extract of *S. oleracea* leaf include flavonoids, saponins, tannins and steroidal saponins (Table 2). Results of quantitative phytochemical screening also confirmed presence of phenol (17.73 ± 0.02 mg GAE/g of extract) and flavonoid (43.52 ± 0.01 mg QE/g of extract) in the methanol extract of *S. oleracea* (Table 3).

Alkaloids are among the largest groups of natural products, with approximately 12,000 compounds recorded so far [27]. Some of the numerous pharmacological activities that have been attributed to alkaloids include analgesic, antiseptic, anticancer, anti-inflammatory, antiulcer and antidiarrheal activities. Others include anti-fungal, cardio-protective, antimalarial, antioxidant, vaso-relaxant, immune-regulatory, cerebro-protective and anxiolytic effects [28].

The alkylamide, spilanthol [(2E, 6Z, 8E)-N-isobutylamide-2, 6, 8-decatrienamide], is widely believed to be responsible for most of the pharmacological effects of *S. oleracea* leaf [29]. Alkylamides are a group of alkaloids that occur naturally in roots, leaves, stems and flowers of medicinal plants, where they regulate their growth and biomass. They could either have an aliphatic, aromatic or cyclic amine residue, with a C8 to C18 saturated or unsaturated fatty acid chain [30].

Aliphatic amines, including N-isobutyl, N-2-methylbutyl and N-phenethyl groups have been observed to be predominant among the Asteraceae family of plants to which *S. oleracea* belongs, with C10, C11 and C12 residues representing approximately 72% of Asteraceae-based alkylamides, while C14 and C18 residues account for about 13% of Asteraceae alkylamides [31]. Furthermore, the aliphatic amines have been associated with the antiseptic, anti-bacterial, anti-inflammatory, immune-stimulatory and analgesic effects of *S. oleracea* plant [32]. Inhibition of peripheral mediators of pain such as prostanoids, including PGE_2 and $PGF_{2\alpha}$ and lipoxygenase products, as well as inhibition of peripheral secretion of neuropeptides are known mechanisms of *S. oleracea*-mediated anti-nociceptive effects [33].

In the present study, methanol leaf extract of *S. oleracea* reduced the average number of acetic acid-induced writhing reactions in mice from 18.5 ± 1.6 to 9.0 ± 0.4, as against 100 mg/kg of standard aspirin which reduced the average number of writhing reactions from 18.5 ± 1.6 to 11.3 ± 0.7, suggesting that this extract could potentially inhibit the secretion and release of prostanoids and neuropeptides (Figure 4).

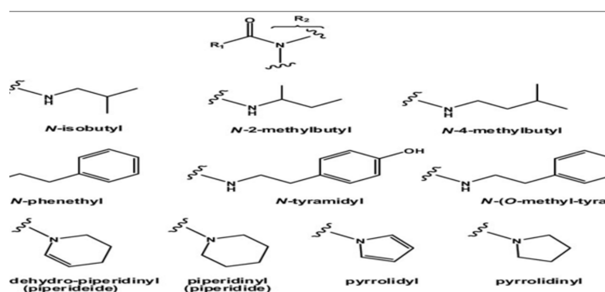

Figure 4: Structures of aliphatic amines of Asteraceae alkylamides [31].

Apart from alkaloids, phenolic compounds, including tannins and flavonoids were also observed to occur in moderate to high concentrations in the methanol extract of *S. oleracea* leaf. There are currently over 8000 reported plant-based phenolic compounds, making them one of the most diverse groups of phytochemicals [34].

Phenolic compounds can broadly be classified into three important groups: Flavonoids, phenolic acids and polyphenols, with flavonoids accounting for more than half of all known phenolics hence, the most widely studied group [35]. They are hydroxyl (-OH) containing compounds, with their OH groups directly bonded to aromatic hydrocarbons. Although widely harnessed for their free-radical scavenging (antioxidant) activities, phenolic compounds, especially flavonoids, have also been observed to possess significant anti-microbial, anti-inflammatory, anti-ulcer, anti-tumor, anti-depressant and cytotoxic activities [36].

According to Saxena and colleagues, the antioxidant effects of phenolic acids and flavonoids are dependent on the number of free hydroxyl groups and their positions in these structures. Studies have also revealed several mechanisms of action of phenolic compounds, including scavenging of reactive oxygen species (ROS) and reactive nitrogen species (RNS), suppression of the formation of free-radical species through enzyme inhibition and enhancement of other antioxidant defense systems of the body [37]. Examples of phenolic compounds that have been isolated from *Spilanthes oleracea* plant include vallinic acid, trans-ferulic acid, trans-isoferulic acid and scopoletin [38].

Terpenoids are another group of phytochemicals that have been consistently associated with the Asteraceae family of plants, including *S. oleracea* species, where they mediate important biological activities such as anti-cancer, analgesic, anti-inflammatory, anti-microbial, anti-fungal, anti-viral, and anti-parasitic activities [39]. They are also widely applied as flavors and additives for food and fragrances for perfumes [40]. Terpenoids such as hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), sesterpenes (C25), triterpenes

(C30), tetraterpenes (C40), and polyterpenes (>C40) are derivatives of isoprene (C5) and occur abundantly in plants, fungi and marine animals [41]. The drugs artemisinin (an anti-malarial) and arglabin (an anti-neoplastic agent) are both sesquiterpenes obtained from *Artemisia* species and approved for treatment of malaria and cancer, respectively. Similarly, the drug paclitaxel is a terpenoid-derived anti-cancer agent used for the management of different types of malignancies [42]. Some of the pharmacologically active terpenoids that have been characterized from *Spilanthes* species include β-Isocomene, lupeyl acetate, caryophyllen-l,l0-epoxide, alantolactone, eudesmanolide [43], N-2-Phenylethylcinnamide, stigmasterol and Taraxasterol acetate [44].

CONCLUSION

The rich phytochemical composition and significant antioxidant properties of the methanol extract of *S. oleracea* are largely responsible for its pharmacological activities including peripheral anti-nociceptive effects, which contribute to its wide application for the traditional management of pain, especially toothaches. However, the specific mechanism of action and biochemical compounds responsible for inhibition of peripheral mediators of pain such as prostanoids are yet to be identified.

DECLARATIONS

Ethical Approval

Ethical approval for this study and use of plant materials was obtained from the Institutional Ethical Review Committee, Life Sciences, University of Benin, Benin City, Edo State, with approval number: LS20017 and dated 25th of March, 2020. All guidelines pertaining to the use of plant materials as spelt out in the ethical approval were duly followed.

Competing Interests

The authors declare no competing interests in the publication of this manuscript.

Authors' Contributions

Author O.O conceived and carried out most of the experiments and wrote most of the draft manuscript.

P.O.U directed and supervised the work and reviewed the draft manuscript.

K.O carried out some of the phytochemical screening and wrote parts of the draft manuscript.

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