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GC-MS Analysis and *in vivo* Anti-Inflammatory, Analgesic Activities of *Phyllanthus niruri* Linn and *Sida acuta* Burm, Used to Treat Malaria in Togo

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Abstract

The use of plants in traditional medicine is an alternative solution to address the adverse effects of conventional anti-inflammatories. This study evaluated the phytochemistry, anti-inflammatory, analgesic and toxicity activities of the hydroethanolic extracts (HE) alone and the combination of *Phyllanthus niruri* Linn and *Sida acuta* Burm. Total phenolics were quantified using the Folin-Ciocalteu method, condensed tannin by the Butanol-HCl method, and flavonoid by the Aluminum chloride method. Gas chromatography coupled with Mass Spectrometry (GC-MS) analysis was used to identify several compounds in the extracts. Anti-inflammatory activity and analgesic activity, respectively, were carried out using the paw edema method in rats and pain method induced by intraperitoneal injection of acetic acid in the animal model. Acute, and subacute oral toxicity of extracts was conducted according to OECD n˚423 for the testing of chemicals and n˚407 for testing chemical products. Screening of *S. acuta* and *P. niruri* revealed that both of them contain alkaloids, phenolic compounds, terpenes and flavonoids, but *P. niruri* is richer in total phenols 14.69 ± 0.22 mg GAE/g; proanthocyanidins 4.42 ± 0.01 mg CE/g; and flavonoids 264.21 ± 3.64 mg RE/g. GC-MS analysis was presented for *S. acuta* 13 and *P. niruri* 19 compounds. The compounds most represented are levoglucosan (16.01%) and neophytadiene (10.48%) for *S. acuta* and 2-[(3,4-Dimethoxyphenyl) methyl]-4-methoxy-4-oxobutanoicacid (24%), and (3R,4R)-3-(Benzo[d][1,3] dioxol-5-ylmethyl)-4-(3,4 dimethoxybenzyl) dihydrofuran-

2(3H)-one (18.34%) for *P. niruri*. The concentration of 400 mg/kg recorded the best anti-inflammatory activity. The analgesic effect's results showed that the hydroethanolic compound at a concentration of 800 mg/kg recorded the highest percentage inhibition (71.60% ± 5.32%) by the oral route. Toxicity results showed that the extracts alone and in combination also did not exhibit signs of toxicity. These results therefore validate the use of *S. acuta* and *P. niruri* in traditional medicine.

Keywords

Sida acuta, *Phyllanthus niruri*, Phytochemistry, Anti-Inflammatory, Analgesic, Toxicity, Togo

1. Introduction

Inflammation is the result of the body's anti-inflammatory response to various stimuli [1] [2], which may be physical, chemical, biological or infectious [3] [4]. To treat these manifestations, a variety of anti-inflammatory drugs are used, including steroids and non-steroids [5]. Although effective, these drugs are often accompanied by adverse effects that limit their long-term use [6].

However, herbal medicines and preparations are generally considered less toxic and less expensive, which has increased their use in alternative medicine and the consumption of these plant materials in many countries around the world [7]. According to the World Health Organization (WHO), nearly 80% of the world's population depend on traditional medicine for primary health care [8]. As such, medicinal plants remain the primary reservoir of new medicines, and are considered an essential source of raw material for the discovery of new molecules needed to develop future drugs [9]. It is therefore essential to explore plant-based medicines that could replace chemical drugs.

What's more, medicinal plants offer a remedy for inflammation and malaria, as several studies have demonstrated [10]-[12]. Indeed, they are well known for their analgesic effects based on phenolic compounds [13] [14]. These molecules are involved in the prevention of diseases such as cancer, diabetes, inflammation, cardiovascular disease and aging [15].

In Togo, two plants, *Sida acuta* Burm and *Phyllanthus niruri* Linn, belonging to the Malvaceae and Phyllanthaceae families respectively, have been identified for use in traditional medicine not only for their anti-inflammatory potential but also to treat malaria. Several studies have explored these compounds and their implication in the treatment of several diseases [16] [17]. Thus, *Sida acuta* Burm is supposed to possess a variety of new and known natural compounds, each conferring a unique biological activity. The most common phytochemical compounds found in *Sida acuta* Burm are alkaloids, flavonoids, steroids, terpenoids, tannins and phenolic compounds [18] [19].

Similarly, it has been noted that *Phyllanthus niruri* is traditionally used as an

anticancer, antioxidant, anti-inflammatory, antimalarial and analgesic [20]. Phytochemical studies have shown that *Phyllanthus niruri* contains important phytochemical compounds useful for eradicating and treating diseases, such as alkaloids, flavonoids, saponins, tannins, vitamin C and steroids [21] [22]. Studies carried out on these two plants, which are also used in traditional medicine in Togo [23]-[25], have given little attention to their anti-inflammatory, analgesic and toxic activity. Thus, the present study was designed to assess the phytochemistry, anti-inflammatory activity, analgesic activity and *in vivo* toxicity of co-administration of the hydroethanolic extract of *S. acuta* and *P. niruri*.

2. Material and Methods

2.1. Study Framework

This research was conducted at the Microbiology and Food Quality Control Laboratory of the Faculty of Food Science and Technology, at ESTBA, University of Lome as the Regional Center of Excellence in Poultry Sciences both located in Togo.

2.2. Chemicals and Reagents

Various chemicals and reagents such as methanol, magnesium turnings, iron trichloride, Dragendorff reagent concentrated sulfuric acid, aluminum chloride, rutin, Folin Ciocalteu reagent, gallic acid, potassium bicarbonate, butanol, HCl, 0.6 M sulfuric acid, 0.1% sodium phosphate, carrageenan and acetic acid were procured from Fisher (USA). Additionally obtained were hexane; pyridine; methoxamine hydrochloride; N methyl N (trimethylsilyl)trifluoroacetamide (MSTFA) from Merck (Sigma Aldrich), in Milan, Italy. Moreover, ketoprofen and paracetamol were obtained from Tongmei (Togo).

2.3. Plant Material

Fresh leafy twigs of *S. acuta* Burm. F. and *P. niruri* L. were collected on September 2021 from the agricultural area of Agoe-Nyive ((6˚14'11.0"N 1˚10'27.3"E) 6,236377, 1.174243) and Anfoin ((6˚19'46.5"N 1˚36'30.4"E) 6.329583, 1.608442), in the maritime region of Togo. Identification and authentication of the plants were carried out at the Botany and Plant Ecology Laboratory of the University of Lome with their respective codes, TOGO 15650 and TOGO 15651. After air-drying for one week at room temperature in the laboratory, the twigs were all pulverized to obtain the powder.

2.4. Extraction

The hydroethanolic extract was prepared by stirring 250 g of the powder for 48 h in 3000 ml of an ethanol-water mixture of 70:30. The mixture was then filtered with Whatman N˚1 filter papers. Alcohol was evaporated under reduced pressure using a rotary evaporator (Heidolph, Hei-VAP Precision, Germany), and then the extract was frozen and freeze-dried. Our previous optimizations tested the mixture

of the two extracts to identify which of the ratios was 3:1 (75% from *S. acuta* and 25% *P. niruri*). For GC-MS analysis, 10 g of each plant powder was dissolved in 100 mL ethanol to prepare their ethanolic extractions. Thereafter, the mixture was filtrated (using Whatman N˚1 filter papers) and freezer-dried after being vaporized under a vacuum using a rotary evaporator. The freeze-dried ethanolic extract was then tested using GC-MS.

2.5. Qualitative Phytochemical Testing of Extracts

To control secondary metabolites, the following main families were determined for HE of plants using classical methods of characterization. The iron trichloride test was used to control phenolic compounds, the interaction of test substances with magnesium paddles and HCl determined flavonoids; for saponosides—foam test, alkaloids—Dragendorff test and triterpenes and steroids-Liebermann-Burchard test. All determinations were carried out in triplicate with control [26].

2.6. Quantitative Phytochemical Testing of Extracts

Polyphenolics assay: Method using Folin Ciocalteu reagent was used for quantifying polyphenolics content [27]. Shortly after making serial dilutions (0.097 to 12.5 µg/ml) of a solution of gallic acid (5 mg/ml) using distilled water, a standard curve was established by extrapolation. Using 50 µl of the sample to be evaluated, another 100 µl of the Folin Ciocalteu Reagent (FCR) 10% was added. Both components were inserted into the wells of a 96-well plate. After 5 minutes of incubation at room temperature and protection from light, 100 µl potassium bicarbonate (75 g/l) was added to the mixture. After shaking, the different solutions were left to stand, protected from light, for 30 min. Optical densities (OD) readings were taken at 760 nm using a plate reader against a negative blank made up of a mixture of 100 µl FCR, 100 µl potassium bicarbonate and distilled water, and a positive blank made up of extract to be determined and distilled water. Three readings were taken per sample.

Proanthocyanidin assay: Proanthocyanidin was quantified using the butanol-HCl method [28]. The assay involved the combination of 0.2 ml of ammoniacal iron sulfate 20 g/l with 7 ml of a butanol/HCl solution 95/5 ml with 50 mg of each extract in tubes. The tubes were incubated for 45 min in a water bath at 95˚C, after which 200 µl of the contents of each tube were transferred to the wells of a microplate. The wells were then read at 550 nm using a plate reader. Each extract's optical densities were measured three times.

$$X = \left(OD \times 1CE/g\right)/0.280$$

OD = 280 g equivalent to 1% catechin; OD = optical density measured at 540 nm.

X = catechin equivalent concentration per gram (%CE /g).

Flavonoid content: The flavonoid content was determined by aluminum chloride method [29]. The extract or rutin at a concentration of 1 mg/ml in the amount of 500 µl was mixed with 500 µl 2% aluminum chloride ($AlCl_3$). After 10 minutes

incubation, the absorption in the UV was read at 415 nm using METASH UV-5200PC UV/VIS spectrophotometer (METASH, Shanghai, Chine). As a standard, rutin was used to create a calibration curve in the concentration range of 0 - 200 µg/ml of methanol. The determination was made based on the slope of the linear regression equation and expressed in µg RE/mg of dry extract. The test was conducted in triplicate.

2.7. Gas Chromatography-Mass Spectrometry Analysis

Sample preparation for CG-MS analysis: To enhance the suitability of polar and volatile metabolites for GC-MS analysis, the freeze-dried extracts underwent derivatization using MSTFA (N-Methyl-N-(trimethylsilyl) trifluoroacetamide), and methoxamine. In this process, 50 mL of pyridine containing 10 mg/ml of methoxamine hydrochloride were carefully added to 10 mg of each freeze-dried extract· Following a 17 h incubation period, 100 mL of MSTFA were added and allowed to react for 1 h. Subsequently, each sample underwent dilution with 600 mL of hexane before GC-MS analysis.

GC-MS analysis: Identification of compounds of ethanolic extract of *S. acuta* and *P. niruri* was determined with the method which used an Agilent Technologies model 7890B gas chromatograph (GC) coupled mass spectrometry (MS), a 5977B MSD also injector which was set at 250˚C (Agilent Technologies Inc., Santa Clara, CA) [30]. One microliter from each derivatized sample was injected in splitless mode into the gas chromatograph attached to a mass spectrometer. The injector temperature was set at 200˚C and gas flows through the column was 1 mL every minute. A DB5 MS column made of fused silica, 0.25 µm thick measuring 30m x 0.25 mm id., from J&M Scientific Inc. in Folsom CA was used. The temperature profile in the oven involved a 3 minute at 50˚C followed by an increase to 250˚C at a rate of 3˚C/min and then maintained at 250˚C for 25 minutes. The transfer line and ion source temperatures were 280˚C and 180˚C respectively. Ions were generated with an electron beam energy of 70 eV in electron impact ionization and recorded and 1.6 scans/s over a mass range of 50 to 550 m/z. Metabolite identification was carried out by comparing their mass spectra with those in the NIST08 mass spectral database.

2.8. Anti-Inflammatory Activity

The carrageenan-induced rat leg edema assay was used to evaluate the anti-inflammatory potential of the combination of plants [31]. According to body weight, the rats were divided into 4 groups of 5 and were subsequently fasted 12 hours before the experiment. The initial volume of the rat leg V0 was then measured using a water plethysmometer before receiving any treatment. The treatment was gently administered through gavage. More specifically, Group 1 rats were administered with physiological water at 10 mg/kg to serve as a control, whereas, rats in Group 2 were given the treatment at 400 mg/kg, Group 3 rats were treated at 800 mg/kg, and those in the fourth group were given the treatment at 20 mg/kg of

ketoprofen.

$$\%AUG = \left(\frac{V_t - V_0}{V_0} \right) \times 100$$

Legend: V_t is the Volume of the leg at time t (T1h, T3h and T6h); V_0 is the Initial volume of the leg.

Anti-inflammatory activity was assessed by calculating the X percent inhibition (%INH) of edema according to the formula:

$$\%INH = \left(\frac{\%AUG\ control - \%AUG\ Processed}{\%\ AUG\ control} \right) \times 100$$

Legend: %AUG Control is the percentage increase in rat leg volume of the control group; %AUG Processed is the percentage increase in rat leg volume of the treated group.

2.9. Analgesic Activity

Assessment of analgesic activity was performed using pain induced by intraperitoneal injection of acetic acid in the animal model [32]. The rats were fasted 17 hours before the experiment. Four groups of 5 rats were used and according to the route of administration of the extract as detailed in Table 1.

Table 1. Composition of groups according to the route of administration.

Oral route			
Group 1	Group 2	Group 3	Group 4
Physiological water (10 mg/kg)	Combined extract (400 mg/kg)	Combined extract (800 mg/kg)	Paracetamol (100 mg/kg)
Intraperitoneal route			
Group 1	Group 2	Group 3	Group 4
Physiological water (10 mg/kg)	Combined extract (400 mg/kg)	Combined extract (800 mg/kg)	Paracetamol (100 mg/kg)

Depending on the route of administration of the extracts to animals, the withdrawal period before intraperitoneal injection of acetic acid (0.6%) will be one and a half an hour respectively for the oral and intraperitoneal routes, respectively. Five minutes after the acetic acid injection, the number of contortions was counted in each rat for 15 minutes. The analgesic effect was assessed by calculating the percent inhibition (PI) according to the following formula:

$$PI = 1 - \left(\frac{Wt}{Wb} \right) \times 100$$

Legend: Wb is the average of the number of contortions of the rats in the control lot; Wt is the average of the number of contortions of the rats in the treated lot.

2.10. Acute Toxicity

The acute single-dose toxicity test was conducted according to the Organization for Economic Cooperation and Development (OECD) Test Guideline 423 [33]. Rats were acclimated, fasted overnight tagged and weighed before the experiment. Animals were randomly assigned to 4 groups (n = 4). Each group received orally: Group 1 (control, physiological water); Group 2 (extract from *S. acuta*) Group 3 (extract from *P. niruri*) and Group 4 (combined extracts of both). A single dose of 5000 mg/kg of the extracts was administered· The experiment was conducted on 4 groups, each receiving a single dose of the drugs· The animals were observed individually for the first five hours and then daily throughout the 14 days of the experiment. Signs and symptoms of toxicity have been recorded· Observations ranged from changes in skin, hair, salivation, and behavior, including various manifestations of tremors, convulsions, diarrhoea, drowsiness, coma, and death.

2.11. Subacute Toxicity

It was conducted by OECD Guideline 407 [34]. Sixteen female rats (109 ± 12 g BW) and sixteen male rats (124 ± 15 g BW) were used. Animals were randomly assigned to 4 groups (n = 8: 4 females and 4 males). Each rat in group 1 (control group) was given only physiological water. Groups 2, 3 and 4 received the hydroethanolic extract of *S. acuta*, *P. niruri* and the combined extracts of both at a dose of 1000 mg/kg BW, respectively, for 28 days. The animals were force-fed at about the same time each day. The animals were monitored for signs of toxicity and mortality twice daily for the entire 28-day experimental period. The weight of each animal was recorded daily throughout the experiment. At the end of the treatment on day 28, the body weight of all the rats was taken. The animals were anesthetized with chloroform, and blood samples were taken retro-orbitally from EDTA tubes and anticoagulant-free tubes. Haematological and biochemical parameters were recorded. As per OECD guidelines, the animals were then slaughtered, and visual gross aspects of the heart, lungs, liver, spleen, kidneys, ovaries, or testes were observed. Blood cell counts were analysed using the Sysmex SN A4201 automated system (Sysmex, Kobe Japan). Biochemical parameters were determined with automatic biochemical analyzers, models Roch/Hitachi Cobas C 311 (Hitachi, Tokyo, Japan) and Selectra PoM (Elitech, Netherlands). The study was approved by the Committee for Animal Experimentation Ethics of ESTBA-UL (N° 0003/2023-07/ESTBA-UL). A minimum number of animals was used to obtain reliable results.

2.12. Statistical Analysis

The statistical study was carried out using the statistical software Graph Pad Prism 8.02 and the Excel 2016 spreadsheet. Mean values were accompanied by the standard error on the mean. The difference between the means was statistically significant at the 5% level $p < 0.05$ and means were separated using the Tukey test.

3. Results

3.1. Phytochemical Composition of Extracts

Qualitative phytochemical tests: Crude extracts from HE were examined for alkaloids, phenolic compounds, saponins, terpenes/sterols and flavonoids. The tested extracts contained alkaloids, phenolics, terpenes and flavonoids. However, both extracts lacked saponins (Table 2).

Table 2. Results of qualitative phytochemical tests.

Extracts	*S. acuta*	*P. niruri*
Phenolic compounds	+	+
Alkaloids	+	+
Saponins	–	–
Terpenes/ sterols	+	+
Flavonoids	+	+

(+: presence; –: absence).

Total phenol, proanthocyanidin and flavonoid contents: Total phenol content was calculated using the equation given by the calibration curve of absorbance versus gallic acid mass. The findings reveal that the HE of *P. niruri* exhibited levels of phenols condensed tannins and flavonoids. Measuring at 14.69 ± 0.22 mg GAE/g extract; 4.42 ± 0.01 mg CE/g; and 264.21 ± 3.64 (µg RE/mg ES) for flavonoid content respectively. HE of *Sida acuta* recorded values of 5.66 ± 0.35 mg AGE/g extract; 3.84 ± 0.21 mg CE/g and 162.75 ± 7.10 (µg RE/mg) respectively for total phenols, proanthocyanidins and flavonoids (Table 3).

Table 3. Results for total phenols, proanthocyanidins and flavonoids in both extracts.

Extracts	Total phenol content (mg GAE/g)	Proanthocyanidin content (mg CE/g)	Flavonoid content (µg RE/mg ES
S. acuta	5.66 ± 0.35	3.84 ± 0.21	162.75 ± 7.10
P. niruri	14.69 ± 0.22	4.42 ± 0.01	264.21 ± 3.64

GAE: gallic acid equivalent; CE: catechin equivalent; RE: rutin equivalent.

3.2. GC-MS Analysis

The GC MS analysis of *S. acuta* identified 13 compounds (Figure 1) while *P. niruri* had 19 compounds (Figure 2). In Table 4 the predominant compounds for *S. acuta* included levoglucosan at 16.01%, neophytadiene at 10.48% and glutamic acid at 9.50%. Meanwhile in Table 5 for *P. niruri*, the compounds that were largely identified include (3R,4R)-3 Benzo[d] [1,3] dioxol-5-ylmethyl4 3,4 dimethoxybenzyl dihydrofuran 2,3H one was found to be at a concentration of 18.34%. while another compound named 2-[(3,4 Dimethoxyphenyl) methyl]-4-methoxy-4-oxobutanoic acid was present, in amount reaching up to 24%.

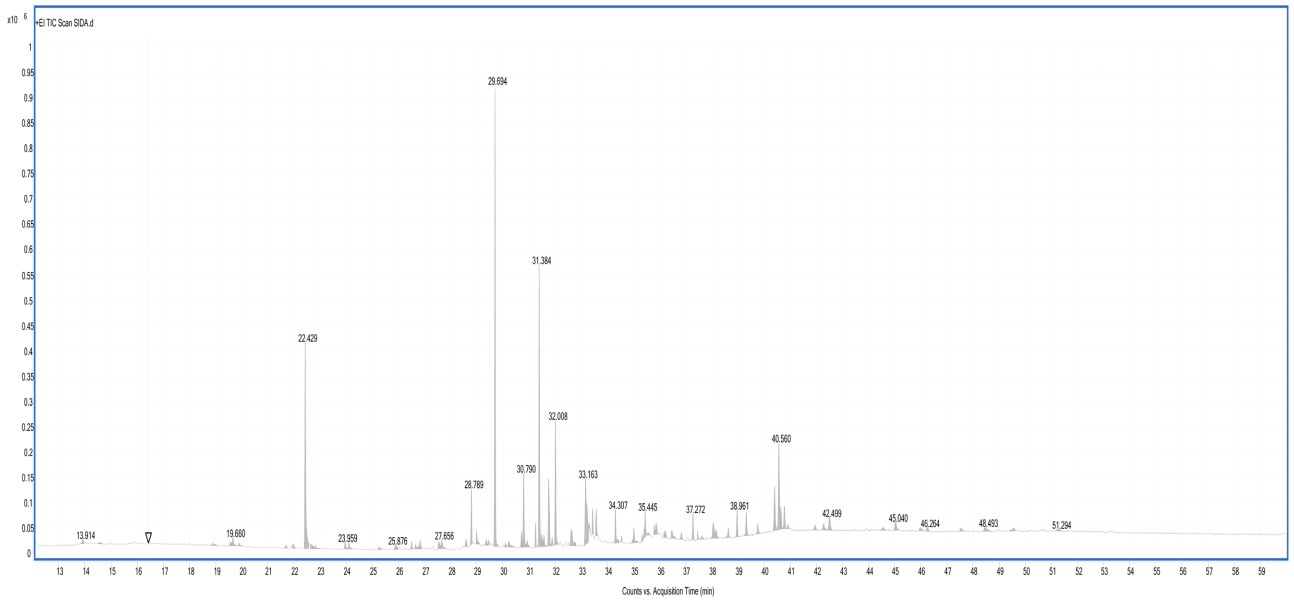


Figure 1. Chromatogram of ethanolic extract of *Sida acuta.* Each peak corresponds to a compound. Spikes appear based on retention time. A higher peak reflects a more abundant compound in the extract.

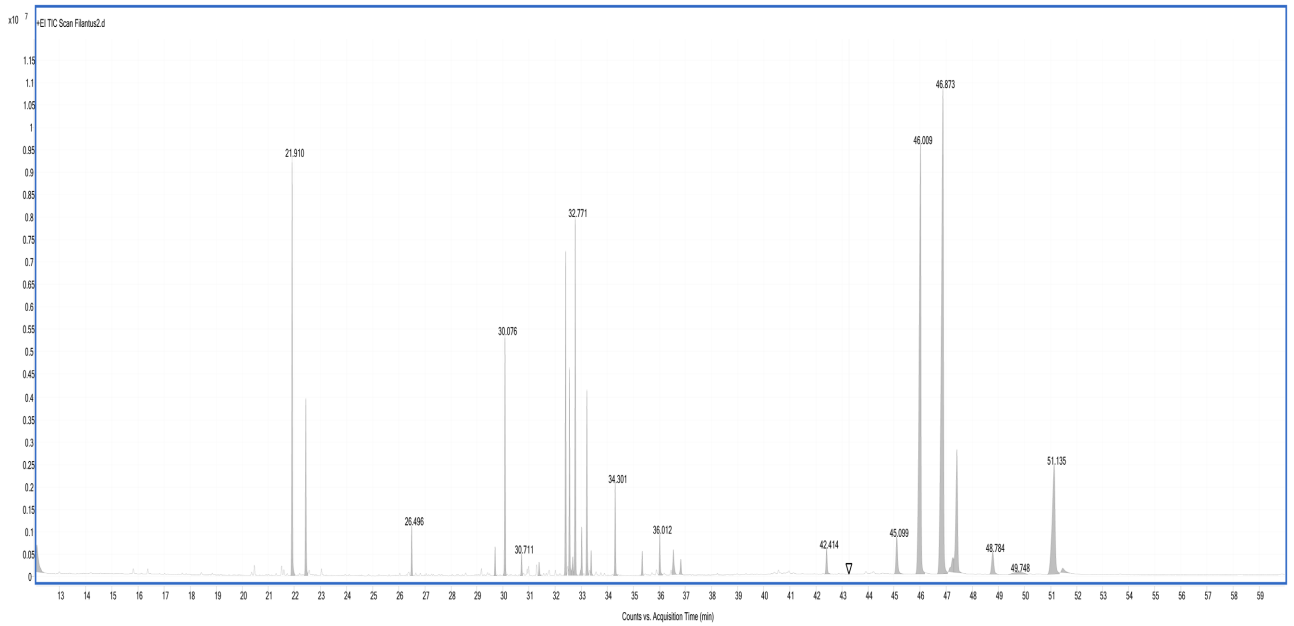


Figure 2. Chromatogram of ethanolic extract of *Phyllanthus niruri.* Each peak corresponds to a compound. Spikes appear based on retention time. A higher peak reflects a more abundant compound in the extract.

Table 4. Chemical composition of extract of *S. acuta.*

N°	*Retention time (minutes)*	*Compounds*	*Percentage (%)*
1	22.43	L-Glutamic acid, N, N-di(3-methylbutyl)-, dimethyl ester	9.50
2	26.83	1-Octanol, 2-butyl-	0.46
3	27.54	1-Tetradecene	0.51

Continued

4	27.66	Hydroxylamine, O-decyl	0.71
5	29.69	Levoglucosan	16.01
6	30.82	4-[1,2-Dimethyl-6-(2-trimethylsilylethoxymethoxy) cyclohexyl] but-3-en-2-one	3.40
7	31.25	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	0.82
8	31.38	Neophytadiene	10.48
9	33.16	N-Hexadecanoic acid	5.82
10	35.86	2-Myristynoyl-glycinamide	0.69
11	36.17	Acetamide, N-(6-acetylaminobenzothiazol-2-yl)-2-(adamantan-1-yl)	0.28
12	38.06	1,10-Cycloeicosanedione	1.31
13	40.56	Cyclohexane, 1,3,5-triphenyl-	4.51

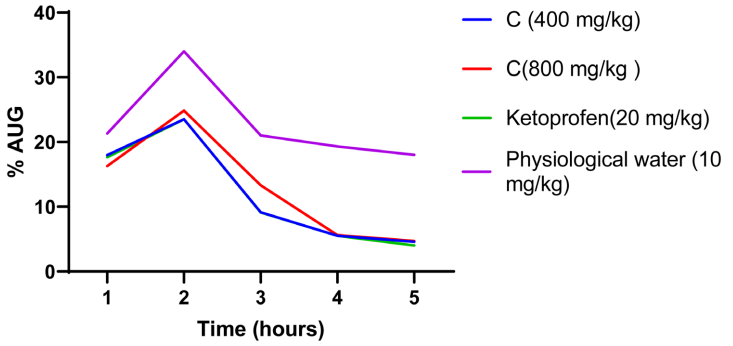
Table 5. Chemical composition of extract of *P. niruri.*

N°	Retention time (minutes)	Compounds	Percentage (%)
1	21.91	Glycerol	6.81
2	22.43	L-Glutamic acid, N, N-di(3-methylbutyl)-, dimethyl ester	3.03
3	26.50	Meso-Erythritol	0.81
4	29.69	Levoglucosan	0.50
5	30.08	D-(+)-Arabitol	3.91
6	32.55	D-Fructose	3.42
7	32.68	D-Mannose	0.45
8	32.77	d-Galactose	5.93
9	33.02	D-Mannitol	0.92
10	33.22	Gallic acid	3.12
11	33.38	Palmitic Acid	0.44
12	35.34	Phytol	0.44
13	36.01	α-Linolenic acid	0.80
14	45.10	(2R,3R)-2,3-bis (Benzo[d][1,3] dioxol-5-ylmethyl)butane-1,4-diol	1.44
15	45.95	(3R,4R)-3-(Benzo[d][1,3]diol-5-ylmethyl)-4-(3,4-dimethoxybenzyl)dihydrofuran-2(3H)-one	18.34
16	46.85	2-[(3,4-Dimethoxyphenyl) methyl]-4-methoxy-4-oxobutanoic acid	24
17	47.25	2,3,3',4'-tetramethoxy-α-methyl-5-(prop-1-enyl) stilbene	0.78
18	47.41	Hydrocortisone 21-acetate	5.13
19	51.14	8-(2,5-Dimethoxyphenyl)-6-methyl-2-(4-methylpent-3-enyl)octa-2,6-dienoic acid, ethyl ester	7.24

3.3. Anti-Inflammatory Activity

A significant increase in rat paw volume was observed in all groups from the first two hours after carrageenan administration, with a higher increase in rats in the physiological water control group. Inhibition percentages ranged from 18.89% at the first hour after injection of carrageenan to 74.61% at the 5th hour for the combined extract (75% *S. acuta* and 25% *P. niruri*) at a concentration of 400 mg/kg. They range from 23.72% in the first hour to 73.72% in the 5th hour for the combined extract at a concentration of 800 mg/kg (Figure 3).

The 400 mg/kg extract showed the highest percentage inhibition at 5 hours after induction of inflammation (Table 6). The evolution of white blood cells (WBC) after administration of the extracts and induction of inflammation was explored. Then, the concentration of 400 mg/kg body weight significantly prevented the onset of inflammation after the 5th hour. Leukocyte counts in all batches returned to normal after 3 days of treatment (Figure 4).



C: Combined extracts.

Figure 3. Percentage increase (%AUG) in rat leg volume as a function of time. C (400 mg/kg) and C (800 mg/kg): Combined extracts at 400 and 800 mg/kg, respectively.

Table 6. Effects of combined extracts administration on carrageenan-induced edema.

Extracts	Percentage of edema inhibition (%)				
	1 h	2 h	3 h	4 h	5 h
Combined extract (400 mg/kg)	18.89 ± 0.59	30.79 ± 1.12	56.43 ± 0.28	70.91 ± 0.63	74.61 ± 1.78
Combined extract (800 mg/kg)	23.72 ± 0.12	26.91 ± 0.27	56.62 ± 0.38	70.93 ± 0.88	73.72 ± 1.28
Ketoprofen (20 mg/kg)	17.29 ± 0.15	30.82 ± 0.75	56.66 ± 0.25	71.55 ± 1.03	77.00 ± 3.63

3.4. Analgesic Activity

The combined extract was administered orally and intraperitoneally. The results are shown in Figure 5 and Figure 6. The extracts are most effective when administered orally. The combined extracts at a concentration of 800 mg/kg BW, recorded the highest percentage of inhibition (71.60% ± 5.32%), while intraperitoneal

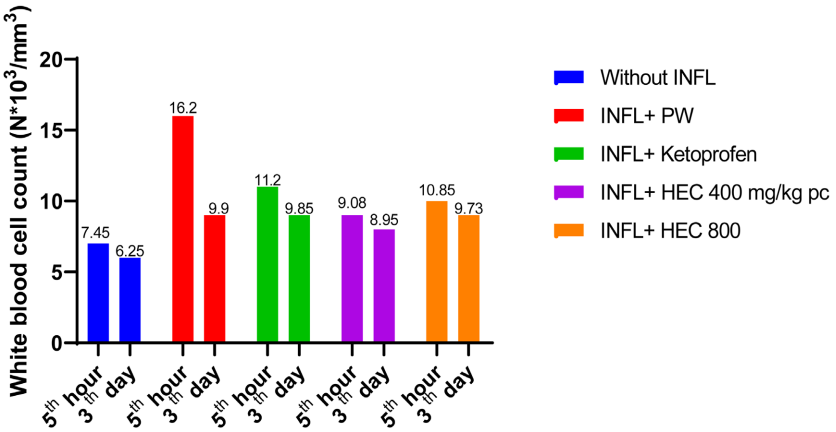


Figure 4. Evolution of white blood cells after administration of extracts. INFL: Inflammation, HEC: Hydroethanolic extract of combined (75% of *S. acuta* /25% of *P. niruri*).

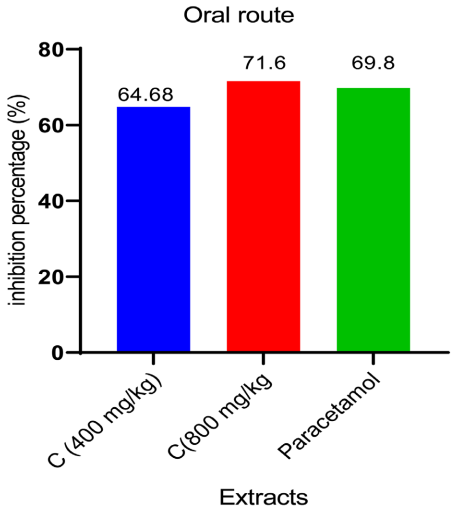


Figure 5. Effect of the combined extract (75% of *S. acuta*/25% of *P. niruri*) administration on acetic acid-induced contortions via the oral route. C (400 mg/kg) and C (800 mg/kg): Combined extracts at 400 and 800 mg/kg, respectively.

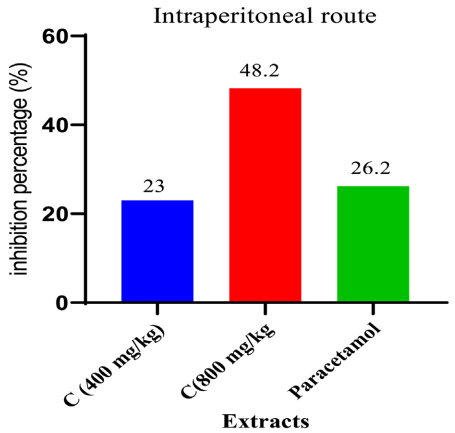


Figure 6. Effect of the combined extract (75% of *S. acuta*/25% of *P. niruri*) administration on acetic acid-induced contortions via the intraperitoneal route. C (400 mg/kg) and C (800 mg/kg): Combined extracts at 400 and 800 mg/kg, respectively.

it recorded a percentage of inhibition of 48.20% ± 2.24%.

3.5. Acute Toxicity

The results showed weight gain in the rats during the 14 days of treatment (Figure 7). No signs of toxicity were observed at 5000 mg/kg BW.

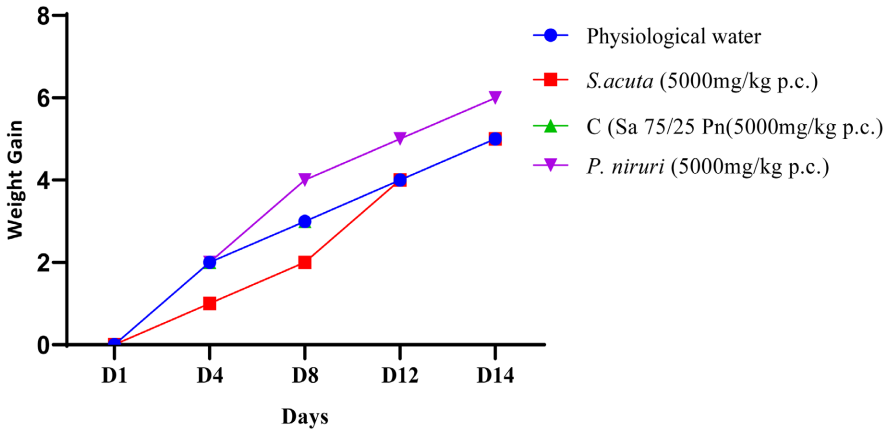


Figure 7. Effect Curve showing the evolution of the animals' weight during the 14 days of the experiment. D: Day.

3.6. Subacute Toxicity

One death was recorded at the female group level of the *S. acuta* extract and one death at the control level. Then, for all other groups, all rats survived. Thus, regardless of sex and dose, the rats gained weight during the test (Figure 8). There are no significant differences in organ weights were observed between treated and control animals (Table 7), and no major variations in haematological (Table 8) and biochemical parameters (Table 9).

Table 7. Effect of extracts on organ weights.

Parameters	Control (physiological water)	*S.acuta* (1000 mg/kg p.c.)	*P. niruri* (1000 mg/kg p.c.)	C (Sa75/25Pn) 1000 mg/kg p.c.
		Male rats		
Heart	0.65 ± 0.02	0.74 ± 0.10	0.58 ± 0.06	0.90 ± 0.15
Lungs	1.19 ± 0.10	1.45 ± 0.22	1.58 ± 0.23	1.79 ± 0.13
Liver	6.75 ± 0.81	6.01 ± 0.34	4.67 ± 0.27	7.10 ± 1.26
Kidneys	1.13 ± 1.12	1.42 ± 0.53	1.12 ± 0.09	1.66 ± 0.20
Spleen	0.80 ± 0.10	0.57 ± 0.07	0.71 ± 0.11	0.97 ± 0.28
Testicles	2.79 ± 0.24	2.51 ± 0.61	2.39 ± 0.14	2.59 ± 0.23
		Female rats		
Heart	0.62 ± 0.03	0.72 ± 0.03	0.79 ± 0.04	0.79 ± 0.04
Lungs	1.13 ± 0.16	1.13 ± 0.04	1.23 ± 0.04	1.19 ± 0.08

Continued

Liver	5.28 ± 0.9	5.91 ± 0.22	5.38 ± 0.38	6.10 ± 1.18
Kidneys	1.13 ± 0.08	1.17 ± 0.05	1.26 ± 0.07	1.24 ± 0.08
Spleen	0.57 ± 0.07	0.79 ± 0.03	0.75 ± 0.03	0.75 ± 0.04
Ovaries	2.17 ± 0.33	1.98 ± 0.10	202 ± 0.14	1.91 ± 0.34

Values represent mean ± SD (n = 4/group). The differences between the treatment group and the treatment group were not significant $P < 0.05$. C (Sa 75/25Pn): combined extract (75% of S. acuta/ 25% of *P. niruri*).

Table 8. Effect of extracts on haematological parameters.

Parameters	Physiological water	*S. acuta* (1000 mg/kg p.c.)	*P. niruri* (1000 mg/kg p.c.)	C (Sa 75/25Pn) 1000 mg/kg p.c.
		Male rats		
WBC (10^3/µL)	8.36 ± 0.65	6.90 ± 0.49	7.82 ± 1.68	7.78 ± 1.74
RBC (10^6/µL)	7.38 ± 0.47	7.36 ± 0.88	7.03 ± 0.75	6.81 ± 0.55
HGB (g/dL)	13.18 ± 0.70	13.44 ± 0.31	12.22 ± 0.13	12.62 ± 0.55
HCT (%)	38.04 ± 2.46	43.6 ± 1.22	37.06 ± 2.29	37.50 ± 3.84
MCV (fl)	51.48 ± 1.12	56.66 ± 3.81	50.74 ± 4.23	54.34 ± 2.12
MCH	17.74 ± 0.73	17.42 ± 1.14	15.88 ± 1.25	1786 ± 0,9.7
MCHC (g/dL)	34.84 ± 1.05	30.93 ± 0.47	31.34 ± 0.73	26.70 ± 12.83
PLT (10^3/µL)	764.2 ± 105.48	641.40 ± 60.11	632.80 ± 103.06	738.00 ± 158.45
		Female rats		
WBC (10^3/µL)	6.86 ± 1.44	6.16 ± 1.88	8.62 ± 1.88	6.68 ± 1.77
RBC (10^6/µL)	7.22 ± 0.27	6.30 ± 0.32	6.26 ± 1.25	6.63 ± 0.57
HGB (g/dL)	13.32 ± 0.34	12.30 ± 0.24	13.26 ± 0.69	12.64 ± 0.35
HCT (%)	39.8 ± 1.59	35.36 ± 3.23	40.44 ± 2.29	38.88 ± 1.55
MCV (fl)	53.86 ± 1.72	55.32 ± 2.87	55.72 ± 1.33	55.22 ± 1.42
MCH	18.28 ± 0.35	18.28 ± 0.28	18.46 ± 1.20	17.96 ± 0.69
MCHC (g/dL)	34.16 ± 0.65	33.02 ± 1.91	32.84 ± 1.70	32.52 ± 0.60
PLT (10^3/µL)	769.6 ± 245.76	691.8 ± 97.55	673.20 ± 89.99	620.40 ± 41.26

Values represent mean ± SD (n = 4/group); C (Sa 75/25Pn): combined extract (75% of S. acuta/ 25% of *P. niruri*); WBC: White Blood Cells; RBC: Red blood cells; HB: haemoglobin; HCT: haematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; PLT: Pads. The differences between the treatment group and the treatment group were not significant $P < 0.05$.

4. Discussion

Medicinal plants are one of the undeniable sources of bioactive substances [35]. Then, *S. acuta* of the family Malvaceae and *P. niruri* of the family Phyllanthaceae

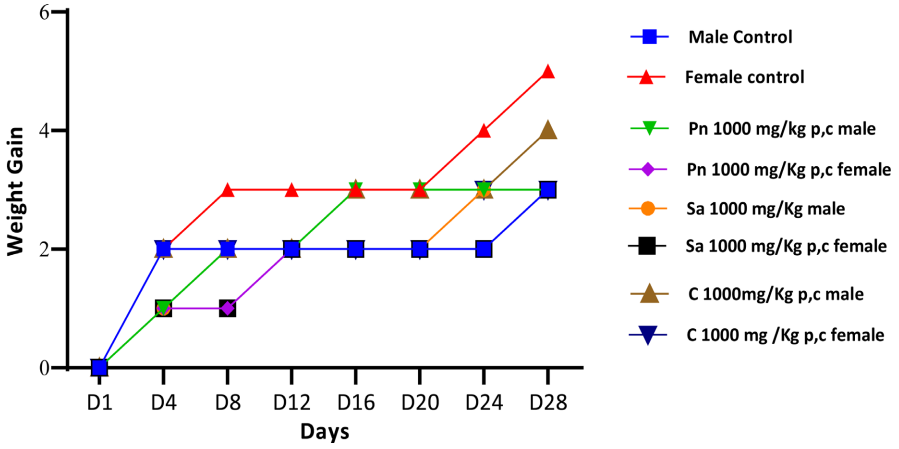


Figure 8. Curve showing the evolution of the rats' weight during the 28 days of the experiment. Sa: *Sida acuta*, Pn: *Phyllanthus niruri*; C (Sa 75/25Pn): combined extract (75% of *S. acuta*/ 25% of *P. niruri*).

Table 9. Effect of extracts on biochemical parameters.

Parameters	Control	*S.acuta* (1000 mg/kg p.c.)	*P. niruri* (1000 mg/kg p.c.)	C (Sa 75/25Pn) 1000 mg/kg p.c.
Male rats				
ALAT (U/L)	59.20 ± 383	43.20 ± 6.10	80.40 ± 5.08	74.40 ± 3.65
ASAT (U/L)	141.80 ± 6.57	131.20 ± 9.6	146.00 ± 8.49	127.40 ± 6.40
APL (U/L)	237.80 ± 3.70	258.80 ± 7.43	253.40 ± 3.36	237.20 ± 10.40
Creatinine (mg/L)	9.00 ± 1.00	5.40 ± 1.52	7.40 ± 1.52	6.00 ± 1.22
Urea (g/L)	0.36 ± 0.05	0.39 ± 0.08	0.40 ± 0.25	0.38 ± 0.07
Glycemia (g/L)	0.77 ± 0.07	0.69 ± 0.24	0.82 ± 0.43	0.73 ± 0.04
Cholesterol (g/L)	0.64 ± 0.03	0.53 ± 0.07	0.55 ± 0.07	0.57 ± 0.04
Triglycerides (g/L)	0.47 ± 0.05	0.69 ± 0.33	1.19 ± 0.57	0.82 ± 0.22
Gamma GT (g/L)	19.20 ± 2.28	22.60 ± 1.52	21.40 ± 2.07	26.20 ± 5.02
Female rats				
ALAT (U/L)	43.20 ± 2.77	53.20 ± 2.77	47.80 ± 4.44	62.80 ± 3.27
ASAT (U/L)	170.60 ± 5.80	151.80 ± 6.46	156.40 ± 4.83	148.20 ± 6.69
APL (U/L)	139.80 ± 7.40	256.80 ± 4.60	316.40 ± 3.21	233.80 ± 8.47
Creatinine (mg/L)	8.40 ± 0.55	5.80 ± 0.45	5.20 ± 0.84	6.20 ± 1.30
Urea (g/L)	0.37 ± 0.01	0.28 ± 0.05	0.31 ± 0.18	0.23 ± 0.13
Glycemia (g/L)	0.68 ± 0.06	0.72 ± 0.05	0.63 ± 0.35	0.73 ± 0.08
Cholesterol (g/L)	0.67 ± 0.04	0.56 ± 0.08	0.69 ± 0.07	0.64 ± 0.03
Triglycerides (g/L)	0.45 ± 0.06	1.17 ± 0.28	1.19 ± 0.13	1.22 ± 0.28
Gamma GT (g/L)	20.20 ± 1.92	29.00 ± 3.39	22.60 ± 2.07	24.40 ± 1.67

Values are averaged ± SD. (n = 4/group); $P < 0.05$. ASAT: Aspartate aminotransferase; ALAT: Alanine aminotransferase; ALP: Alkaline Phosphatase. C (Sa 75/25Pn): combined extract (75% of S. acuta/ 25% of *P. niruri*).

are among the plants that are used as anti-inflammatory and analgesic drugs [36] [37]. The purpose of the present studies is to investigate pharmacological phytochemistry, toxicity, anti-inflammatory, and analgesic effects of the combination of the extract of both plants the first time we report the components of their ethanolic extracts. The qualitative phytochemical test results obtained for *S. acuta* are in line with the findings of work in India which revealed the presence of the same compounds [38]. Also in India, other authors also found that HE of *S. acuta* contained alkaloids, flavonoids and terpenes [39]. The results obtained for HE of *P. niruri* corroborate the work of other authors, carried out in Malaysia and in Indonesia respectively [40] [41]. The presence of saponins found in Malaysia would be due to edaphic and climatic differences, as the content of compounds can vary from one region to another [42]. The results obtained from the phytochemical screening confirm the medicinal benefits for which these two plants have been used in recent years and support their traditional uses for the management of various health problems [43].

The choice of quantifying these classes of polyphenols among the various phytochemicals, results from the fact that these compounds have very important biological activities [44]. Determination of total polyphenols, condensed tannins and flavonoids revealed the presence of these compounds, but in greater proportion in *P. niruri*. The total phenol and flavonoid contents obtained for *S. acuta* are respectively in line with the work of authors in Burkina-Faso [45], and in India [46] who found flavonoid contents of 6.22 ± 0.07 and 0.84 ± 0.04 mg quercetin equivalent/g respectively [45] [46]. In Costa Rica, similar phenolic and proanthocyanidin contents of *P. niruri* were reported [47]. These results are similar to those obtained during the qualitative phytochemical screening.

The analysis of the ethanolic extract of the two plants using GC-MS indicates families of various compounds, which include carbohydrates, terpenoids, steroids, fatty alcohol, fatty acids, ketones, alkanes, and phenolic compounds. The most represented compounds in *S. acuta* extract are levoglucosan 16.01%, neophytadiene 10.48%, and glutamic acid 9.50%. A comparative literature search shows that other studies in India found 45 compounds and 71 compounds [48] [49].

As for *P. niruri* extract, the majority compounds are 2-[(3,4-Dimethoxyphenyl)methyl]-4-methoxy-4-oxobutanoic acid (24%), (3R,4R)-3-(Benzo [d][1,3] dioxol-5-ylmethyl)-4-(3,4-dimethoxybenzyl) dihydrofuran-2(3H)-one(18.34%), 8-(2,5-Dimethoxyphenyl)-6-methyl-2-(4-methylpent-3-enyl)octa-2,6-dienoic acid, ethyl ester, (7.24%), hydrocortisone 21-acetate (5.13%). Similar results were found in Indonesia [50].

The effectiveness of the combined extract, in reducing inflammation was tested by inducing swelling using carrageenan as a model. Carrageenan was chosen due to its ability to trigger responses as it is a polysaccharide obtained from red algae. The formation of edema over time is differentiated into two phases [51]. The first phase lasts one hour and is mediated by histamine, serotonin, and bradykinin; the second phase (more than an hour) is due to the biosynthesis of prostaglandins

[52]. The combined extracts inhibited edema in a dose-dependent manner and all these phases. The highest inhibition value was obtained at the fifth hour. This suggests that the inhibitory action of the combined extracts may have acted more on cyclooxygenases which are involved in the synthesis of prostaglandins [53]. These results would probably be related to the synergistic effect of the molecules contained in the two extracts. Indeed, the results of GC-MS analysis showed compounds with anti-inflammatory activities, in particular gallic acid and hydrocortisone in *P. niruri*, and neophytadiene and acid oxobutanoic in *S. acuta.* Similar results in terms of the carrageenan-induced leg edema process in rats, proving the anti-inflammatory activity of the methanolic extract of *P. niruri* at the recommended dose of 400 mg/kg [54]. A study carried out on crude extracts of *S. acuta* also showed considerable analgesic and anti-inflammatory activity in mice [13]. As shown by acetic acid-induced pain inhibition, this suggests the extracts are likely to inhibit the release of inflammatory mediators such as prostaglandin, bradykinin, and histamine [55]. Oral administration of the extract combination produced significant inhibition of acetic acid-induced abdominal torsion in a dose-dependent manner. This inhibition was higher than that produced by paracetamol. When administered via the oral route, the extracts at a concentration of 800 mg/kg showed a higher percentage of inhibition relative to the intraperitoneal route. The difference might be related to the solubility and metabolism of the extracts, making them highly bioavailable after their metabolism. These results suggest that the combined extracts can produce a peripheral analgesic effect by inhibiting chemical mediators and/or cytokines. Previous studies have shown that analgesic activity can result from high flavonoid content as well as free radical scavenging activity. Free radicals are involved in pain stimulation and antioxidants are known to inhibit pain [56].

In an attempt to identify specific analgesic and anti-inflammatory bioactive agents, one study showed that analgesic properties appeared to be a function of gallic acid concentration [57]. Corilagin found in abundance in *P. niruri* extracts has been shown to reduce the contortion response of acetic acid in a dose-dependent manner and has also shown significant neurogenic analgesia, suggesting the possibility that corilagin attenuates the release of inflammatory endogenous mediators into the peripheric circulation or induces analgesia via direct interaction with peripheral nociceptors or bradykinins [58]. In addition, recently discovered substances that have analgesic properties include alkaloids, flavonoids, and terpenoid compounds [59]. The presence of flavonoids and tannins in *S. acuta* and *P. niruri* extracts may explain the effects observed with their combination [13] [14].

Understanding plant toxicity information such as lethal doses is crucial, for their utilization. Acute toxicity showed no evidence of toxicity at 5000 mg/kg for the extracts and their combinations. The amount of substance needed to cause death in 50% of rats (LD_{50}) is greater than this dose. The findings from *S. acuta* and *P. niruri* align with other studies [60] [61]. Additionally, the proportionate organ weight serves as a marker, for assessing the health status of both humans

and animals.

The heart, liver, spleen, kidneys and lungs are commonly impacted by the metabolic responses triggered by substances [62]. Subacute toxicity results suggest that hydroethanolic extracts and their combination are almost non-toxic to these organs. There were no distinctions observed in blood-related and biochemical factors between the groups that received treatment and those, in the control group. These findings align with a research conducted in Burkina Faso [60].

5. Conclusion

The present work made a scientific evaluation of the extracts of *S. acuta* and *P. niruri*. Toxicity tests showed that the two extracts in their combination were non-toxic. The combined extracts recorded potent anti-inflammatory and analgesic activities. Phytochemical screening showed that the extracts contain all the same compounds, except saponosides, which were absent in the two extracts. The hydroethanolic extract of *P. niruri* is richer in phenols, proanthocyanidins and flavonoids. Analysis at GC-MS showed compounds with anti-inflammatory, analgesic, antioxidant and antimicrobial activities. The results of this work confirm the use of these plants in traditional medicine and merit further research. The identified molecules must be purified and exploited. The side effects of extracts when administered orally or by injection into the abdomen will be examined.

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Data Availability

The datasets analysed in this study are available in this article.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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