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Phytochemical screening of ethanol extract of *Ziziphus spina-christi* (leaf) and *Euphorbia hirta* (whole plant) and their analgesic activity in Wistar albino mice

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Abstract

Background and Objective: Pain is a significant global healthcare problem, usually resulting from either medical procedure, injury, or illness. It can be chronic or acute. In this study, the analgesic effect of the ethanol extract of *Euphorbia hirta* and *Ziziphus spina-christi* at different concentrations in Wistar albino mice was determined. In addition, the phytochemical composition of the extracts was studied in the laboratory.

Materials and Methods: Ethanol extracts of *Euphorbia hirta* and *Ziziphus spina-christi* at concentrations of 250, 500, and 1000 mg/kg were administered to mice, and the results were compared with negative (10 ml/kg distilled water) and positive (30 mg/kg paracetamol) controls using hot plate method. The experiments were done by 24 mice for lethal dose (LD_{50}) study, and 24 mice for the main study. The administration was done via oral route, and the mice were placed on hot plate after every 30 min of administration (six mice in each group).

Results and Conclusion: The plants contained tannins, flavonoids, carbohydrates, anthraquinone, saponins, and alkaloids, but triterpenoids were not detected. No acute toxicity or mortality was found in each group after 24 h administration of 10, 100, 1000, 1600, 2900, and 5000 mg/kg body weight of the extracts. The plants had lethal dose (LD_{50}) of $\geq$ 5000 mg/kg, and *Euphorbia hirta* had more significant analgesic effects than *Ziziphus spina-christi.* After administration, the mice were be placed immediately on a hot plate to observe how many minutes they will jump off the hot plate due to the heat. The highest extended mean time reaction for *Euphorbia hirta* was at the 30th min at a dose of 1000 mg/kg (3.97 ±0.27 min), while that of *Ziziphus spina-christi* was at the 90th min at a dose of 1000 mg/kg (2.22 ±0.41 min). We conclude that both extracts are potent candidate as natural analgesic agent in pain alleviation. However, more toxicological studies are required.

Keywords: Analgesic effect, *Euphorbia hirta*, Phytochemical screening, Wistar strain albino mice, *Ziziphus spina-christi*

1. Introduction

The most common health issue is pain, and how the pain is described is crucial to both diagnosis and treatment selection [1]. In clinical practice, the characterization of pain is complicated by systemic reactions like fever and general malaise, as well as various indicators of the underlying disease or complaints pertaining to psychological, cognitive, and social elements of the condition [2]. Pain is an enormous

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problem globally. Nevertheless, the pain has primarily been regarded as a medical problem, and has been little addressed by the field of public health [3]. Globally, it has been estimated that 1 in 5 adults suffer from pain, and that another 1 in 10 adults are diagnosed with chronic pain each year. While pain affects all populations, regardless of age, sex, income, race/ethnicity, or geography, it is not distributed equally across the globe [3]. The term medicinal plant includes the herbs used in herbalism. These medicinal plants are considered rich ingredient resources that can be used in drug development and synthesis. These plants also play a critical role in the development of human cultures around the world. Medicinal plants have a promising future because there are about half a million plants around the world. Most of them have medicinal activities that have yet to be investigated, and their medicinal activities could be advantageous in treating diseases [4].

The World Health Organization (WHO) recently estimated that 80% of people worldwide rely on herbal medicines for some aspect of their primary healthcare needs [5]. According to WHO, around 21,000 plant species have the potential of being used as medicinal plants [5]. Treatment with medicinal plants is safe at very low dosages as there are no or minimal side effects due to the absence of additional chemical agents. These remedies are compatible with nature, which is the biggest advantage. The golden fact is that herbal treatments are independent of age group and sex. Ancient scholars believed that herbs are the only solution to cure health-related problems and diseases [6].

Euphorbia hirta originates from tropical and subtropical North America, and belongs to the plant family Euphorbiaceae and genus Euphorbia. It is an annual hairy plant with a thin stem that is reddish or purplish in color, and has several branches from the base to the top, reaching a height of 40 cm. The opposing leaves are elliptic, oblong to oblong-lanceolate, acute or subacute, pale underneath, dark green above, and 1-2.5 cm long. They have toothed edges with a purple splotch in the center. The yellow, three-celled, hairy, keeled fruits have a 1-2 mm diameter, and contain three brown, wrinkled, angular, four-sided seeds. Organoleptic attributes of the ethanolic extract of the plant are presented in Table 1 [7]. *Euphorbia hirta* L. (Euphorbiaceae) is a valuable medicinal plant, and has strong antimalarial, antifungal, antifertility, antispasmodic, sedative, antiasthmatic, anthelmintic, and antibacterial activities [8]. Furthermore, the plant has demonstrated strong anticancer properties against a range of aggressive cancer cells [9].

Ziziphus spina-christi originated from Sudan. It is a shrub, sometimes a tall tree, reaching a height of 20 m and a diameter of 60 cm, which has light grey, very cracked, and scaly bark, twisted and much branched trunk, thick crown, whitish and flexible shoots, with pairs of thorns, one straight and the other curved. Organoleptic attributes of the ethanolic extract of the plant leaves are presented in Table 1. The leaves contain various alkaloids, including ziziphine, jubanine, and amphibine, alpha terpinol linalool, and diverse saponins [10]. Diabetes, malaria, typhoid, liver problems, weakness, skin infections, urinary diseases, obesity, diarrhea, and insomnia have been treated using different parts of the plant, particularly the leaves [11]. In this study, we aimed to carry out the qualitative phytochemical screening of the ethanol plant extracts, determine the acute toxicity (LD_{50}) of the ethanol extract in mice, and evaluate and compare the analgesic effect of the plant extracts in Wistar strain albino mice.

Table 1- Organoleptic attributes of the ethanolic extract of *Ziziphus spina-christi* leaf and *Euphorbia hirta* whole plant

Attribute	***Ziziphus spina-christi***	***Euphorbia hirta***
Color	Dark blue	Dark blue
Texture	Gummy	Gummy
Odor	Odorless	Odorless
Taste	Slightly bitter	Bitter
Solubility in water	Insoluble	Insoluble

2. Materials and Methods

2.1. Materials and devices

BDH AnalaR Ethanol 95%, distilled water, tray, pestle and mortar, conical flask, beaker, syringe and NG tube, hot plate, glass rod, glass and rubber funnel, spatula, filter paper and cotton wool, weighing balance, wire cage and rubber bowl, plants, stop watch, and thermometer were used in the laboratory.

2.2. Plant collection and identification

Euphorbia hirta plant (Figure 1) was collected at the Faculty of Pharmacy Medicinal Garden on November 22-23, 2017. *Ziziphus spina-christi* plant (Figure 2) was collected from the University of Maiduguri, near the Faculty of Veterinary Medicine on November 24, 2017.

The plants were identified by a taxonomist in the Department of Biological Science, Faculty of Science, University of Maiduguri. The plant materials were shade-dried during six days for *Ziziphus spina-christi* and 15 days for *Euphorbia hirta.*

Figure 1- Plant of *Euphorbia hirta*

Figure 2- Plant of *Ziziphus spina-christi*

2.3. Extraction

The dried plant materials were powdered by mortar and pestle. Ethanol extract of the plants was prepared by subjecting the powders to cold maceration using ethanol 95% as solvent. It was filtered further using a filter paper placed in a glass beaker. The extract was then left on a tray in the laboratory to dry at room temperature (approximately for seven days). Yield of the plant extracts was calculated according to the Equation 1 as follows.

$$\text{Yield (\%)} = \frac{\text{Weight of dried extract}}{\text{Weight of powdered sample}} \times 100 \qquad \text{Equation 1}$$

2.4. Phytochemical screening

2.4.1. Test for carbohydrates

The extract (4 g) was dissolved in distilled water. The mixture was filtered with filter paper, and the filtrate used for the following tests.

2.4.1.1. Molisch's test

A few drops of the Molisch reagent were added to 2 ml of the extract, and 1 ml of concentrated sulfuric acid was added to the mixture. The tube was left without shaking to form a layer. The mixture was then allowed to stand for 2 min, and diluted further with 5 ml distilled water. A purple color observed at the interface indicated the presence of carbohydrates like fructose, glucose, and sucrose [12].

2.4.1.2. Fehling test (for free reducing sugars)

The filtrate in section 2.4.1 was heated with 2.5 ml of Fehling solution A and 2.5 ml of Fehling solution B. Formation of a brick-red cuprous oxide (Cu_2O) precipitate indicated the presence of free reducing sugars like fructose and glucose [12].

2.4.2. Tests for tannins

Following section 2.4.1, a few drops of ferric chloride solution 10% was added to the filtrate. Occurrence of a blue-black/green or blue-green precipitate showed the presence of tannins [12].

2.4.3. Test for anthraquinone derivatives

2.4.3.1. General test for glycosides

The glycosides sample (0.2 g) was put in a test tube and 5 ml of dilute sulfuric acid was added followed by boiling on a water bath for 10-15 min. Then, it was cooled and neutralized with 20% (w/v) KOH. The mixture was divided into two portions. To the first portion, 5 ml of a mixture of Fehling solutions A and B was added and boiled. The appearance of a brick red precipitate indicated the release of a reducing sugar as a result of glycoside hydrolysis. To the second portion, 3 ml of Ferric chloride solution was added and the appearance of a green to blue color was an indication of phenolic glycoside release due to hydrolysis [13].

2.4.4. Test for cardiac glycosides

2.4.4.1. Liebermann-burchard test for unsaturated steroids (steroidal nucleus) and triterpenes (terpenoids)

For steroids, 2 ml of acetic anhydride was added to 0.5 g of the extract, and then cooled well in ice. Concentrated H_2SO_4 was carefully added, and development of color from violet to blue black or bluish-green was an indication of the presence of steroids i.e. aglycone portion of cardiac glycosides [14].

For triterpenoids, 5 ml of ethanol was added to 0.5 g of the extract, followed by addition of 1 ml acetic anhydride. Then, 1 ml of concentrated H_2SO_4 was carefully added by the side of the test tube to form a lower layer. A color change from pink to violet indicated the presence of triterpenoids [14].

2.4.5. Tests for saponins or saponin glycosides

Frothing test: 5 ml of the filtrate was mixed with 10 ml distilled water in a test tube. The test tube was stoppered and shaken vigorously for 5 min. The occurrence of a foam color that was persistent for over 5 min showed the presence of saponins [14].

Fehling solution test: 2 ml of the filtrate was mixed with 2.5 ml of Fehling solutions A and B (at equal volumes), and heated further. Appearance of a brick red precipitate showed the presence of saponin glycosides [12].

2.4.6. Test for flavonoids

Ferric chloride test: The extract was boiled with distilled water and then filtered. To the filtrate (2 ml), a few drops of 10 % of Ferric chloride solution was then added. The appearance of the green blue or violet coloration indicated the presence of phenolic hydroxyl group [12].

2.4.7. Tests for alkaloids

First, 0.5 g of the extract was stirred with 5 ml of aqueous hydrochloric acid 10% on a water bath, and then filtered. In the next step, 3.5 ml of the filtrate was taken and divided into three portions in the test tube. The first portion was used for Dragendoff test. For this, 3 drops of Dragendoff reagent were added to the test tube. A positive result was obtained with the formation of orange-red precipitate. The second portion was used for Mayer test. In this step, 3 drops of Mayer reagent were added to the test tube. A positive result was obtained with the formation of a buff-colored precipitate. The third portion was used for Wagner test. This step was done by addition of 3 drops of Wagner reagent to the third test tube. A positive result was obtained with the formation of a dark-brown precipitate [14].

2.5. Experimental animals

Wistar strain albino mice weighing 17-27 g and aged between 6-12 weeks in both sexes were used for the experiment. For LD50 analysis, 24 mice were used. In addition, the main study was done by an additional 24 mice (six mice for negative control, six mice for positive control, and 12 mice for the two treatment groups). Therefore, a total of 48 mice were administered in total study. The animals were housed in an aluminum cage and fed vital feeds (growers and finishers) and water in the morning and evening. They were moved to the animal house after each experiment.

2.6. Acute toxicity in animal models

This experiment was done using the modified Lorke Method. It includes two phases. In phase

1, six animals were used for each of the treatment groups (ethanol extract of *Ziziphus spina-christi* (ETHOZ) and ethanol extract of *Euphorbia hirta* (ETHOE)). Doses of 10, 100, and 1000 mg/kg were administered separately to two mice in each group (ETHOZ at three doses for one group and ETHOE at three doses for another group). The animals were monitored for 24 h for their behavior and mortality. The experiment was done similarly in phase 2, except for the doses of 1600, 2900, and 5000 mg/kg of each extract used in the second phase [15]. LD_{50} was calculated according to Equation 2.

$$LD_{50} = \sqrt{(D_0 \times D_{100})} \quad \text{Equation 2}$$

D_0 = The highest dose leading mortality

D_{100} = The lowest dose leading mortality

2.7. Analgesic effect

Temperature of the hot plate was set at 56 ±1 °C. The mice were placed on the hot plate, and they were monitored until the time they will jump off.

2.8. Animal grouping

Group 1: NC (negative control); treated with distilled water (10 ml/kg)

Group 2: PC (positive control); treated with 30 mg/kg paracetamol (PCM)

Group 3: ETHOZ –Ethanol leaf extract of *Ziziphus spina-christi* (250 mg/kg)

Group 4: ETHOZ –Ethanol leaf extract of *Ziziphus spina-christi* (500 mg/kg)

Group 5: ETHOZ –Ethanol leaf extract of *Ziziphus spina-christi* (1000 mg/kg)

Group 6: ETHOE –Ethanol leaf extract of *Euphorbia hirta* (250 mg/kg)

Group 7: ETHOE –Ethanol leaf extract of *Euphorbia hirta* (500 mg/kg)

Group 8: ETHOE –Ethanol leaf extract of *Euphorbia hirta* (1000 mg/kg)

Administration was done via oral route (P.O), and the time was taken every 30 min (i.e., 0, 30, 60, 90, and 120 min).

2.9. Statistical analysis

The data are expressed as mean ±standard error. One way analysis of variance (ANOVA) was used for statistical analysis using the software IBM SPSS version 20. P-value less than 0.05 was considered as significant difference.

3. Results and Discussion

3.1. Yield and phytochemical constituents of the extracts

The yield was calculated as 4.71% and 4.92% for *Ziziphus spina-christi* and *Euphorbia hirta*, respectively. The ethanol extract of *Ziziphus spina-christi* leaf and *Euphorbia hirta* whole plant contained carbohydrates, tannins, steroids, saponins, flavonoids, anthraquinones, and alkaloids, but triterpenoids were not detected (Table 2).

Table 2- Phytochemical analysis of *Ziziphus spina-christi* and *Euphorbia hirta* extracts

Ingredient	Test	*Ziziphus spina-christi*	*Euphorbia hirta*
Carbohydrates	Molisch test	+	+
	Fehling test	+	+
Tannins	Ferric chloride 10%	+	+
Anthraquinone	Glycosides	+	+
Cardiac glycosides	Steroids	+	+
	Triterpenoids	-	-
Saponins		+	+
	Frothing	+	+
	Fehling	+	+
Flavonoids	Ferric chloride	+	+
Alkaloids		+	+
	Dragendoff	-	-
	Mayer	+	+
	Wagner	+	+

+ = present; - = absent

3.2. Acute toxicity (LD_{50})

The ethanol extract of *Ziziphus spina-christi* leaf and *Euphorbia hirta* whole plant showed no death in the treated mice within 24 h administration in both phase I and phase II. Therefore, LD_{50} was calculated ≥ 5000 mg/kg for both extracts (Table 3).

Table 3- Acute toxicity (LD_{50}) of the ethanolic extracts of *Ziziphus spina-christi* leaf and *Euphorbia hirta* whole plant.

Experimental phases	Dose (mg/kg)	Number of death within 24 h/ total number of mice
Phase I	0	0/2
	100	0/2
	1000	0/2
Phase II	1600	0/2
	2900	0/2
	5000	0/2
LD_{50} ≥ 5000 mg/kg		

3.3. Analgesic effect of the ethanolic extracts

Ethanol extract of *Ziziphus spina-christi* leaf and *Euphorbia hirta* whole plant (250, 500, and 1000 mg/kg) showed a dose-dependent activity, so that the highest dose had more analgesic effect than the lower doses. Compared with the negative control, the effect of the two extracts showed a significant difference, as indicated by the higher reaction time. The positive control (paracetamol) and *Euphorbia hirta* whole plant extract showed their peak activity at 30 min, that was 90 min for *Ziziphus spina-christi* leaf extract (Table 4).

Table 4- The analgesic effect of the ethanol extracts of *Ziziphus spina-christi* leaf and *Euphorbia hirta* whole plant in hot plate test.

Treatment	Dose	Reaction time (min)				
		0	30	60	90	120
NC (DW)	10 ml/kg	1.10 ±0.22	1.08 ±0.21*	0.73 ±0.21	0.54 ±0.01	0.53 ±0.00
PC (PCM)	30 mg/kg	1.11 ±0.10	3.53 ±0.36♣	1.12 ±0.09♣	0.93 ±0.12♣	0.99 ±0.09♣
ETHOZ	250 mg/kg	0.95 ±0.13	0.95 ±0.06*	0.91 ±0.16	1.07 ±0.08	0.83 ±0.09
ETHOE	250 mg/kg	0.88 ±0.01	3.34 ±0.38♣	0.89 ±0.15	0.77 ±0.08	0.75 ±0.3
ETHOZ	500 mg/kg	1.16 ±0.24	1.20 ±0.26*	1.09 ±0.06	2.02 ±0.26♣*	1.20 ±0.15♣
ETHOE	500 mg/kg	0.78 ±0.02	3.69 ±0.82♣	0.82 ±0.13	0.83 ±0.07	0.75 ±0.03
ETHOZ	1000 mg/kg	1.12 ±0.25	1.08 ±0.28*	1.61 ±0.45	2.22 ±0.41♣*	1.09 ±0.11♣
ETHOE	1000 mg/kg	1.17 ±0.07	3.97 ±0.27♣	1.26 ±0.38	1.20 ±0.22	1.26 ±0.38♣

n=3; DW = distilled water; PCM = paracetamol; ♣ = $p < 0.05$ (when compared with DW); * = $p < 0.05$ (when compared with PCM); ETHOZ = Ethanol extract of *Ziziphus spina-christi* leaf; ETHOE = Ethanol extract of *Euphorbia hirta* whole plant.

The phytochemical screening for the ethanol extract of *Ziziphus spina-christi* leaf and *Euphorbia hirta* plant revealed the presence of carbohydrates, tannins, steroids, saponins, flavonoids, anthraquinones, and alkaloids, which agreed with the study of Dangoggo et al. [16] on *Ziziphus spina-christi*, and study of Waseem et al. [17] and Asha et al. [18] on *Euphorbia hirta.*

Tannins and flavonoids have been found to possess analgesic and anti-inflammatory activities, with flavonoids playing a significant role in analgesic activity targeting the prostaglandins.

The acute toxicity of the ethanol extract of *Ziziphus spina-christi* and *Euphorbia hirta* was found to be greater than 5000 mg/kg, suggesting that LD_{50} is higher than 5000 mg/kg.

Pain is a multifaceted sensation involving the entire nervous system. Pain processes usually start in the periphery, where tissue or nerve damage arises as a consequence of trauma, disease, or lesion, including the growth of a tumor within tissue or nerves [19]. The analgesic activity of the ethanol leaf and whole plant extracts of *Ziziphus spina-christi* and *Euphorbia hirta*, respectively, have been determined using the hot plate method. It was observed that *Ziziphus spina-christi* showed its peak analgesic activity at the highest dose of 1000 mg/kg at 90th min (time of 2.22 ±0.41 min). When compared with the positive control (PCM), there was a significant difference. However, PCM showed a very high mean time at 30th min (time of 3.53 ±0.36 min). *Euphorbia hirta* also showed analgesic effect, and its peak activity was seen at dose of 1000 mg/kg at 30th min (time of 3.97 ±0.27 min). Interestingly, it showed a longer reaction time at the highest dose compared to PCM. Although, their difference was not significant ($p > 0.05$). We have found that *Euphorbia hirta* whole plant has more analgesic activity than *Ziziphus spina-christi* leaf at the same dose, and there is a significant difference between analgesic activity of their ethanolic extracts *in vivo*. However, similar phytochemicals have been found qualitatively in both extracts, and their different analgesic activity might be related to different levels of phytochemicals.

4. Conclusion

In conclusion, the ethanol extract of *Ziziphus spina-christi* leaf and *Euphorbia hirta* whole plant contained some pharmacologically active ingredients (such as tannins and flavonoids), which is assumed to involve in pain management. Acute toxicity (LD_{50}) was calculated as equal or greater than 5000 mg/kg. *Euphorbia hirta* whole plant extract showed a more analgesic effect than *Ziziphus spina-christi* leaf extract. Both *Euphorbia hirta* whole plant extract and the positive control (PCM) showed their peak analgesic activity at 30th min (3.97±0.27 min reaction time at dose of 1000 mg/kg for *Euphorbia hirta*, and 3.53 ±0.36 min reaction time at dose of 30 mg/kg for PCM), while *Ziziphus spina-christi* leaf extract showed its peak analgesic activity at 90th min (2.22 ±0.41 min reaction time at dose of 1000 mg/kg).

5. Conflict of Interest

The authors declare no conflict of interest.

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