Original Article

*Thai Journal of Pharmaceutical Sciences*

# Subacute Toxicity Study and Clinical Trials for *Ziziphus spina-christi* Leaves Extract as an Anti-dandruff Shampoo

**Abdulkarim K. Y. Alzomor[1,2], Wafa M. Al-Madhagi[2,3], Nahlah M. Sallam[1], Haytham Mojamel[2], Mostafa M. Alawar[2], Abdulfattah G. Al-Hetari[2]**

*[1]Department of Pharmacy, Faculty of Medicine and Health Sciences, Thamar University, Dhamar, Yemen, [2]Department of Pharmacy, Faculty of Medical Sciences, Al-Nasser University, Sana'a, Yemen, [3]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Sana'a University, Sana'a, Yemen*

**Corresponding Author:**
Abdulkarim K. Y. Alzomor, Department of Pharmacy, Faculty of Medicine and Health Sciences, Thamar University, Dhamar, Yemen.
Tel.: +967-773418635/ +967-736558282.
E-mail: al_zomor1974@tu.edu.ye

**Received:** Aug 04, 2020
**Accepted:** Nov 13, 2020
**Published:** Feb 13, 2021

## ABSTRACT

In light of recent developments in the scientific and technological world, herbs are widely used as remedial agents. This study was aimed to formulate and evaluate the safety and the efficacy of herbal antidandruff shampoo from Ziziphus spina-christi (Sidr) leaves extract, which is used traditionally for several medical and cosmetic purposes. Sidr leaves were extracted by maceration using ethanol as a solvent. The antifungal activity of the plant extract was tested against *M. furfur*. In-vivo study of the extract has been evaluated with daily oral doses (50, 100, and 200 mg/kg) for 28 days for tested rats, followed by, evaluation of the biochemical and histological parameters. Subsequently, the plant extract was formulated as a shampoo and tested for its antidandruff efficiency on 80 volunteers, with dandruff, for four consecutive weeks. An obvious antifungal activity against *M. furfur* has been displayed by sidr extract, where 86% of the tested volunteers substantially ameliorated from dandruff with sidr shampoo formulation. The toxicity of the extract was not shown aberrant results as compared with the control group in biochemical analysis. However, mild histological harmful effects on the liver and kidney tissues appeared at high doses of the extract, 100 and 200mg/kg, when used orally. As a conclusion, sidr extract could be used topically as anti-dandruff shampoo formulation with high efficiency and good safety

**Keywords:** Antifungal, dandruff, formulation, hair treatment, safety, *Ziziphus spina-christi*

## INTRODUCTION

*Ziziphus spina-christi* (L.) is a member of Rhamnaceae that is also known as sidr in Arabic countries. Sidr is an indigenous tree to the Middle East and its leaves are used traditionally for hair cleaning and promoting its growth.[1] *Z. spina-christi* exhibited antibacterial, antifungal, anti-nociceptive, antioxidant, antidiabetic, anti-plasmodium, anti-schistosomiasis, analgesic, and anticonvulsant activities.[2-9] Several saponin glycosides have been isolated from sidr that are proven to help in removing the excess sebum and have antibacterial and antifungal activities which increase their immense value and to be used as cosmetic preparation.[7] Petal et al. stated that christinin-A is the foremost saponin of sidr's leaves.[10] In addition to, flavonoids, lipids, protein, and mucilage; these include butic acid, ceanothic acid, and cyclopeptides [11,12] were found in *Z. spina-christi* extract.

*Malassezia* fungus is the main causative agent of dandruff that use lipase enzymes to break down the sebum to oleic acid. The latter penetrates the upper layer of skin then causes increased skin cell turnover in susceptible people and this in turn produces the dandruff flakes,[13] Other studies indicated that two other species of the fungus-Malassezia restricta and *M. globosa* are also causal organisms of the dandruff.[14] In drug discovery and development stage of the pharmaceutical industry, on average, only one in five thousand screened compounds in research reach the market. The most failure to the screened compounds is related to drug safety and a lack of efficacy.[15] Therefore, the present study is designed to develop a new shampoo formula of

sidr leaves' extract to be used as anti-dandruff and to study the sidr toxicity on albino rats followed by clinical trials on humans to confirm the safety and efficacy of this extract.

## METHODOLOGY

### Study Area

The study was carried out at Al-Nasser University, Department of Pharmacy, Pharmaceutical and Histology Laboratories. While, the toxicology study was performed in Sana'a University, Faculty of Sciences and the pathological study in Forty-Eight Hospital, Department of Histopathology and the accelerated stability study was performed in Global Pharma Company, Sana'a – Yemen.

### Ethical Consideration

Ethical clearance and approval of the study protocols obtained from the Ethical Research Committee of Thamar University, Faculty of Medicine and Health Sciences in 25/09/2018 Reference No. TUMEC-17016 and the study followed the ethical principles in phytochemical, experimental pharmacology research, and the clinical trials. The animals involved in this study were investigated and housed according to European community guidelines and Guidelines for the Housing of Rats in Scientific Institutions. In terms of clinical trials, all volunteers were informed about the research and the drug to be used, then they were asked to sign a written informed consent for participation in the study.

### Preparation of Crude Extract

Initially, *Z. spina christi* leaves were collected from Wesab Mountains, Yemen in November 2018 and identified by Dr. Hassan Ibrahim; Plant Taxonomy Department, Agricultural Research Centre, Sana'a University. The dried plant was then kept with voucher number NU192018 in the Herbarium Unite of Al-Nasser University, Pharmacognosy Lab. Once the leaves dried at 25°C and milled, 140 g of the sample was soaked in 500 ml absolute ethanol in shaking apparatus for 24 h.[16] The extract was filtered before being concentrated through a rotary evaporator (Telstar, model no.: CRYODOS-50), followed by freeze-drying and kept in the refrigerator at 4°C.

### Toxicology Study

Twelve female albino rats with average weight 120–150 g and age 60 days were obtained from the animal house of Sana'a University's (ventilated environment and controlled humidity). During the study for 28 days, animals were fed on specific diet formula consists of White corn, soybean, wheat bran, melt bran, yellow corn, nd dried fish (Wazef) as a source of animal protein vegetable oil mixed together with multivitamins and minerals (Vitamin-M, Aman Veterinary Manufacturing Company, Sana'a-Yemen). The yielded paste was rolled into cylindrical pellets then each rat received 100 g/day of the dried pellets. During the subacute toxicity study, the rats were divided into four groups, 1st group was received distilled water and served as a control, while the 2nd, 3rd, and 4th were treated with Sidr extract as 50, 100, and 200 mg/kg of body weight, respectively, for 28 days. At the end of the experiment, rats from each group were anesthetized through inhaled chloroform, then slaughtered to identify gross lesions. Then, specimens from liver, kidney, heart, spleen, brain, and small intestine were taken from each group followed by immediate fixation in 10% neutral buffered formalin and processed for histopathology. Conversely, the blood samples were collected at slaughter for hematology and serum analysis.

### Analysis Parameters

*Growth changes*

Average body weights and weight gain for each group of rats were measured on a weekly basis throughout the period of study.

*Hematologic analysis*

First, the blood samples were collected in test tubes containing ethylene diamine tetra-acetic acid (EDTA) following by measuring the hemoglobin (Hb), the blood cells, packed cell volume (PCV), mean cell volume (MCV), mean corpuscular Hb (MCH), and MCH concentration (MCHC) using a hematology analyzer (HumaCount plus, German).

### Biochemical Analysis

Blood samples were centrifuged at 3000 RPM for 5 min, then the serum was kept at (−20°C) for analyzing the aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP).

### Histopathological Study

The specimens were preserved from the isolated vital organs of the desiccated rats before being fixed in formalin 10% and after processing embedded in paraffin wax. Successively, the tissues were trimmed into 5 $mm^2$ pieces then stained with hematoxylin and eosin to be examined through microscope.

### Statistical Analysis

The results of the biochemical estimations were reported as mean ± SD of rats in each group. The data were analyzed by one-way analysis of variance (ANOVA) using AGRES statistical package (Version 3.1) with a level of confidence 95%.

### Formulation and Evaluation of Shampoo

*Formulations of sidr leaves extract*

Five formulas of sidr shampoo were prepared for an anti-dandruff purpose as illustrated in Table 1.

*Preparation of the formulas*

F1, F2, F3, and F4 were prepared starting by dissolving the sodium lauryl sulfate in a half amount of the distilled water and the remaining ingredients were combined in the other half. Next, sidr extracts were dispersed in 10 ml of ethanol and all parts were mixed until homogenous shampoo formed. Finally, a sufficient quantity of NaCl was added until the viscosity adjusted. Alternatively, F5 was formulated by heating 20 g of texapon in water bath 60°C for 5 min prior to adding hot distilled water at 80°C with continuous stirring until complete dissolve. Second, 3 ml of glycerine, 3 g of cocamide, and a sufficient quantity of NaCl were supplied

**Table 1:** Different formulations of sidr shampoo was prepared and the different additives were included

| Excipients | Quantity | | | | |
|---|---|---|---|---|---|
| | F1 | F2 | F3 | F4 | F5 |
| Sidr extract | 5% | 5% | 5% | 5% | 5% |
| Bentonite | 5% | - | - | - | - |
| Sodium lauryl sulphate | 20% | 20% | 20% | 20% | - |
| Citric acid | QS | QS | QS | QS | QS |
| Tragacanth | - | - | 4% | - | - |
| Gum Arabica | - | 3% | - | - | - |
| Cocamide | 3% | 3% | 3% | 3% | 3% |
| NaCl | QS | QS | QS | QS | QS |
| Texapon | - | - | - | - | 20% |
| Glycerin | - | - | - | 3% | 3% |
| EDTA | 0.15% | 0.15% | 0.15% | 0.15% | 0.15% |
| Ethanol | 10% | 10% | 10% | 5% | 10% |
| Methyl paraben sodium | 0.18% | 0.18% | 0.18% | 0.18% | 0.18% |
| Propyl paraben sodium | 0.02% | 0.02% | 0.02% | 0.02% | 0.02% |
| Distilled Water up to | 100 ml | 100 ml | 100 ml | 100 ml | 100 ml |

gradually to adjust the viscosity. After that, 50 mg of sidr extract was dissolved in 10 ml of ethanol and combined with the mixture. At last, an adequate quantity of citric acid used to adjust the pH.

## Shampoo Evaluation Tests

*Physical appearance/visual inspection*

The formulations were evaluated in terms of color, odor, appearance, wash-ability, and texture. The viscosity and homogeneity tests were carried out through taking a small quantity of the product then pressed between the thumb and the index finger.

*Foam stability*

Fifty milliliters of the 1% formulated shampoo solution were transferred to a 250 ml graduated cylinder and shaken. Foam stability was evaluated by recording the foam volume after 1 min and 4 min of shake test.[17]

*Measurement of pH*

Digital pH meter was used to determine the pH of the shampoo formulas by diluting 10 ml of shampoo into 50 ml with distilled water.

*Contamination test*

Mueller–Hinton agar was prepared, according to manufacturer's instructions, and poured in two petri dishes, one control and the other was mixed with 10 ml of shampoo product and incubated at 37°C for 48 h.

*Surface tension*

Surface tension could be tested through calculating the amount of surfactant found in shampoos to reduce the surface tension.[17] Measurement of surface tension was performed after using 10% of shampoo solution in distilled water at conventional conditions using a clean dropper. The calculations were carried out using the following equation: $R_2 = ([W_3\text{-}W_1]\ n_1)/([W_2\text{-}W_1]\ n_2) * R_1$.

$R_2$ is the surface tension of the shampoo solution, $R_1$ is the surface tension of water, $W_1$ is the weight of the empty beaker, $W_2$ is the weight of the beaker with the distilled water, $W_3$ is the weight of the beaker with the shampoo solution, $n_1$ is the number of drops of distilled water, and $n_2$ is the number of drops of the shampoo solution.

*Packing of the final product*

Shampoo formulations were packed in 100 ml transparent plastic containers.

## Stability Study of the Shampoo

Stability study was carried out for shampoo formula according to International Conference on Harmonization guidelines. Amongst all prepared shampoo formulas, F5 showed the best physical properties. Therefore, this formula was divided into two samples, those samples were kept at different storage conditions, that is, at 25°C and at 40°C with 75% relative humidity (RH) with intensive light in stability chambers (airtight and transparent plastic containers), and observed for a period of 3 months at definite time intervals. A sufficient quantity of each formula in suitable containers was stored under 40 ± 2°C and 75% ± 5% RH and the quality tests were done for the samples monthly through 3 months.[13]

## Antifungal Assay

*Malassezia furfur* samples were collected from the scalp of the volunteers and kept in a thermal sack at room temperature, then examined under the microscope by taking a specimen of scalp dandruff on a slide with drops of potassium hydroxide, and identified by the objective lens (40×). Second, sabouraud agar media was prepared and sterilized by chloramphenicol

cycloheximide supplement (CCS), the subcultures from the growing colony of *M. furfur* were transferred and incubated at 32°C for 72 h. Next, one colony was taken from each subculture which was stained by lactophenol to be examined under the oily objective lens (100×) of the microscope. A clinical isolate was treated in the same way**.**

Likewise, the fungicidal activity of shampoo against *M. furfur* was carried out by dissolving 4.7 g sabouraud dextrose agar in 100 ml distilled water with heating before transferring it to five sterilized test tubes. Later, the media injected by CCS to prevent the growth of bacteria and the isolated fungi was inoculated into four tubes containing the media. Three concentrations of the prepared shampoo (1 ml, 2 ml, and 3 ml, respectively) have been added to the tubes. In addition, a control (media + shampoo without extract) and pure media have been tested. Then, all samples have been incubated at 32°C for 72 h.

## Clinical Study

The sample size was calculated according to the population size using Australian statistical analysis (National Statistical Service). Confidence level = 95%, proportion = 0.5, standard error = 0.05, population size = 800, and consequently the sample was obtained to be 89 ± 10 volunteers. In the current study, the effectiveness of *Z. spina christi* leaves extract shampoo was evaluated using 80 volunteers who entered the study in preclinical stages. All volunteers were exposed to clinical diagnosis for the yeast presence from *Malassezia* by direct examination under the microscope during 1 week and the results are summarized in Table 2.

## Inclusion Criteria

Eighty Yemeni volunteers (Arab ethnic group) with age equal or higher than 15 years old where clinically diagnosed with moderate to severe dandruff. Volunteers were agreed to give informed consent and enrolled in the study, disposing to attend all the visits punctually and acceptance for no using other treatment or hair cosmetics while they are involved in the study.

**Table 2:** Characteristic conditions of the volunteers in the study including sex, yeast presence, and morphology and previous treatment

| Parameters | No. of patients | Age (years) average=28±12 | |
|---|---|---|---|
| | | 15–25 | ≥5± |
| Sex | | | |
| Males | 53 | 39 | 14 |
| Females | 27 | 19 | 8 |
| Diagnosis of yeast | | | |
| +V | 79 | 58 | 21 |
| −V | 1 | 0 | 1 |
| Morphology of the dandruff | | | |
| Severe | 51 | 46 | 15 |
| Mild | 29 | 22 | 7 |
| Previous treatment | | | |
| Yes | 31 | 18 | 13 |
| No | 49 | 40 | 9 |

## Exclusion Criteria

Pregnant women; volunteers with any disorder may compromise immunologically (diabetes, cancer, etc.); those with the history of hepatic, renal or cardiovascular disease; use another anti-dandruff agent; and mentally or neurologically disabled volunteers that are considered not fit to approve their participation in the study.

*Clinical protocol*

Mycology diagnosis for *Malassezia* species and dermatophytes was performed to confirm the infection. Volunteers with positive results were recruited for the study and they were prevented from using any cosmetics, emollients, or any antifungal drug during treatment.

*Clinical examination*

Hair scarping tests of the infected area were performed before, during, and after treatment for 3 weeks. The efficacy of the product was assessed by the direct examination of microorganism under the microscope using KOH 20%.

## Study Design

This study is a random experimental trial and the volunteers, who had fulfilled the criteria, were selected randomly from the university. The procedure was explained to volunteers to sign the written informed consent and received 100 ml of the shampoo with sufficient clarification on the instructions of use. They were advised to apply the shampoo on moist hair once daily for 5 min then rinse it for 3 weeks and avoid any other medicals or cosmetics during their participation in the study.

## Evaluating Treatment and Follow Up

Each volunteer was followed up weekly for 4 weeks to evaluate the efficacy of the product according to the improvement of the dandruff status. Mycological tests were performed through two steps; first is the direct examination with potassium hydroxide and the second is the culture of scaly/hair obtained from the affected area of the hair using sabouraud agar. After 1 week of treatment, 20 volunteers were selected randomly for direct examination and volunteers with improved status (complete cured, excessive, moderate, and mild dandruff) were determined.

# RESULTS AND DISCUSSION

## Analysis of the Extract

The results of phytochemical tests of *Z. spina christi* extract confirmed the presence of tannins, glycosides, alkaloids, phenolic compound, saponins, reducing sugar, and flavonoids.

## Formulation Evaluation

All formulated preparations were evaluated for physical characteristics such as color, odor, homogeneity, surface tension, consistency, skin irritation, pH, and transparency as demonstrated in Table 3. The different formulas revealed good physical properties; however, the most stable formula physically was F5.

**Table 3:** Physicochemical tests of the shampoo formulations.

| Parameter | Shampoo formulations | | | | |
|---|---|---|---|---|---|
| | F1 | F2 | F3 | F4 | F5 |
| Viscosity (CPS) | 3000 | 3000 | 3400 | 3000 | 3500 |
| pH | 5.62 | 5.50 | 5.52 | 5.38 | 5.84 |
| Homogeneity | Good | Good | Good | Good | Good |
| Consistency | Conform | Conform | Conform | Conform | Conform |
| Skin Irritation | Non | Non | Non | Non | Non |
| Surface tension(dyn/cm) | 34 | 32 | 32 | 30 | 30 |
| Foam stability($cm^3$±SD) | 98±5.6 | 100±9.3 | 104±5.1 | 103±4.6 | 105±4.0 |

It has been proved that shampoo pH plays an important role for enhancing and improving the hair quality, minimizing the eye irritation, and stabilizing the scalp's ecological balance. All prepared formulas displayed maintained pH value range between 5 and 6 that help to prevent the swelling and promote tightening of the scales, thereby inducing shine and minimizing the hair damage.[17] The pH value of the prepared shampoo was close to the recommended value from previous study to be close to skin pH.[13] Regarding the foam testing, foam is of paramount important criteria in evaluating shampoos, even though it has no correlation with the cleansing ability of the shampoo.[17] In the present study, all the formulas have good foam volume more than 100 ml, especially F3 and F5, and it is stable for 5 min. Turning to surface tension measurement, whenever the surface tension is lower, the cleaning ability of the shampoo is improved.[17] The good quality of the shampoo evaluated by the capability of the shampoo to reduce the surface tension of pure water from 72.28 dyn/cm to about 40 dyn/cm.[18] Accordingly, all the tested shampoos prepared in this study showed reductions in surface tension ranging from 30 to 34 dyn/cm and this is confirmed to the pharmacopeia's standards. F5 has the lowest surface tension; therefore, it is indicated to have the strongest cleansing ability. Moreover, the formulas did not present noticeable changes in the vital tests of the shampoo under stress conditions 40°C, 75% RH during the 3 months and the most suitable and satisfied formula was F5. In conclusion, F5 is the most suitable and stable formula of sidr shampoo during the 3-month study.

## Antifungal Assay

The antifungal activity of F5 shampoo has been tested and compared to negative control (media without shampoo). After 72 h, no growth has been detected in compared to the growth from the negative control to indicate the antifungal activity of the Sidra shampoo as antifungal, as summarized in Table 4.

**Table 4:** Antifungal activity of the shampoo after 1 week from treatment: the level of dandruff was estimated as ***: Excessive, **: Moderate *: Mild, 0: Complete cure

| Fungal growth | Dandruff level of the scalp | | | |
|---|---|---|---|---|
| | *** | ** | * | 0 |
| Yes | 3 | 2 | 1 | 0 |
| No | 2 | 3 | 4 | 5 |

## Toxicology Study

No sign of toxicity, behavioral changes, or mortality were observed in the test groups of rats having a dose of 50, 100, and 200 mg/kg dose in compared to the controls throughout the dosing period of 28 days.

*Effects of the extract on the hematological parameters*

The mean results of blood samples parameters are calculated in Table 5 for the treated rats and control. Evaluating the hematological parameters is crucial in such studies since these parameters are considered as sensitive biomarkers of the physiological changes in response to any external effects including toxic stress in testing animals.[19] This study has shown that sub-acute treatment with the extract did not cause any change in hematological parameters except the significant increase in the platelets level with P-value 0.0061. There were no significant changes in Hb, red blood cells, white blood cells, PCV, neutrophils, lymphocytes, monocytes, eosinophil, and basophil in all the treated groups as compared to the control group with different doses of sidr extract as exhibited in Table 5. As the hematological alterations such as anemia are mostly accompaniments of bone marrow toxicity, the analysis of hematology parameters in the animal studies has a high relevance and predictive value for humans.[20] Furthermore, the lack-of-effect on polymorphonuclear leukocyte levels indicate that the extract may not have induced an inflammatory process, since these cells are usually elevated in the course of inflammations. The platelets have an essential role in the coagulation process and this study proved significant elevated levels of platelets count indicating hemostatic activity of tested extract samples.[21]

*Effects of the extract on the liver functions*

AST, ALP, and ALT enzymes serve as biomarkers capable of predicting toxicity. AST is present in several tissues, which include heart, kidney, skeletal muscle, and liver, whereas ALP presence in bone, kidney, intestine, bile duct, and liver, and ALT is predominantly localized in the liver.[22]

Apparently, serum levels of ALP, ALT, and AST of treated rats were not significantly different than control especially at 50 and 100 mg/kg dose; however, the levels of all these enzymes significantly increased with boosting the dose to 200 mg/kg as summarized in Table 6 and these are often diagnostic signs of underlying cellular injuries.[23]

*Effects of the extract on the weight of rats*

The table below demonstrates the effects of a given dose of the extract to the test animals on the overall body weight throughout the study period. As displayed in Table 7, the extract has no effect on the body weight of the rats, even at a dose of 200 mg/kg, and food and water intake was normal

**Table 5:** Effect of oral administration of sidr extract on hematological parameters in rats

| Parameters | Dose of Sidr extract (mg/kg) | | | |
|---|---|---|---|---|
| | 50 | 100 | 200 | Control |
| | Mean±SD | Mean±SD | Mean±SD | Mean±SD |
| White blood cells s | 4.4±1.7 | 6.943±2.11 | 5.986±0.775 | 8.077±2.153 |
| Red blood cells | 8.3±0.05 | 8.213±0.1419 | 8.33±1.55796 | 8.876±0.644 |
| PLT** | 673.3±34.5 | 1016.67±108.61 | 938.3±90.51 | 684.0±100.50 |
| Neutrophil | 20±0.00 | 22.00±2.646 | 19.3333±3.786 | 24.00±9.539 |
| Lymphocyte | 72±3 | 73.66±2.309 | 70.3333±1.527 | 69.00±10.535 |
| Monocyte | 2.4±0.6 | 2.20±1.411 | 4.60±1.058 | 2.967±1.222 |
| Eosinophil | 4.6±2.3 | 2.30±0.721 | 5.63±4.113 | 4.1667±1.778 |
| Basophil | 0.00±0.00 | 0.033±0.058 | 0.033±0.058 | 0.100±0.100 |
| Hb | 16.6±0.15 | 15.9±1.179 | 15.83±3.023 | 17.20±1.374 |
| PCV | 49.5±0.058 | 47.93±2.401 | 48.63±7.144 | 51.93±2.358 |
| MCV | 60±0.53 | 58.36±1.901 | 58.70±2.718 | 58.60±2.152 |
| MCH | 20.2±0.44 | 19.36±1.137 | 19.0±0.5567 | 19.366±0.551 |
| MCHC | 33.5±0.25 | 33.16±0.929 | 32.40±1.947 | 33.10±1.916 |

The Data are expressed as mean±SD, $n=12$, *$P<0.05$, **$P<0.01$.

**Table 6:** Effect of oral administration of sidr extracts on liver function of the rats

| Enzyme | Sidr extract dose (mg/kg) | | | |
|---|---|---|---|---|
| | 50 | 100 | 200 | Control |
| | Mean±SD | Mean±SD | Mean±SD | Mean±SD |
| AST** | 186.3±10.5 | 191±15.7 | 268.67±54.2 | 180.0±35.5 |
| ALT** | 38±8 | 67.33±12 | 79.3±14.2 | 51.0±9.1 |
| ALP** | 139.3±31.9 | 183.00±8.6 | 206.0±40.7 | 106.0±23.89 |

Liver enzymes were measured as mean±SD., $n=12$, *$P<0.05$, **$P<0.01$

**Table 7:** Effect of oral administration of sidr extracts on body weight of rats

| Body weight | Dose of sidr extract (mg/kg) | | | |
|---|---|---|---|---|
| | 50 | 100 | 200 | Control |
| | Mean±SD | Mean±SD | Mean±SD | Mean±SD |
| Before administration | 122±20 | 96.333±11.50362 | 109.667±35.7258 | 102.6667±32.47050 |
| After administration of | 142.7±27.5 | 107.667±25.54082 | 122.333±38.3710 | 130.0000±35.55278 |

The data are expressed as mean± SD., $n=12$, *$P<0.05$, **$P<0.01$S

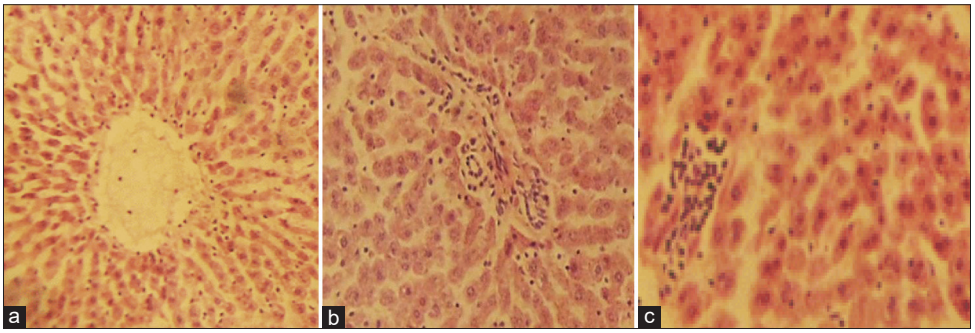


**Figure 1:** Photomicrograph of the liver sections of the control and rat treated with dose 50 mg/kg of Sidr extract. (a) Control rat normal hepatocytes with normal central vein. (b) Infiltration of mononuclear cells. (c) Inflammatory region

throughout the study timeframe. As a result, this suggests the normal processing and metabolism of proteins, carbohydrates, and lipids as it plays a key role in the physiology of the body.[24]

## Histopathological Results

At the end of the study, the vital organs of the scarified testing animals including liver, heart, kidney, lungs, spleen,

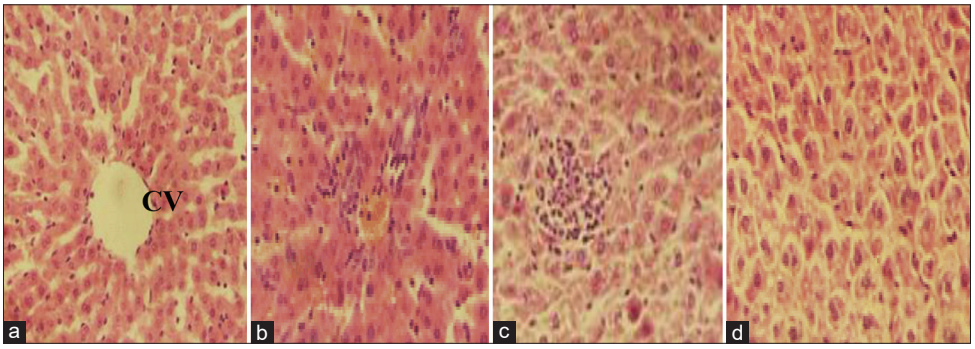


**Figure 2:** Photomicrograph of the liver sections of the control and rat treated with dose 100 mg/kg of Sidr extract. (a) Control rat normal hepatocytes with normal central vein (CV). (b) Infiltration of mononuclear cells. (c) Inflammatory region. (d) Hydropic changes

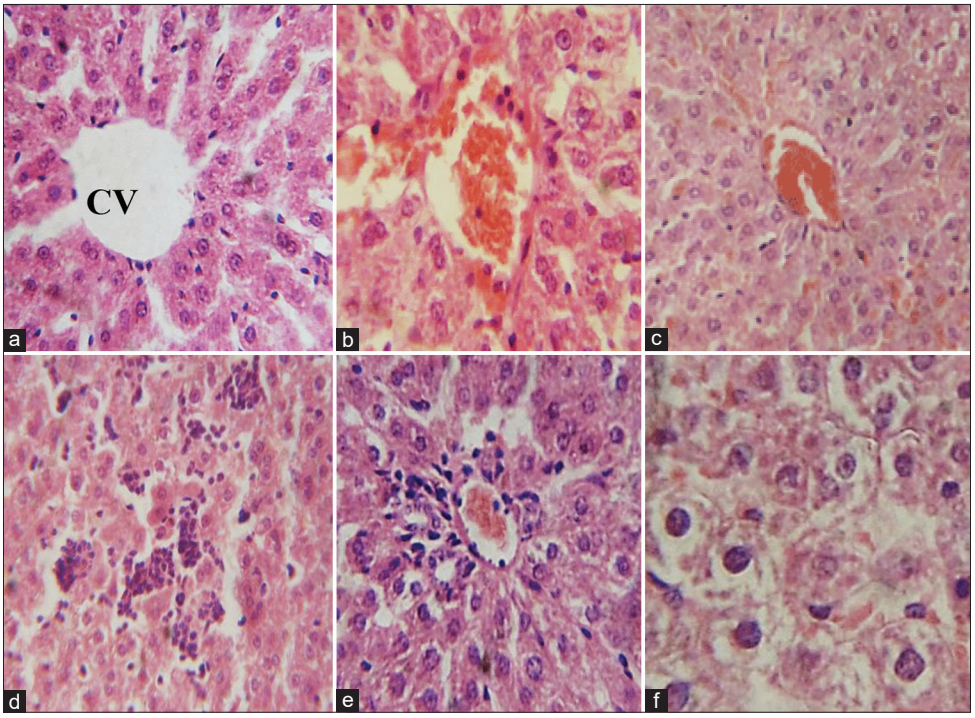


**Figure 3:** Photomicrograph of the liver sections the control and rat treated with dose 200 mg/kg of sidr extract. (a) Control rat showing normal hepatocytes with normal central vein (CV). (b) Congested central vein. (c) Liver hemorrhage. (d): Inflammatory region. (e) Infiltration of mononuclear cells. (f) Hydropic changes

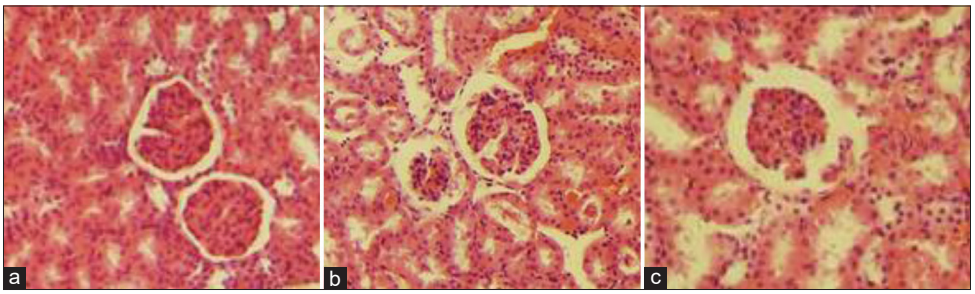


**Figure 4:** Photomicrograph of the kidney sections the control and rat treated with dose with 50 mg/kg of Sidr extract. (a) Control rat showing normal structure of glomerulus and tubules. (b) Glomerular shrinkage and tubular hemorrhage. (c) Glomerular degeneration

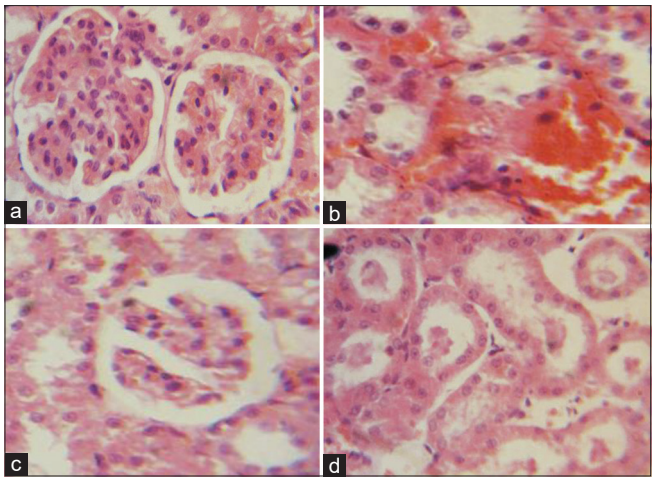


**Figure 5:** Kidney sections the control and rat treated with dose 100 mg/kg of Sidr extract. (a) Control rat showing normal structure of glomerulus and tubules. (b) Hemorrhage. (c) Glomerular degeneration. (d) Tubular casts

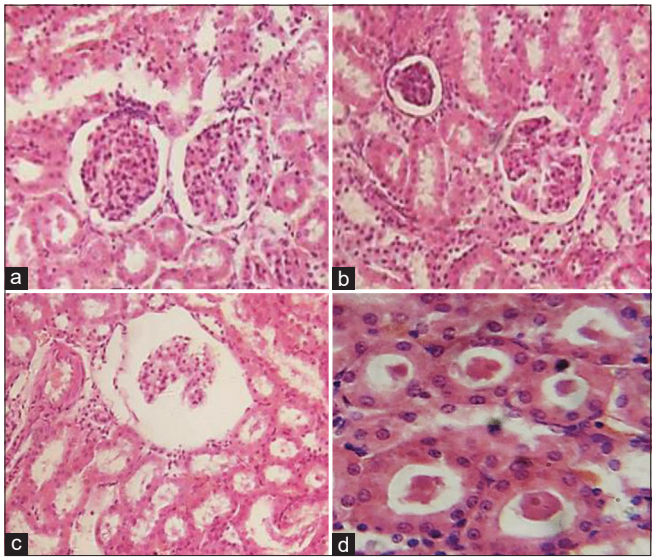


**Figure 6:** Photomicrograph of the kidney sections the control and rat treated with dose 200 mg/kg of plant extract. (a) Control rat showing normal structure of glomerulus and tubules. (b) and (c) Glomerular shrinkage. (d) Medullary tubular casts

and brain which are the most targeted area of the toxic substances metabolically, were isolated and there were no lesions found on the macroscopic examination of these tissues when were compared with the control group,[25] as displayed in Figures 1-12. Moreover, the microscopic examination of the vital organs of the treated rats with sidr extract showed only some effect for both hepatic and renal tissues and no effect to other organs. As shown in Figure 1, the hepatic cells of the control have a round nucleus in the center and the central vein (CV) is surrounded by flat endothelial cells without any other changes. Whereas, on the liver of rats treated with 50 mg/kg of the extract, there were some mild histopathological changes such as inflammation and infiltration of the mononuclear cells from the blood vessels

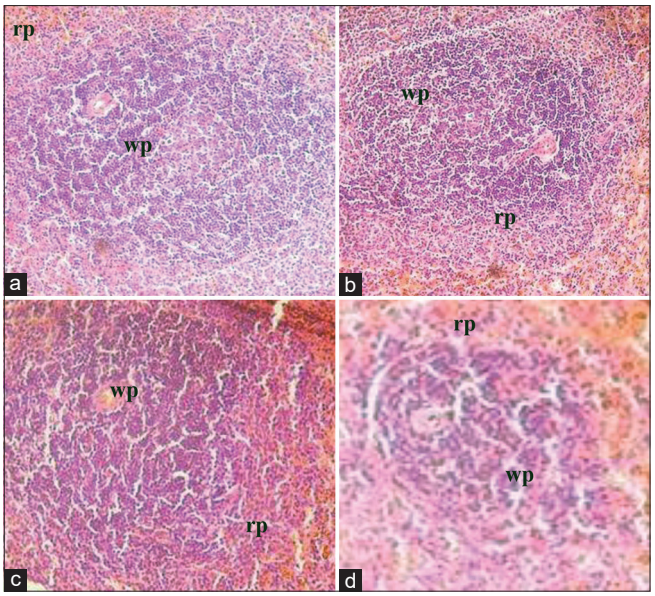


**Figure 7:** Photomicrograph of the spleen sections. (a) Control spleen with normal white pulp (WP) and red pulp (RP). (b) Spleen administrated with 50 mg/kg of plant extract with normal WP and RP. (c) Spleen administrated with 100 mg/kg of plant extract with normal WP and (RP). (d) Spleen administrated with 200 mg/kg of plant extract showing normal white pulp (WP) and normal red pulp (RP) structure

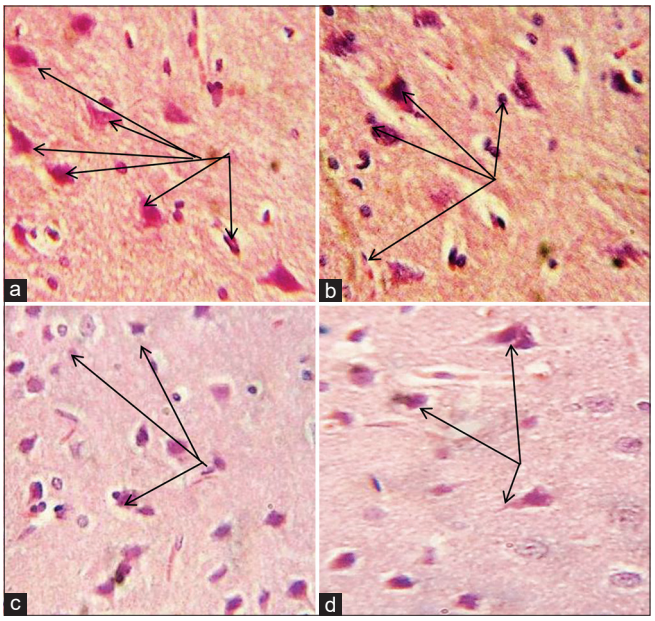


**Figure 8:** Photomicrograph of the brain sections. (a) Control brain showing normal neuron cell bodies (arrows). (b) Brain administrated with 50 mg/kg of plant extract showing normal neuron cell bodies. (c) Brain administrated with 100 mg/kg of plant extract showing normal neuron cell bodies. (d) Brain administrated with 200 mg/kg of plant extract showing normal neuron cell bodies

and with higher dose the detrimental effects increased at 200mg/kg dose of sidr extract including hemorrhage in Figure 3c, congestion, inflammation, and hydropic changes in Figures 3b-f, respectively.

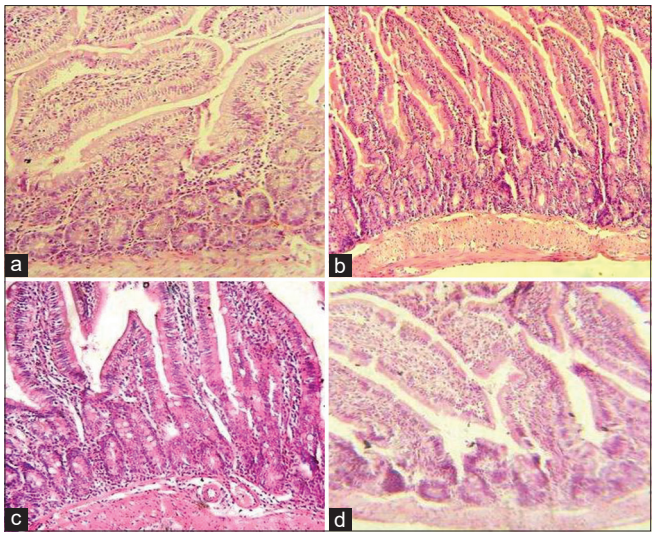


**Figure 9:** Photomicrograph of the intestine sections. (a) Control intestine showing normal villi. (b) Intestine administrated with 50 mg/kg, (c) 100 mg/kg, and (d) 200 mg/kg of sidr extract all are showing normal villi architecture

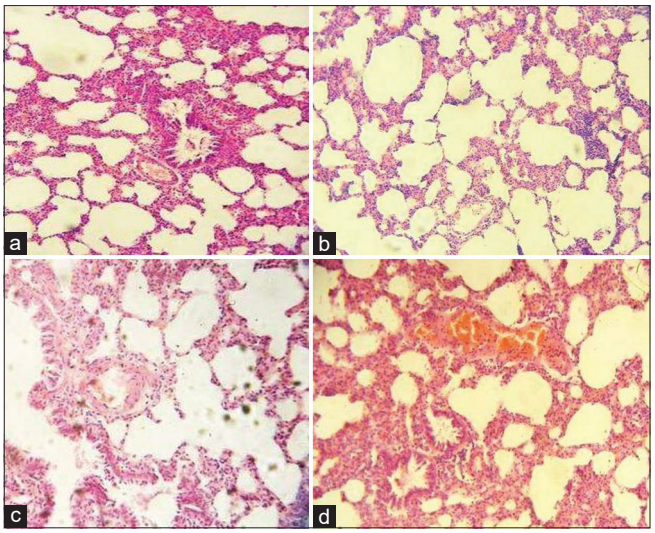


**Figure 11:** Photomicrograph of the lung sections. (a) Control lung display normal alveoli, (b) lung the rat treated with dose 50 mg/kg, (c) 100 mg/kg, and (d) 200 mg/kg of sidr extract show normal alveoli

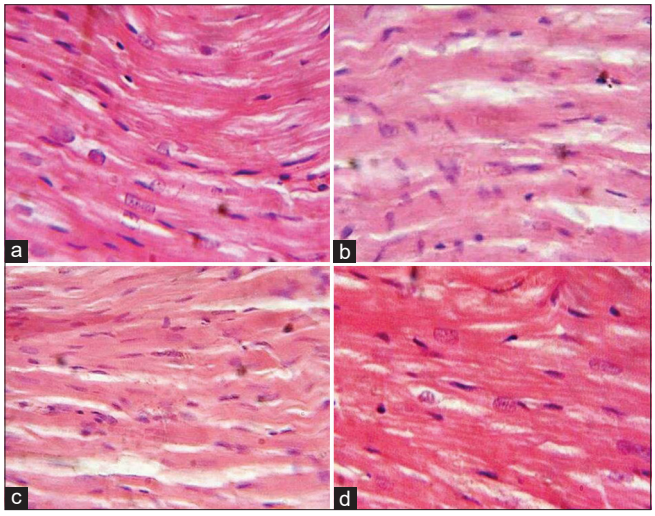


**Figure 10:** Photomicrograph of the skeletal muscle sections. (a) Control muscle showing normal myofibers. (b) Intestine muscles at dose 50 mg/kg, (c) 100 mg/kg, and (d) 200 mg/kg of sidr extract all keep normal myofibers

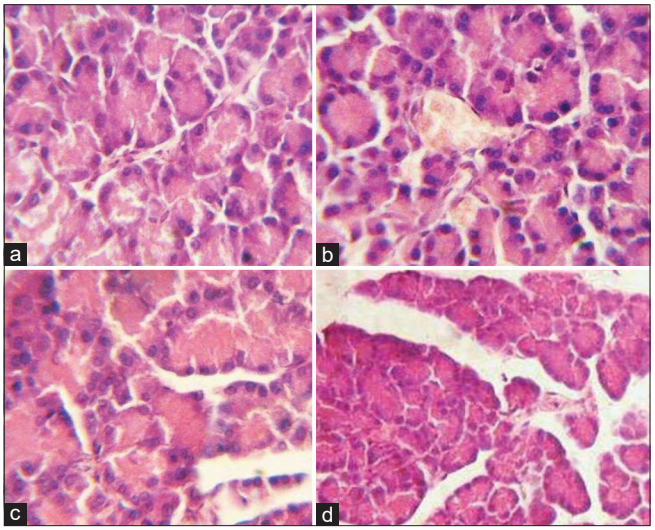


**Figure 12:** Photomicrograph of the pancreas sections. At last, Figure, all a, b, c, and d show normal pancreatic acini; (a) control, (b) pancreas administrated 50 mg/kg of sidr extract, (c) at dose 100 mg/kg, and (d) pancreas administrated 200 mg/kg of sidr extract

Concerning the kidney sections, the control rats revealed normal glomerulus and tubules as represented in Figure 4a, though, there were glomerular shrinkage and degeneration in 4B and 4C in case of the rats treated with 50 mg/kg of the extract. More severe effects appeared at 100 mg/kg of oral administered extract such as hemorrhage between kidney tubules as represented in Figure 5b and tubular casts in 5D and the major devastating changes obtained at the dose of 200 mg/kg such as the glomerular shrinkage in 6B and 6C, and modularly tubular casts in 6D.

In conclusion, histopathological changes occurred in liver and kidney of rats at low dose of sidr extract (50 mg/kg) could be considered as mild, while the highest dose 200 mg/kg severely destructed the liver and kidney tissues. The three different doses of sidr extract elucidated no histopathological effects on spleen, brain, intestine, skeletal muscle, lungs, and pancreas.

## Results of Clinical Trails

*Direct examination*

The results of the shampoo effectiveness as anti-dandruff, after using for 14 days as described in the methods above, evaluated by direct examination under the microscope once a week, as shown in Table 8.

The table above highlights that after 1 week from the treatment roughly more than the half of the volunteers were completely cured, although the scalp dandruff status was assessed in 7.5%, 28.75%, and 11.25% as excessive, moderate, and mild, respectively. After 2 weeks of continuous treatment, the majority of the volunteers (83.75%) were completely cured, while out of 8.75%

**Table 8:** Clinical effectiveness of the shampoo; the number of volunteers that showed presence of dandruff and its level in the scalps

| Parameters | Presence of dandruff | | Level of dandruff in the scalp | | | |
|---|---|---|---|---|---|---|
| | +V | −V | *** | ** | * | 0 |
| After 1 week | 38 | 42 | 6 | 23 | 9 | 42 |
| After 2 weeks | 11 | 69 | 7 | 4 | 2 | 67 |

***: Excessive, **: Moderate, *: Mild, 0: Complete cure

illustrated no improvement and 7.5% were in with mild and moderate dandruff status.

After 4 weeks of the treatment, the outcomes confirmed that less than the tenth of the volunteers were not benefited from the sidr shampoo and approximately 86% were entirely cured of this disorder.

*Cultural investigation*

The cultural antifungal assay is used to assure the effectiveness of the sidr shampoo as shown in Table 8.

Under the optimum conditions of the fungal growth, a total of 65% of the samples established no growth when cultured on sabouraud agar; however, the remaining samples 35% were demonstrated as variable growth of the fungi. It might be supposed that either the resistant samples indicate to the inactivity of the extract on these volunteers because of the previous treatments or the etiologic of the dandruff is a species other than *M. furfur*. Previous studies have approved the antifungal activity of the aqueous extract of *Z. spina-christi* against *Candida albicans in vivo* where the extract showed MIC of 6.25 mg/ml against *C. albicans*.[26] Moreover, the methanolic extract of the roots of *Z. spina-christi* revealed an antifungal effect against dermatophyte especially *Microsporum canis, Aspergillus fumigatus*, *Trichophyton rubrum*, and *Trichophyton mentagrophytes*.[4] Others reported that the fruits were also active against *C. albicans*.[27] Different extracts and fractions of the leaves, fruits, and seeds of *Ziziphus* showed considerable antiviral, antifungal, and antibacterial activities.[28]

## CONCLUSION

Formulated shampoo of *Z. spina-christi* extract is safe and effective at 50 mg/kg and 100 mg/kg according to the sub-acute toxicity study using albino rats. Although at higher doses, this extract did not cause many adverse effects on the testing animals except some observable histopathological and biochemical changes on the liver and kidney.

## ACKNOWLEDGMENT

The authors express their gratitude to Pharmaceutics and Histology Laboratories in Al-Nasser University, Biology Department – Faculty of Applied Sciences – Sana'a University, Forty-Eight Hospital, Department of Histopathology and Global Pharma Company, Sana'a – Yemen; for supplying all materials and equipment used in this research**.**

## DECLARATION OF INTEREST

The authors declare no conflicts of interest.

## REFERENCES

1. Saad AH, Kadhim RB, Rasool BA. Formulation and evaluation of herbal shampoo from *Ziziphus spina* Christi leaves extract. Int J Res Ayurveda Pharm 2011;2:1802-6.
2. Abalaka M, Mann A, Adeyemo S. Studies on *in-vitro* antioxidant and free radical scavenging potential and phytochemical screening of leaves of *Ziziphus mauritiana* L. and *Ziziphus spinachristi* L. compared with Ascorbic acid. J Med Genet Genomics 2011;3:28-34.
3. Abdel-Zaher AO, Salim SY, Assaf MH, Abdel-Hady RH. Antidiabetic activity and toxicity of *Zizyphus spina-christi* leaves. J Ethnopharmacol 2005;101:129-38.
4. Adamu HM, Abayeh O, Ibok N, Kafu SE. Antifungal activity of extracts of some Cassia, Detarium and Ziziphus species against dermatophytes. 2006;5:357-60.
5. Adzu B, Haruna AK. Studies on the use of *Zizyphus spina-christi* against pain in rats and mice. Afr J Biotechnol 2007;6:1317-24.
6. Adzu B, Haruna AK, Ilyas M, Pateh UU, Tarfa FD, Chindo BA, *et al*. Structural characterization of ZS-2A: An antiplasmodial compound isolated from *Zizyphus spina-christi* root bark. J Pharm Nutr Sci 2011;1:48-53.
7. Chen YF, Yang CH, Chang MS, Ciou YP, Huang YC. Foam properties and detergent abilities of the saponins from *Camellia oleifera*. Int J Mol Sci 2010;11:4417-25.
8. El-Kamali HH, Mahjoub SA. Antibacterial activity of *Francoeuria crispa*, *Pulicaria undulata*, *Ziziphus spina-christi* and *Cucurbita pepo* against seven standard pathogenic bacteria. Ethnobot Leaflets 2009;6:6.
9. Waggas AM, Al-Hasani RH. Neurophysiological study on possible protective and therapeutic effects of sidr (*Zizyphus spina-christi* L.) leaf extract in male albino rats treated with pentylenetetrazol. Saudi J Biol Sci 2010;17:269-74.
10. Patel D, Prasad SK, Kumar R, Hemalatha S. An overview on antidiabetic medicinal plants having insulin mimetic property. Asian Pac J Trop Biomed 2012;2:320-30.
11. Adzu B, Amos S, Amizan M, Gamaniel K. Evaluation of the antidiarrhoeal effects of *Zizyphus spina-christi* stem bark in rats. Acta Trop 2003;87:245-50.
12. Ali SA, Hamed MA. Effect of *Ailanthus altissima* and *Zizyphus spina* Christi on bilharzial infestation in mice: Histological and histopathological Studies. J Appl Sci 2006;6:1437-46.
13. Krunali T, Dhara P, Meshram D, Mitesh P. Evaluation of standards of some selected shampoo preparation. World J Pharm Pharm Sci 2013;2:3622-30.
14. Dikshit A, Tiwari AK, Mishra RK, Kamran A, Pandey A, Kumar A, *et al*. Botanicals for the management of dandruff. Med Plants Int J Phytomed Relat Ind 2012;4:55-64.
15. Gibson M. Pharmaceutical Preformulation and Formulation: A Practical Guide from Candidate Drug Selection to Commercial Dosage Form. 2nd ed., Vol. 199. Boca Raton: CRC Press; 2009. p. 2.
16. Goci1 E, Haloçi E, Vide K, Malaj L. Application and comparison of three different extraction methods of capsaicin from capsicum fruits. Albanian J Pharm Sci 2013;1:16-9.
17. AlQuadeib BT, Eltahir EK, Banafa RA, Al-Hadhairi LA. Pharmaceutical evaluation of different shampoo brands in local Saudi market. Saudi Pharm J 2018;26:98-106.
18. Ireland S, Carlino K, Gould L, Frazier F, Haycock P, Ilton S, *et al*. Shampoo after craniotomy: A pilot study. Can J Neurosci Nurs 2007;29:14-8.
19. Jain N, Sharma P, Sharma N, Joshi SC. Haemato-biochemical profile following sub-acute toxicity of malathion in male albino rats. Avicenna J Phytomed 2009;2:500-6.
20. Rhiouani H, El-Hilaly J, Israili ZH, Lyoussi B. Acute and sub-chronic toxicity of an aqueous extract of the leaves of *Herniaria glabra* in rodents. J Ethnopharmacol 2008;118:378-86.
21. Li M, Jia Z, Hu Z, Zhang R, Shen T. Experimental study on the

hemostatic activity of the Tibetan medicinal herb *Lamiophlomis rotata*. Phytother Res 2008;22:759-65.
22. Mukinda JT, Syce JA. Acute and chronic toxicity of the aqueous extract of *Artemisia afra* in rodents. J Ethnopharmacol 2007;112:138-44.
23. Ozolua R, Anaka O, Okpo S, Idogun S. Acute and sub-acute toxicological assessment of the aqueous seed extract of *Persea americana* Mill (*Lauraceae*) in rats. Afr J Tradit Complement Altern Med 2009;6:573-8.
24. Saleem U, Amin S, Ahmad B, Azeem H, Anwar F, Mary S. Acute oral toxicity evaluation of aqueous ethanolic extract of *Saccharum munja* Roxb. roots in albino mice as per OECD 425 TG. Toxicol Rep 2017:4;580-5.
25. Ibrahim KE, Al-Mutary MG, Bakhiet AO, Khan HA. Histopathology of the liver, kidney, and spleen of mice exposed to gold nanoparticles. Molecules 2018;23:1848.
26. Tom GM, Yesufu HB, Abdulrahman FI. Antimicrobial screening and effect of the pulp extracts of *Zizyphus spina-christi* (Linnaeus Desf) on some biochemical parameters in rats. J Pharm Bioresour 2009;6:63329.
27. Pirbalouti AG, Bahmani M, Avijgan M. Anti-candida activity of some of the Iranian medicinal plants. Elect J Biol 2009;5:85-8.
28. Shahat AA, Pieters L, Apers S, Nazeif NM, Abdel-Azim NS, Berghe DV, *et al*. Chemical and biological investigations on *Zizyphus spina-christi* L. Phytother Res 2001;15:593-7.