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Comparative evaluation of the hepatoprotective activity of methanol leaf extracts of *Pterocarpus santalinoides* DC obtained by cold maceration and Soxhlet extraction techniques

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ABSTRACT

Objectives: This study compared the hepatoprotective activity of *Pterocarpus santalinoides* cold maceration extract (PSCME) with *P. santalinoides* Soxhlet extract (PSSE). **Materials and Methods:** Phytochemical constituents of both extracts were determined. 45 albino rats, randomly assigned into 9 groups (1–9) of 5 rats each, were used for the study. Liver damage was induced in groups 1–8 using carbon tetrachloride (CCl_4). Group 1 served as untreated control, Groups 2–7 were treated with 50, 250 or 500 mg/kg PSCME or PSSE, Group 8 was treated with 100 mg/kg silymarin, while Group 9 served as normal control. Treatment was done orally for 15 days. Serum enzyme activities, proteins, cholesterol and bilirubin levels, relative liver weight and liver histomorphology were evaluated. **Results:** There were higher concentrations of phenols and saponins in PSCME than in PSSE. 250 mg/kg dose of both PSCME and PSSE significantly ($P < 0.05$) protected hepatocellular integrity, enhanced hepatic bilirubin clearance, ameliorated hepatomegaly, and also led to less severe vacoulation and necrosis of hepatocytes in the rats given CCl_4. **Conclusion:** Extracts of *P. santalinoides* obtained by cold maceration and Soxhlet extraction techniques are of nearly equal hepatoprotective potency. The heat applied during Soxhlet extraction had no adverse effect.

Keywords: Cold maceration extraction, hepatoprotection, *Pterocarpus santalinoides* methanol leaf extract, soxhlet extraction, thermostability

INTRODUCTION

The liver is the key organ of metabolism and excretion in the body. It performs more than 500 vital metabolic functions which include synthesis of glucose, urea, plasma proteins, clotting factors, and regulation of blood levels of amino acids. The liver is also responsible for the detoxification of drugs and xenobiotics in the body; it is thus constantly and variedly exposed to xenobiotics which may induce toxic injury or hepatotoxicity.[1] Toxins and toxicants absorbed from the intestinal tract will first of all pass through liver and may elicit responses such as inflammation, degeneration, and/or neoplasia of the liver. Some common hepatotoxins or hepatotoxicants include a wide variety of pharmaceutical agents, microbial toxins, and industrial chemicals that pollute the environment.[2]

Extraction, as used pharmaceutically and in pharmacology, involves the separation of medicinally active components of plants from the inactive or inert components using selective solvents in standard extraction procedures.[3] It is an important step used in the discovery of bioactive components

of medicinal plants. The extraction process is basically for separation of one component from another using extracting solvent. The products obtained from plants are relatively impure liquids, semisolids, or powders intended only for oral or external use. Nowadays, a variety of extraction methods with differences in terms of methodology, time, temperature, pressure, and solvents have been developed. These extraction methods include cold maceration, Soxhlet extraction, infusion, digestion, decoction, and percolation.[3] They can also be used in combination with other techniques in order to improve the recovery, and consequently, the pharmacological profile of the extracts. However, as a consequence of the differences between the extractive processes, there may be differences and discrepancies in the qualitative and quantitative composition of bioactive components in the extracts obtained from the same plant. This accounts for the differences in clinical efficacy and beneficial effects following treatment with extracts; thus, selection of a suitable extraction method is usually vital to obtaining a viable extract.[3,4]

Cold maceration extraction involves placing the powdered crude plant material in a stoppered container with the solvent, and allowing it to stand at room temperature for a period of at least 3 days with frequent agitation until the soluble matter is dissolved. The mixture is then strained, the marc (the damp solid material) is pressed, and the combined liquids are clarified by filtration after standing.[3,5] Cold maceration is considered ideal for thermolabile constituents.[3]

Soxhlet extraction also known as hot continuous extraction, involves the heating of the extracting solvent such that its condensed vapor drips into a thimble (porous bag made of strong filter paper) containing the finely ground crude plant material in the Soxhlet apparatus, and extracts it by contact. The process of Soxhlet extraction is continuous and has the comparative advantage of enabling the extraction of large amounts of drug with a relatively smaller quantity of solvent.[3,6] Soxhlet extraction, however, can only be used for thermostable constituents because boiling temperatures above 78°C is commonly involved.

Bioactive compounds from plants are often used in medicine in the treatment of several pathologies amongst which is liver disorders. *Pterocarpus santalinoides* DC [Figure 1] is ever-green tree species of the family *Papilionaceae*, native to Tropical West Africa and South America.[7] It's English name is "red sandal wood," and in other languages; it is known as nturukpa in Igbo; gunduru, gyadar, or kurmi in Hausa; gbengbe in Yoruba; akumeze in Edo; nja in Efik; kereke in Tiv; maganchi in Nupe, Ouokisse in French; and mututi in India.[8] The leaves of *P. santalinoides* are traditionally used as food (vegetable) and as medicine in the treatment of various ailments which includes liver diseases amongst others.[9] Previous studies showed that the methanol leaf extract of *P. santalinoides* obtained by cold maceration extraction technique was effective in ameliorating acetaminophen and carbon tetrachloride (CCl_4)-induced hepatotoxicity in albino rats.[10,11] Although extracts obtained by cold maceration extraction method was used in the previous studies,[10,11] the necessity to compare the efficacy of the hepatoprotective principle(s) present in the methanol leaf extract of *P. santalinoides* obtained by cold maceration extraction method with that obtained by the hot continuous extraction that occurs in Soxhlet extraction technique which involves heating, prompted the present study. This is because the leaves of *P. santalinoides* are traditionally used as cooked vegetable, and also administered as medicine in form of hot soup; hence, the present study which evaluated and compared the hepatoprotective activity of methanol leaf extract of *P. santalinoides* obtained by cold maceration extraction technique with that obtained by Soxhlet extraction technique.

Figure 1: Picture showing leaves of *Pterocarpus santalinoides*

MATERIALS AND METHODS

Chemicals, Reagents and Assay Kits

CCl_4, methanol and silymarin were purchased from Sigma-Aldrich, St. Louis, Missouri, USA, while thiopentone sodium was obtained from Chandra Bhagat Pharma Pvt., Ltd., Mumbai, India. The clinical biochemistry assay kits for the evaluation of the serum enzyme activity concentration of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and serum levels of total proteins, albumins and total cholesterol (TC) were procured from Quimica Clinica Applicada (QCA), Spain, while the clinical biochemistry assay kit for the evaluation of serum total bilirubin was sourced from Randox Laboratories Ltd, County Antrim, United Kingdom. All other routine reagents and chemicals were of analytical grade.

Plant Collection, Identification and Extract Preparation

Fresh leaves of *P. santalinoides* DC were collected from Nsukka (6.8903°N, 7.4111°E) in Enugu state, Nigeria, in February 2018. *P. santalinoides* leaves are freely available commercially, and there are no restrictions on its use for research. The plant was identified and authenticated by a plant taxonomist Mr. A.O. Ozioko of the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka. A voucher Specimen of the plant (UNH/2018/No. 2) was deposited at the University of Nigeria, Nsukka herbarium. The leaves were air dried under shade and ground into powder. 500 g of the powdered leaves were extracted by the cold maceration extraction technique using 80% methanol, while another 500 g were extracted by

the Soxhlet extraction technique using 80% methanol. The resulting extracts were concentrated to dryness in a rotary evaporator (Buchi, Switzerland), and labeled as *P. santalinoides* cold maceration extract (PSCME) and *P. santalinoides* Soxhlet extract (PSSE), respectively. The percentage yield of each extract was calculated.

Experimental Animals

69 adult male Sprague-Dawley albino rats (*Rattus norvegicus*) of 12 weeks of age obtained from the Laboratory Animal Unit of the Department of Veterinary Physiology and Pharmacology, University of Nigeria, Nsukka, were used for the study. 24 of the albino rats were used for the acute toxicity test (12 rats for each extract), while 45 were used for the sub-acute hepatoprotection study to compare the effects of PSCME and PSSE on blood levels of markers of hepatotoxicity in CCl_4-induced liver damage. The rats were kept in stainless steel cages in a fly-proof animal house at room temperature (24–28°C), and acclimatized for 2 weeks before the study commenced. They were fed commercial pelletized feed (Grand Cereals Ltd, Jos, Nigeria), and provided with clean drinking water *ad libitum* all through the study. The laboratory animal study was conducted in accordance with the European Community Guidelines (EEC Directive of 1986; 86/609/EEC), and the experimental animal study protocol was approved by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine, University of Nigeria (Approval No: FVM-UNN-IACUC-2018-079).

Phytochemical Analysis

Phytochemical analyses were done on PSCME and PSSE, following standard procedures.[12,13] 1 g of each extract was dissolved in 100 ml of distilled water and filtered with Whatman no 1 filter paper. The clear filtrates obtained were used to test for alkaloids, carbohydrates, flavonoids, glycosides, phenols, saponins, sterols, tannins, and terpenes. Results of the phytochemical analyses were presented as semi-quantitative scores as follows: presence of high level of each phytochemical was scored (+++), moderate levels (++), and low levels (+).[12,13]

Acute Toxicity Test

The oral acute toxicity of PSCME and PSSE was determined by the Organization for Economic Cooperation and Development (OECD) Acute Toxic Class method.[14] The Annex 2b OECD test procedure was followed. Three rats each were used for the 50, 300, 2000 and 5000 mg/kg test doses for each extract. The albino rats were fasted for 12 h before the commencement of the test, but clean drinking water was made available to them *ad libitum*. Each dose of the extracts was dissolved in 1 ml of distilled water and given orally with a gastric tube. The rats were observed closely for 48 h for any signs of toxicity or mortality, and further for up to 14 days post-administration of the extract.

Evaluation of the Effects of PSCME and PSSE on Blood Levels of Markers of Liver Damage

The 45 male albino rats used for the sub-acute hepatoprotection study were randomly assigned to nine groups (1–9) of five rats each. Sub-acute hepatotoxicity was induced in rats in groups 1–8 by intraperitoneal injection of 1 ml/kg CCl_4 in equal volume of olive oil (50% v/v), at the beginning of the experiment (day 0), and after every 72 h for 12 days (days 3, 6, 9 and 12).[10,11,15] CCl_4 was used as a model chemical for the induction of hepatotoxicity because of its reported consistency and reliability.[10,11,15,16] Treatment with PSCME and PSSE was done *per os* twice daily for 15 days starting from the day after the initial CCl_4 injection (day 1) and continuing as co-treatment and ending 3 days after the last CCl_4 injection. The treatment for the groups were as follows: group 1 rats served as negative control (treated with 10 ml/kg distilled water placebo); groups 2, 3 and 4 rats were treated with 50, 250, and 500 mg/kg PSCME, respectively; groups 5, 6 and 7 rats were treated with 50, 250, and 500 mg/kg PSSE, respectively; group 8 rats were treated with silymarin (a known hepatoprotective drug) at the dose of 100 mg/kg *per os* twice daily for the 15 days as positive control, while group 9 rats were treated with 10 ml/kg distilled water placebo and served as normal control.

Blood samples were collected from the albino rats on day 15 (at the end of the experiment) for serum biochemistry assay. Blood sample collection was by the orbital technique.[17] The blood samples were allowed to stand at room temperature for 45 min to clot; they were then centrifuged at 3000 revolutions per minute for 10 min using a table centrifuge (Jenalab Medical, England), after which the serum was harvested. The serum biochemistry assay was done immediately upon separation of the serum from blood clot, following standard procedures.

Assay of Serum Enzyme Activities

Serum ALT and serum AST activities were evaluated using the QCA ALT and AST test kits based on the Reitman-Frankel colorimetric method,[18] and quantified at 505 nm wavelengths, using a semi-automated blood biochemistry analyzer (Diatek Instruments, Wuxi, China). The serum ALP activity was evaluated using the QCA ALP test kit, based on the phenolphthalein monophosphate method,[18] and quantified at 546 nm wavelength, using a semi-automated blood biochemistry analyzer (Diatek Instruments, Wuxi, China).

Assay of Serum Proteins

Serum total protein levels were determined using the QCA total protein test kit which was based on the direct Biuret method,[19] and was quantified at 546 nm wavelength using a semi-automated blood biochemistry analyzer (Diatek Instruments, Wuxi, China). Assay of serum albumin level was done using the QCA albumin test kit based on the bromocresol green method[19] and was quantified at 630 nm wavelength using a semi-automated blood biochemistry analyzer (Diatek Instruments, Wuxi, China). The serum globulin levels were calculated by subtracting the serum albumin levels from the serum total protein levels.[19]

Determination of Serum TC and Serum Total Bilirubin Levels

The serum TC levels were determined using the QCA TC test kit, which is based on the enzymatic colorimetric method,[20]

and was quantified at 505 nm wavelength using a semi-automated blood biochemistry analyzer (Diatek Instruments, Wuxi, China). The serum total bilirubin levels in the serum samples were assayed using the Randox® Bilirubin test kit (Randox Laboratories Ltd, County Antrim, United Kingdom), which is based on the Jendrassik and Grof method,[21] and was quantified at 578 nm using a semi-automated blood biochemistry analyzer (Diatek Instruments, Wuxi, China).

Determination of Relative Liver Weight

After blood sample collection on the 15th day, the rats were euthanized by intra-peritoneal injection of 250 mg/kg of thiopentone sodium.[22] The liver of each rat was carefully eviscerated and weighed, and relative liver weight of individual rats was calculated.

Processing of Liver Sections for Histology

Thin slices of the liver of albino rats from each group were fixed in 10% buffered formal saline and further processed for histology. First, they were serially dehydrated through progressive concentrations of alcohol, and then cleared with xylene. After clearing, the tissues were embedded in paraffin wax, and then thin sections of about 5 microns were made with the microtome. Each section was mounted on a clean glass slide and stained with hematoxylin and eosin. A mounting medium (Canada balsam) was dropped on each tissue section and cover slips were placed on them and they were allowed to dry,[23] The slides were then examined with a light microscope. Photomicrographs were captured using a Moticam Images Plus 2.0 (Motic China Group Ltd.) digital camera attached to the microscope.

Statistical Analysis

Data obtained from the study were analyzed using a one way analysis of variance. Variant means were separated post hoc using the least significant difference method. Statistical Package of the Social Sciences version 16.0 was used for the analysis. Significance was accepted at $P < 0.05$. Summaries of the results were presented as tables of means with standard error, and figures.

RESULTS

Yield of PSCME and PSSE upon Extraction

The PSCME and PSSE obtained were dark brown in color and soluble in water. The percentage yield of PSCME was 10.33% w/w, while the percentage yield of PSSE was 12.8 % w/w.

Phytochemical Analyses

The phytochemical analyses showed that PSCME has high levels (+++) of tannins, saponins, carbohydrates, glycosides, phenols, terpenes and sterols, moderate level (++) of flavonoids, and low level (+) of alkaloids, while PSSE has high levels (+++) of tannins, carbohydrates, glycosides, terpenes and sterols, moderate levels (++) of flavonoids, and low level (+) of alkaloids, saponins, and phenol. Polyuronides, starch, phytic acid, and resins were absent in both extracts [Table 1].

Acute Toxicity Study

Results of the acute toxicity test showed that there were neither mortality nor any sign of toxicity in all the rats treated with PSCME and PSSE at all doses used. Rats tolerated both extracts up to 5000 mg/kg; therefore, the LD_{50} of both extracts is above 5000 mg/kg ("Category 5–Unclassified" of the Global Harmonized Classification System).[14]

Effects of PSCME and PSSE on Blood Levels of Markers of Liver Damage

The serum ALT and AST activity levels of rats treated with 250 mg/kg PSCME and PSSE (group 3 and 6), 100 mg/kg silymarin (group 8) and the normal control (group 9) were significantly lower ($P < 0.05$) than and about half of those of the untreated negative control (group 1) and of those treated with 50 mg/kg and 500 mg/kg of both PSCME and PSSE (groups 2, 4, 5 and 7) [Table 2]. The serum ALP activity level of only rats in the normal control group (group 9) was significantly lower ($P < 0.05$) than those of the untreated negative control (group 1) and the rats treated with 50 mg/kg PSCME and PSSE (groups 2 and 5), 250 mg/kg PSCME (group 3) and 500 mg/kg PSCME and PSSE (groups 4 and 7) [Table 2]. The serum total protein levels of rats treated with 250 mg/kg PSSE (group 6), 100 mg/kg silymarin (group 8) and the normal control (group 9) were significantly higher ($P < 0.05$) than that of the untreated control (group 1) and that of those treated with 50 mg/kg and 500 mg/kg PSCME (groups 2 and 4), but the serum protein levels of rats treated with 250 mg/kg PSCME (group 3), 50 mg/kg PSSE (group 5) and 500 mg/kg PSSE (group 7) were not significantly ($P > 0.05$) different from that of the untreated negative control (group 1) and the normal control (group 9 rats) [Table 3]. There were no significant variations ($P > 0.05$) in the serum albumin levels among the groups [Table 3]. The serum globulin levels of the untreated negative control (group 1) and the groups treated with 50 and 500 mg/kg PSCME (groups 2 and 4) were significantly lower ($P < 0.05$) than that of the normal control (group 9), but that of rats treated with 250 mg/kg PSCME and PSSE (groups 3 and 6), 50 mg/kg PSSE (group 5), 500 mg/kg PSSE (group 7) and 100 mg/kg silymarin (group 8) were not significantly different from that of both the untreated negative

Table 1: Phytochemical constituents of PSCME and PSSE

Phytochemical constitents	Semi-quantitative composition	
	PSCME	PSSE
Tannins	+++	+++
Terpenes	+++	+++
Sterols	+++	+++
Alkaloids	+	+
Flavonoids	++	++
Saponins	+++	+
Carbohydrates	+++	+++
Glycosides	+++	+++
Phenol	+++	+

+++: High levels present, ++: Moderate levels present, +: Low levels present, PSCME: Pterocarpus santalinoides cold maceration extract, PSSE: *Pterocarpus santalinoides* Soxhlet extract

Table 2: Comparison of the effects of oral administration of *Pterocarpus santalinoides* cold maceration extract (PSCME) and *Pterocarpus santalinoides* Soxhlet extract (PSSE) on serum enzyme activity concentration of albino rats given sub-acute toxic doses of CCl4.

Groups*	Mean±standard error		
	Alanine aminotransferase (IU/L)	Aspartate aminotransferase (IU/L)	Alkaline phosphatase (IU/L)
Group 1	107.72±5.11[a]	111.44±7.87[ac]	379.22±77.87[a]
Group 2	106.13±7.48[a]	118.94±6.48[ac]	420.62±30.07[a]
Group 3	52.36±5.81[b]	79.31±8.35[b]	361.97±9.81[a]
Group 4	102.10±5.69 [a]	117.00±2.65[ac]	335.31±20.95[a]
Group 5	107.83±4.37[a]	118.10±5.38[ac]	406.54±15.82[a]
Group 6	48.54±8.70[b]	75.47±6.49[b]	272.60±26.15[ab]
Group 7	85.73±17.39[a]	95.37±12.33[bc]	315.71±39.72[a]
Group 8	47.34±9.36[b]	76.83±7.78[b]	284.32±24.12[ab]
Group 9	30.49±3.46[b]	59.91±6.58[b]	107.11±25.22[b]

*Groups: Group 1–CCl4 alone, no treatment, Group 2–CCl4+50 mg/kg PSCME, Group 3–CCl4+250 mg/kg PSCME, Group 4–CCl4+500 mg/kg PSCME, Group 5–CCl4+50 mg/kg PSSE, Group 6–CCl4+250 mg/kg PSSE, Group 7–CCl4+500 mg/kg PSSE, Group 8–CCl4+100 mg/kg Silymarin, Group 9–No CCl4, no treatment. [a, b, c]: Alphabetical superscripts in a column indicate significant differences between the groups, $P<0.05$

Table 3: Comparison of the effects of oral administration of *Pterocarpus santalinoides* cold maceration extract (PSCME) and *Pterocarpus santalinoides* Soxhlet extract (PSSE) on the serum protein levels of albino rats given sub-acute toxic doses of CCl4

Groups*	Mean±standard error		
	Total proteins (g/dl)	Albumins (g/dl)	Globulins (g/dl)
Group 1	7.17±0.37[a]	3.77±0.25	3.40±0.13[a]
Group 2	6.88±0.18[a]	3.40±0.14	3.48±0.16[a]
Group 3	7.33±0.12[ab]	3.46±0.14	3.86±0.23[ab]
Group 4	6.87±0.16[a]	3.44±0.02	3.44±0.14[a]
Group 5	7.37±0.15[ab]	3.70±0.20	3.67±0.08[ab]
Group 6	7.45±0.12[b]	3.64±0.13	3.69±0.22[ab]
Group 7	7.34±0.15[ab]	3.60±0.03	3.74±0.15[ab]
Group 8	7.48±0.18[b]	3.66±0.15	3.82±0.21[ab]
Group 9	7.73±0.19[b]	3.71±0.10	4.03±0.20[b]

*Groups: Group 1–CCl4 alone, no treatment, Group 2–CCl4+50 mg/kg PSCME, Group 3–CCl4+250 mg/kg PSCME, Group 4–CCl4+500 mg/kg PSCME, Group 5–CCl4+50 mg/kg PSSE, Group 6–CCl4+250 mg/kg PSSE, Group 7–CCl4+500 mg/kg PSSE, Group 8–CCl4+100 mg/kg Silymarin, Group 9 - No CCl4, no treatment. [a, b, c]: Alphabetical superscripts in a column indicate significant differences between the groups, $P<0.05$

control (group 1) and the normal control (group 9) [Table 3]. The serum TC of the rats treated with 250 mg/kg PSCME and PSSE (groups 3 and 6), 500 mg/kg PSCME (group 4) and the normal control (group 9) were significantly lower ($P < 0.05$) than those of the untreated negative control (group 1) and those treated with 50 mg/kg PSSE [Table 4]. Further, the serum TC of the rats treated with 250 mg/kg PSCME and PSSE (groups 3 and 6) and those treated with 500 mg/kg PSCME (group 4) were not significantly ($P > 0.05$) different from that of the normal control (group 9) [Table 4]. The serum total bilirubin of rats treated with 250 mg/kg PSCME and PSSE (groups 3 and 6), 100 mg/kg silymarin (group 8) and the normal control (group 9) were significantly ($P < 0.05$) lower than those of the untreated negative control (group 1), the groups treated with 250 mg/kg PSCME and PSSE (groups 2 and 5) and the group treated with 500 mg/kg PSCME (group 4) [Table 4].

Effects of PSCME and PSSE on Relative Liver Weight

The relative liver weight of the rats treated with 50 mg/kg PSCME (group 2), 250 mg/kg PSCME and PSSE (groups 3 and 6), 100 mg/kg silymarin (group 8) and the normal control (group 9) were significantly ($P < 0.05$) lower than that of the untreated negative control (group 1) and those of the rats treated with 500 mg/kg of PSCME and PSSE (groups 4 and 7) [Table 4]. Further, the relative liver weights of the rats treated with 250 mg/kg PSCME and PSSE and the ones treated with 100 mg/kg silymarin (group 8) were statistically comparable with/not significantly different ($P > 0.05$) from that of the normal control rats (group 9) [Table 4].

Histology of the Liver Sections

Representative histological sections of the liver of all the rat groups are presented in Figure 2. Specifically, there were necrosis of some hepatocytes and vacuolar degeneration (small sized vacuoles) of some others especially around the centrilobular area in the untreated negative control (group 1) rats (arrowed on group 1 liver section). The liver section of the rats treated with 250 mg/kg PSCME (group 2) showed large sized cytoplasmic vacuoles widely spread in the liver parenchyma (arrowed in group 2 liver section), with necrosis of some of the hepatocytes and hyperemia of

Table 4: Comparison of the effects of oral administration of *Pterocarpus santalinoides* cold maceration extract (PSCME) and *Pterocarpus santalinoides* Soxhlet extract (PSSE) on total cholesterol, bilirubin and relative liver weight of albino rats given sub-acute toxic doses of CCl4

Groups*	Mean±standard error		
	Total cholesterol (mg/dl)	Total bilirubin (mg/dl)	Relative liver weight (%)
Group 1	79.19±7.52[a]	0.68±0.05[ac]	5.20±0.15[a]
Group 2	62.67±6.85[ab]	0.62±0.15[ac]	4.37±0.16[b]
Group 3	60.43±1.70[b]	0.34±0.08[b]	4.13±0.09[bc]
Group 4	55.14±1.45[b]	0.69±0.02[ac]	5.16±0.20[a]
Group 5	78.71±7.07[a]	0.58±0.03[c]	4.68±0.21[ab]
Group 6	61.72±6.34[b]	0.42±0.03[b]	4.13±0.09[bc]
Group 7	65.14±7.21[ab]	0.47±0.02[bc]	5.19±0.26[a]
Group 8	63.29±6.87[ab]	0.39±0.03[b]	4.10±0.10[bc]
Group 9	61.86±2.61[b]	0.41±0.03[b]	3.97±0.28[c]

*Groups: Group 1–CCL4 alone, no treatment, Group 2–CCl4+50 mg/kg PSCME, Group 3–CCl4+250 mg/kg PSCME, Group 4–CCl4+500 mg/kg PSCME, Group 5–CCl4+50 mg/kg PSSE, Group 6–CCl4+250 mg/kg PSSE, Group 7–CCl4+500 mg/kg PSSE, Group 8–CCl4+100 mg/kg Silymarin, Group 9 - No CCl4, no treatment. [a, b, c]: Alphabetical superscripts in a column indicate significant differences between the groups, $P<0.05$

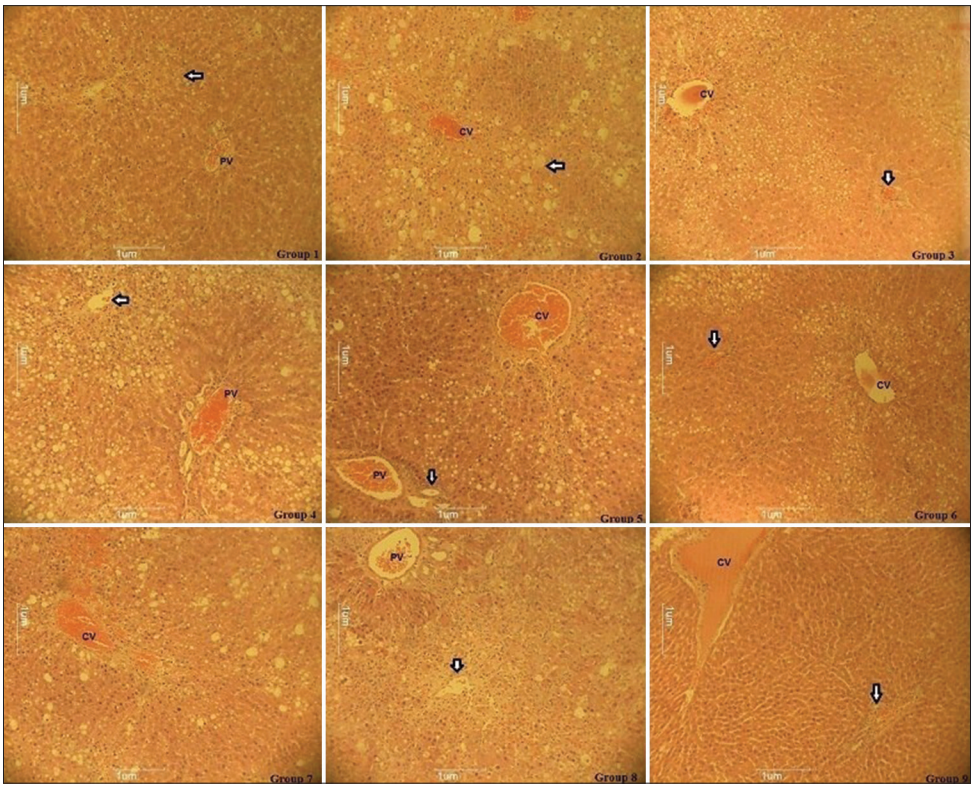

Figure 2: Liver sections of rat groups* given CCl_4 and treated with varied doses of *Pterocarpus santalinoides* cold maceration extract (PSCME) or *Pterocarpus santalinoides* Soxhlet extract (PSSE), compared to control group not given CCl_4 (H and E, 100×). PV: Portal vein, CV: Central vein. *Groups: Group 1–CCl_4 alone, no treatment, Group 2–CCl_4+50 mg/kg PSCME, Group 3–CCl_4+250 mg/kg PSCME, Group 4–CCl_4+500 mg/kg PSCME, Group 5–CCl_4+50 mg/kg PSSE, Group 6–CCl_4+250 mg/kg PSSE, Group 7–CCl_4+500 mg/kg PSSE, Group 8–CCl_4+100 mg/kg Silymarin, Group 9–No CCl_4, no treatment

the central vein (CV) and sinusoids. Liver section of the rats treated with 250 mg/kg PSCME (group 3) also showed vacuolar degeneration of hepatocytes, but with smaller sized cytoplasmic vacuoles observed mainly in the centrilobular and mid-zonal areas of the liver section, with milder hepatocyte necrosis and mild infiltration of lymphocytes in the portal area (arrowed on the liver section of group 3 rats). The liver section of the rats treated with 500 mg/kg PSCME (group 4) exhibited numerous cytoplasmic vacuoles of varied sizes in the centrilobular (arrowed in the group 4 liver section) and mid-zonal areas. In the liver section of the rats treated with 50 mg/kg PSSE (group 5), there was necrosis and vacuolar degeneration of hepatocytes especially in the midzonal area, with relatively less vacuoles but more necrotic hepatocytes (the bile duct is arrowed). The liver section of rats treated with 250 mg/kg PSSE (group 6) had vacuolar degeneration of

hepatocytes in some areas with other areas (around the portal vein) free of vacuoles (arrowed on liver section of group 6 rats), and relatively small number of necrotic hepatocytes. Liver section of the rats treated with 500 mg/kg PSSE (group 7) exhibited necrosis of hepatocytes, with cytoplasmic vacuoles of varied sizes widely spread in the liver section, and also hyperemia of the CV and sinusoids; while in liver sections of the rats treated with 100 mg/kg silymarin (group 8), there were areas of vacuolar degeneration and mild necrosis of hepatocytes especially around the CV (arrowed in the group 8 section). Liver sections of the normal control rats (group 9) showed normal hepatocytes with healthy cords of hepatocytes radiating around the CV and the portal area (arrowed in the group 9 liver sections). In summary, the histopathological evaluation showed that sections of the liver of all the rat groups given CCl_4 showed varying degrees of vacuolar degeneration and necrosis of hepatocytes, with variations in the size and distribution of the vacuoles, and severity of the necrosis, and that liver sections from rats treated with 250 mg/kg PSCME and PSSE (groups 3 and 6) and those treated with 100 mg/kg silymarin (group 8) showed relatively less severe lesions, while no lesions were observed on the liver sections of the normal control (group 9) rats that were not given CCl_4.

DISCUSSION

The relatively higher yield of PSSE (12.8 %) when compared to PSCME (10.33 %) in this study is in agreement with earlier reports that Soxhlet extraction technique yields more extract than cold maceration extraction technique.[3]

The findings in phytochemical analyses of the presence of varied levels of tannins, alkaloids, flavonoids, carbohydrates, glycosides, terpenes and sterols, phenols, and saponins in both PSCME and PSSE are in agreement with previous reports on the phytochemical constituents of methanol and ethanol leaf extracts of *P. santalinoides*.[10,11,24-26] These phytochemical constituents observed in *P. santalinoides* leaf extract are some of the bioactive compounds commonly found in various herbs used for medicinal purposes. The very high levels of saponins and phenols in PSCME and contrasting low levels of these two phytochemicals in PSSE suggests that the concentration of these phytochemicals may have been affected adversely by the heat used in Soxhlet extraction, which is one of the demerits of Soxhlet extraction technique.[3,6] Some phytochemical constituents of medicinal plants are thermolabile and are denatured when exposed to heat such as is used during Soxhlet extraction.[3,6]

In the acute toxicity test, the highest dose of 5000 mg/kg in both PSCME and PSSE did not cause any toxicity to the rats, thereby placing both extracts on "Category 5–Unclassified" of the Global Harmonized Classification System.[14] This implies that leaf extract of *P. santalinoides* is acutely non toxic irrespective of the extraction process, thus the two different extraction methods did not yield extracts that differ in terms of acute toxicity and safety. Both PSCME and PSSE are therefore considered safe for acute use as treatment of ailments and diseases for which they are effective. This is in agreement with previous reports that *P. santalinoides* leaf extracts are acutely non-toxic.[10,11,24-26]

The significantly higher serum enzyme activity level of ALT, AST and ALP in the untreated negative control (group 1) rats and other groups that were given CCl_4 is indication of the ability of CCl_4 to induce liver damage by disrupting hepatocellular integrity and liver function.[15] The findings in the present study that rats in group 3 which were given CCl_4 and treated with 250 mg/kg PSCME, and rats in group 6 which were given CCl_4 and treated with 250 mg/kg PSSE, had serum ALT and AST activities significantly lower than that of the untreated negative control (group 1), and comparable to that recorded for the silymarin treated positive control (groups 8) and the normal control (group 9), implied that the administration of PSCME and PSSE at the dose of 250 mg/kg was protective of the hepatocellular integrity of the liver, and compared effectively with silymarin (a known hepatoprotective drug) at the dose of 100 mg/kg. The findings in this present study that the serum ALP activity of the group 6 rats treated with 250 mg/kg of PSSE was comparable and not significantly different from that of the silymarin treated positive control (group 8) and the normal control (group 9) imply that PSSE at 250 mg/kg ameliorated irritation and damage to the biliary epithelium more than other doses/extract. These findings are in agreement with the reports of Offor *et al*.[27] who recorded decreases in ALT, AST, and ALP in albino rats treated with ethanol leaf extract of *P. santalinoides*. Ihedioha *et al*.[10,11] also reported decreases in ALT and AST activities in hepatotoxic albino rats treated with methanol leaf extract of *P. santalinoides*. It is thought that the hepatoprotective activity of PSCME and PSSE on hepatocellular integrity may be due to the presence in these extracts of phytochemicals like flavonoids, alkaloids and tannins which are well known natural antioxidants,[11] as CCl_4-induced hepatotoxicity is known to be produced mainly by oxidative stress.[15] Ihedioha *et al*.[11] also reported the hepatoprotective and antioxidant activities of methanol leaf extract of *P. santalinoides* in CCl_4-induced hepatotoxicity in albino rats.

The depletion of serum total protein in rats that were given CCl_4 is a further indication of dysfunction of hepatic synthesis associated with CCl_4 administration.[28] The findings in this present study that treatment with PSCME at the dose of 250 mg/kg (group 3), and PSSE at the doses of 50, 250, and 500 mg/kg (groups 5, 6, and 7) led to higher levels of serum total protein comparable to the levels seen in rats in the silymarin treated positive control (group 8) and normal control (group 9), indicates enhanced hepatic synthetic activity. Specifically, treatment with PSSE at the dose of 250 mg/kg (group 6) significantly enhanced the synthesis of total proteins. Stimulation of protein synthesis has been recognized as a contributory hepatoprotective mechanism which accelerates the regeneration process and the production of replacement liver cells.[29] The lack of significant variation between the rat groups in their serum levels of albumins indicates that sub-acute administration of CCl_4 as used in the study did not significantly affect the albumin level. This may be attributed to the fact that the duration of the study (sub-acute) was not long enough for the serum albumin to be severely affected, as hypoalbuminemia has been reported to occur mainly in chronic liver diseases.[29] The significantly lower serum globulin levels of the untreated negative control (group 1) rats and the rats treated with 50 mg/kg and 500 mg/kg PSCME (groups 2 and 4) when compared to the normal control (group 9) rats may be attributed to possible negative effects of CCl_4 on other body organs (including the lymphoid organs), apart from the liver.

There was no significant variation ($P > 0.05$) in globulin levels between PSCME at the dose of 250 mg/kg (group 3) and PSSE at all doses (groups 5, 6 and 7), and these compared favorably with silymarin (group 8).

In the present study, it was observed that administration of CCl_4 altered the lipid profile by increasing the serum level of TC of rats in the untreated negative control (group 1) and others that were given CCl_4. The fact that treatment with PSCME at 250 and 500 mg/kg (groups 3 and 4), and PSSE at 500 mg/kg (group 6), led to significantly lower serum TC levels, implied that these extracts at the doses stated may have ameliorated dyslipidemia induced by the CCl_4 administration. Also, the findings in this study that treatment with PSCME and PSSE at 250 mg/kg (groups 3 and 6) led to significantly lower serum total bilirubin levels is suggestive that PSCME and PSSE at this dose enhanced hepatic clearance of bilirubin.

The higher relative liver weight of rats in groups 1–8 (all groups given CCl_4) is an indication of hepatomegaly induced by inflammation and/or degeneration which accompanies CCl_4 hepatotoxicity.[30] The significantly lower relative liver weight of rats treated with PSCME at the doses of 50 and 250 mg/kg (groups 2 and 3), PSSE at the dose of 250 mg/kg (group 6), and silymarin at 100 mg/kg (group 8) implied their amelioration of the hepatomegaly induced by CCl_4 toxicity.

The vacuolar degeneration and necrosis of hepatocytes observed on the liver sections of all the rat groups given CCl_4 is a typical finding in CCl_4-induced liver damage.[31] The vacuoles on the hepatocytes are fat vacuoles and their infiltration of hepatocytes in CCl_4-induced liver damage has been reported to be caused by the ability of CCl_4 to bring about peroxidative degradation in the adipose tissue that leads to fatty infiltration of hepatocytes.[31] The lower level of severity of the lesions observed in the liver sections of rats treated with 250 mg/kg PSCME and PSSE (groups 3 and 6) and those treated with 100 mg/kg silymarin (group 8) is an indication that treatment with both extracts (PSCME and PSSE) at 250 mg/kg and also with silymarin (100 mg/kg), successfully ameliorated the severity of the histopathological lesions associated with CCl_4-induced liver damage.

Combined, the results of the evaluation of the phytochemical components of the extracts and the *in vivo* hepatoprotective bioassay in rats suggest that the active hepatoprotective principle in the *P. santalinoides* extracts may be one or more of those components that were not affected by the heat applied during Soxhlet extraction (tannins, terpenes, sterols, flavonoids, glycosides and/or alkaloids), but not the saponins and phenols that were high in PSCME and low in the PSSE.

The advantage of a relatively higher yield when Soxhlet extraction is used is in favor of PSSE, but the additional cost of Soxhlet extraction equipment may counterbalance this benefit. In contrast, cold maceration can be done without any specialized equipment, and produces an equally hepatoprotective extract. None of the two extracts (PSCME and PSSE) at 250 mg/kg dose was clearly more hepatoprotective than the other in the *in vivo* bioassay with albino rats. The probable consequences of the decrease in the level of saponins and phenol (which may be heat labile) in the PSSE relative to their levels in PSCME is not known, but it is obvious that these two phytochemicals do not play any major role in the hepatoprotective activity of the extracts.

In summary, methanol leaf extract of *P. santalinoides* obtained by both cold maceration and Soxhlet extraction techniques at the dose of 250 mg/kg, significantly and equally protected the hepatocellular integrity of the liver of rats that were given CCl_4; their serum ALT and AST activity levels were significantly lower and almost half of the untreated control group, and comparable to the silymarin treated group and the normal control group (not given CCl_4). Treatment with PSSE at 250 mg/kg significantly enhanced hepatic protein synthetic ability. Treatment with PSCME at 250 and 500 mg/kg doses and with PSSE at 250 mg/kg dose ameliorated dyslipidemia, while treatment with both extracts at 250 mg/kg enhanced hepatic bilirubin clearance. Treatment with PSCME at 50 and 250 mg/kg and PSSE at 250 mg/kg minimized gross inflammatory enlargement (hepatomegaly).

CONCLUSION

Extracts of *P. santalinoides* obtained by cold maceration and Soxhlet extraction techniques are of nearly equal hepatoprotective potency, mainly at the dose of 250 mg/kg. The above results strongly suggests that the hepatoprotective principle(s) in the methanol leaf extract of *P. santalinoides* is thermostable since the heat applied during Soxhlet extraction did not adversely affect its hepatoprotective activity. These findings further validate the traditional use of hot soups made from leaves of *P. santalinoides* as medication for the treatment of liver disorders.

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COMPETING INTERESTS

The authors declare no conflict of interest.

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