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The use of Phytochemical, GC-MS Analysis and Hepatoprotective Effect of the Methanol Leaf Extract of Camellia Sinensis (L.) Kuntze on Paracetamol-Induced Liver Injury in Wistar Rats

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Funding: No specific funding was received for this work.

Potential competing interests: No potential competing interests to declare.

Abstract

Background and objectives: Camellia sinensis is a tree popularly called green tea used as a strong antioxidant and for treating diseases such as cancer. This present study was aimed at assessing the phytochemicals and evaluating the hepatoprotective activity of leaf extract in Wistar rats. **Methods:** The phytochemical screening was performed by standard methods, acute toxicity study and hepatoprotective activity were evaluated in randomly groups of five rats or six rats in paracetamol-induced liver toxicity. **Results:** The phytochemical screening of leaf extract revealed the presence of major metabolites. The GC-MS analysis showed 18 bioactive compounds which are mainly fatty acids with retention times between 7.436 and 18.462 min. A high total phenolic content (TPC) of 1425.22 mg GAE/g and a total flavonoid content (TFC) of 802.01 mg QE/g were obtained. The acute toxicity did not produce any sign of toxicity at a dose of 5,000 mg/kg of extract. The extract produced a dose-dependent activity on the liver. These protections were statistically significant ($p<0.05$) when compared to the standard drug silymarin. There were no elevated values in liver function biomarkers in all the animal groups as well as histopathological damages to the liver after eight weeks of treatment at 200 and 400 mg/kg b.w. of extract doses. **Conclusion:** In conclusion, the study showed that C. sinensis methanol leaf extract contains important phytochemicals that are protective against paracetamol-induced liver injuries, thus, the plant was considered safe for use as ethnomedicinal remedies for liver disease in traditional medicine.

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Keywords: *Camellia sinensis*, GC-MS, Toxicity studies, Total phenolic, Total flavonoid.

1. Introduction

The use of plants for human healthcare needs dates back many centuries. Studies have shown that more than 25% of conventional drugs were derived from plants with many bioactive compounds from plants acting as lead agents (Florence et al., 2014). Medicinal plants have been used and are still being used to treat diseases such as cancers, ulcers, hypertension, diabetes, infections, inflammations, wounds, pains, etc. in traditional medicine (Sofowora et al., 2013).

Many plants have been reported as remedies for both acute and chronic liver problems by various researchers of natural products. This is because plants contain phytochemicals which possess various pharmacological potentials when used. For example, *Camellia sinensis* (Plate 1) is also a traditional remedy for a variety of diseases. It is used traditionally as an alternative for treating cancer (Tchimene et al., 2016). It is sedative, anticarcinogenic, anti-bacterial, anti-diabetic, anti-tumour, and antiviral. *Camellia sinensis* is tolerant of poor soil and prefers lowland areas between the altitudes of 1,200 m (3,900 ft. It is distributed in South American countries, North America, as well as Central and West African countries (Ukwubile et al., 2020).

The plant grows well in Mambilla Beverages Ltd Taraba State where it is found in the Nguroje town of the State. In terms of phytochemical compositions, the leaf contains alkaloids, amino acids, and flavonoids just like those of the Annonaceae family (Florence et al., 2014). The Family Theaceae with about 450 species and more than 50 genera is a family of flowering plants made up of trees, shrubs or in rare cases lianas. This family is unique because of its numerous uses as antioxidants, anticancer, antidiabetic and analgesic.

The present study aimed to assess the phytochemicals, and GC-MS analysis of crude leaf methanol extract of*C. sinensis*, and evaluate the toxicities on Wistar rats.

Plate 1. Pictorial view of *Camellia sinensis* in its natural habitat at Mambila Beverages Ltd., Nguroje (Ukwubile et al., 2020).

2. Materials and Methods

2.1. *Collection and identification of plant*

Healthy, fresh and disease-free leaves and stembark of *C. sinensis* were collected from Mambila Beverages Ltd in Taraba State in December 2023 and were identified by Dr. Cletus A. Ukwubile of the Department of Pharmacognosy, Faculty of Pharmacy, University of Maiduguri, where a voucher specimen number UMM/FPH/THC/001 was deposited for the plant. A plant press was done and deposited in the herbarium of the Department of Pharmacognosy, University of Maiduguri, Nigeria.

2.2. *Preparation of plant material and extraction*

The leaves and stembarks of *C. sinensis* were air dried at room temperature at 25^0C for 2 weeks and pounded using an electronic blender. The dried and ground powder (469.0 g) was extracted with aqueous methanol (1600 mL) in an air-tight separate funnel for two days at room temperature with occasional stirring and shaking. The extract was then filtered using a Whatman number 1 filter paper. The filtrate was concentrated using a water bath at room temperature. The weight of the aqueous methanol leaf extract was 215.6 g (% yield = 45.97).

2.3. *Preliminary phytochemical of C. sinensis methanol leaf extract*

The qualitative phytochemical screening was carried out on the extractsusing standard procedures to identify major constituents such as alkaloids, flavonoids, tannins, terpenoids, saponins, anthraquinones, cardiac glycosides, sterols and phytosterols.

2.4. *GC-MS analysis of the methanol leaf extract*

The phytoconstituents of compounds present in the crude methanol extract of stembark were evaluated in Agilent Technologies 7890A gas chromatography (GC) coupled to a mass spectrum detector (MSD) (Agilent Technologies, USA). The carrier gas was helium with column velocity flow of 1.0 L/min, ion-source temperature was 250 C, interface temperature was 300 C, operating pressure was 16.2 psi, out time was 1.5 min, injection temperature was 300 C in split mode at 1 µL injector, while the temperature of the column was initially 50 C for 5 min and raised to 250 C at the rate of 20 C/min for 5 min (Ukwubile et al., 2019). The total elution time was 25 min, and each compound was calculated in terms of relative abundance, peak areas and retention times while the identification of compounds was done by comparing with data from the NIST library (Olivia et al., 2021).

2.5. *Total phenolic content (TPC)*

The total phenolic content was determined using the Folin-Ciocalteu (FC) method with slight modifications. Briefly, the FC stock solution was prepared by dissolving 10 g of FC in 10 mL deionized water (i.e., 1:10) before the commencement of the experiment. Similarly, 7.5 % (w/v) Na_2CO_3 (sodium carbonate) was dissolved in 10 mL of deionized water, while the stock solution of extract was prepared by dissolving 10 g of the extract in 10 mL of 98.1 % (v/v) methanol (i.e., 1000 µg/mL). After this, the three stock solutions were all mixed and allowed to stand for 6 min. In the same way, a gallic acid (500 µg/mL) standard solution was prepared and the concentrations of 50, 100, 200, 400 and 800 µg/mL were used as diluting concentrations for plotting gallic acid calibration curve (Molole et al., 2022). The absorbance of each solution was taken at 765 nm using the UV-Vis spectrophotometer (ThermoFisher, UK). The concentration of gallic acid (GA) in each extract was then calculated from the linear equation from the calibration curve using their absorbance. The TPC was then calculated and expressed as mg GAE/g using the formula below:

$$TPC = C \times V/m$$

Where TPC is total phenolic contents (in mg gallic acid equipment; GAE per gram), C is the gallic acid concentration (but C = x/1000 mg/g), V is the volume of the extract per solvent (i.e., 1 mL) and m is the weight of extract (in gram). (Ukwubile et al., 2024).

2.6. *Determination of flavonoid content (TFC)*

The TFC of the extract was evaluated using an aluminium chloride ($AlCl_3$) colourimetry assay with slight modifications. Briefly, 0.5 mL of the avocado methanol seed extract was added to a test tube containing 2 mL of 98.1 % (v/v) methanol.

To the test tube, 3 mL of 10 % $NaNO_2$ was added and kept in the dark for 5 min. Then 3 mL of 10 % $AlCl_3$ was added to the mixture and left for 1 min. Thereafter, 1 mL of 1 M NaOH solution was added and made up to 5 mL volume with distilled water and kept for 10 min. Finally, the absorbance of the solution was measured at 510 nm (Ayele et al., 2022). Rutin was used as standard and the result obtained for TFC was expressed as mg rutin equivalent per gram of the sample (i.e., mg RE/ g). The concentrations of rutin were taken from 25 to 800 µg/mL. The TFC was derived from the rutin standard curve and calculated from the formula below. All the readings were taken in triplicates.

$$TFC = C \times V/m$$

Where TFC is total flavonoid contents (in mg rutin equipment; RE per gram), C is rutin concentration (but C = x/1000 mg/g), V is the volume of the extract per solvent (i.e., 1 mL) and m is the weight of extract (in gram) (Ukwubile et al., 2024).

2.7. *Experimental animals*

Thirty-five (35) healthy Wistar rats of both sexes weighing between 100 and 150 g were purchased from PJ Rat Farm Ltd, Jos, Nigeria. The animals were allowed to acclimatize before the experiment at room temperature in the animal house for one week with free access to food and water. Local and international ethical guidelines for the use of these animals were strictly followed.

2.8. *Acute oral toxicity study of C. sinensis methanol leaf extract in rats*

The acute oral toxicity study was carried out following OECD guideline 452, which stipulates the use of only five animals. Five rats of either sex (3 males plus 2 females) were weighed and fasted overnight. Test doses of CSE were calculated in terms of their body weight and administered via oral gavaged at 5,000 mg/kg body weight (b.w) maximum dose at once. The animals were regularly and individually observed for behavioural changes and general toxicity signs after dosing for the first 24 h, with special attention being given during the first 4 h. After these, observations continued daily for a total of 14 days (Ukwubile et al., 2024).

2.9. *Experimental design*

The animals were randomly grouped into five groups of six as shown in Table 1 below:

Group	Dose	Route
I (normal control)	10 mL/kg	Oral (p.o.)
II (disease control)	200 mg/kg PCM	p.o. twice/8 weeks
III (standard)	100 mg/kg/day silymarin	p.o. twice/8 weeks
IV (treatment)	200 mg/kg CSE	p.o. twice/8 weeks
V (treatment)	400 mg/kg CSE	p.o. twice/8 weeks

Table 1. Experimental design for hepatoprotective effect of CSE

The animals in groups II to V were each given 200 mg/kg PCM (paracetamol) twice weekly for eight weeks and CSE: *Camellia sinensis* extract. The animals were then sacrificed 24 hours after the last treatment, and the blood was collected by cardiac puncturing into an EDTA sample and serum was used for the analysis of liver function parameters.

2.10. *Liver function parameters*

The liver function markers such as AST (aspartate amino transaminase), ALT (alanine amino transaminase), ALP (alkaline phosphatase), TC (total cholesterol), ALB (albumin), SBL (serum bilirubin), TP (total proteins), creatinine and biochemical parameters were evaluated using BC-2800 auto haematology analyzer and Reflotron plus apparatus with their appropriate kits.

2.11. *Histopathological examination of the liver*

The liver of all animals in each group was carefully dissected for histopathological study using hematoxylin (H) and eosin (E) stains. Liver sections from the animals were prepared following standard procedure and then stained with H and E stains and observed using the microscope at 400x.

2.12. *Statistical Analysis*

Results obtained were expressed as means ± SD (n=6). The value of $p<0.05$ was taken as statistically significant versus control (one-way ANOVA followed by Dunnett's post hoc test). Analysis was done using GraphPad Prism version 9 statistical software.

3. Results

3.1. *Phytochemical contents*

The results of the phytochemical screening methanol leaf extract revealed the presence of alkaloids, flavonoids, tannins, triterpenes/steroids, fats/oils and phenols while anthracenes, tannins and saponins were not detected (Table 2).

Constituents	Test	Inferences
Alkaloids	Dragendorff's	+
	Wagner	+
Flavonoids	Shinoda's	+
	NaOH	+
Tannins	$FeCl_3$	-
	Goldbeater's	-
Triterpenes/Steroids	Liebermann's	+
Anthracenes	Bontrager	-
Fats/Oils	Spot	+
	Sudan III	+
Phenols	$FeCl_3$	+
Saponins	Frothing	-

Table 2. Phytochemical contents of *C. sinensis* methanol leaf extract

Note: + denotes present, and – denotes absence

3.2. GC-MS analysis

The GC-MS analysis of the methanol leaf extract showed the presence of eighteen (18) compounds belonging to different classes. It showed mainly nine saturated and unsaturated fatty acids, two fatty acid derivatives, one polyphenol, and two alcohols as well as others with their respective retention times (RT), mass-to-charge ratio (m/z), peak areas (PA) and chemical formula (Fig. 1; Table 3).

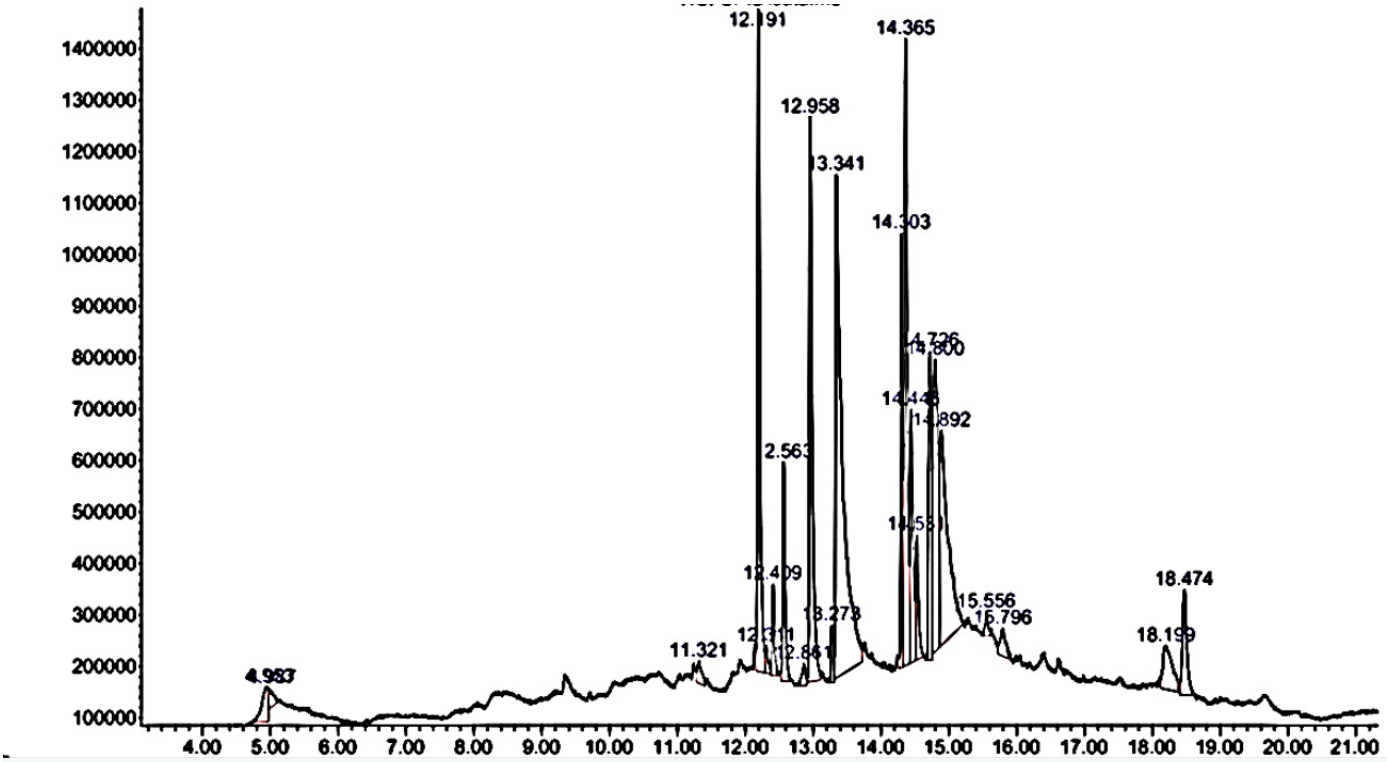

Fig. 1. Mass chromatogram of *C. sinensis* crude leaf methanol extract

Peak #	Compound	RT (min)	PA (%)	Formula	m/z (g/mol)	Class
1.	5,2-Benzofuran	6.43	1.73	C_8H_6O	118.14	FA+
2.	1,3-bendicarboxylic	8.32	0.82	$C_8H_6O_2$	166.13	FA
3.	2-Propanedioic acid	9.90	1.36	$C_3H_4O_4$	104.06	PP
4.	β-D-Galactopyranoside	10.88	16.54	$C_7H_{14}O_6$	194.18	CHO
5.	β-D-Glucopyranoside	11.25	1.28	$C_7H_{14}O$	194.18	CHO
6.	β-Styracitol	11.35	4.69	$C_6H_{12}O_{25}$	164.16	POH
7.	Bicyclo[1.2]heptane	12.18	1.27	$C_{10}H_{18}$	138.25	Alkane
8.	Pentadecanoic acid	11.92	2.80	$C_{15}H_{30}O_2$	242.40	FA
9.	n-Hexadecanoic acid	13.05	4.40	$C_{16}H_{32}O_2$	256.42	FA
10.	9,12-Octadecadienoic	14.30	3.79	$C_{18}H_{32}O_2$	280.45	FA
11.	Linolenic acid	15.34	8.28	$C_{18}H_{30}O_2$	278.44	FA
12.	Phytol-EE	13.43	7.62	$C_{20}H_{40}O$	296.53	ROH
13.	Heptadecanoic acid	17.51	1.05	$C_{17}H_{34}O_2$	270.45	FA
14.	Linoleic acid-E	14.69	1.03	$C_{18}H_{32}O_2$	280.45	FA
15.	9,12-Octadecatrienal	12.75	12.53	$C_{18}H_{30}O$	262.43	FA+
16.	9-hexadecatrienoate	14.88	0.52	$C_{16}H_{26}O_2$	250.38	FA
17.	Docosanoic acid	18.24	8.61	$C_{22}H_{44}O_2$	340.58	FA
18.	Phthalic acid	17.46	8.67	$C_8H_6O_4$	166.14	Di-CA

Table 3. GC-MS analysis of *C. sinensis* crude leaf methanol extract

Note: FA+: fatty acid derivatives, FA: Fatty acids, PP: Polyphenols, RT: retention time, PA: peak area and m/z: molecular ion, CHO: Carbohydrates, POH: Polyhydroxylated alcohol, ROH: methyl alcohol, and Di-CA: Dicarboxylic acid.

3.3. Total phenolic and total flavonoid contents

The total phenolic content (TPC) of methanol leaf extract was determined to be 1425.22 mg GAE/g obtained from the gallic acid calibration curve with linear equation y = 0.0087x + 0.064; R^2 = 0.9811 while the total flavonoid content (TFC) was 802.01 mg QE/g obtained from quercetin calibration curve with linear equation y = 0.0052x + 0.2476; R^2 = 0.9659 (Fig.2 a and b).

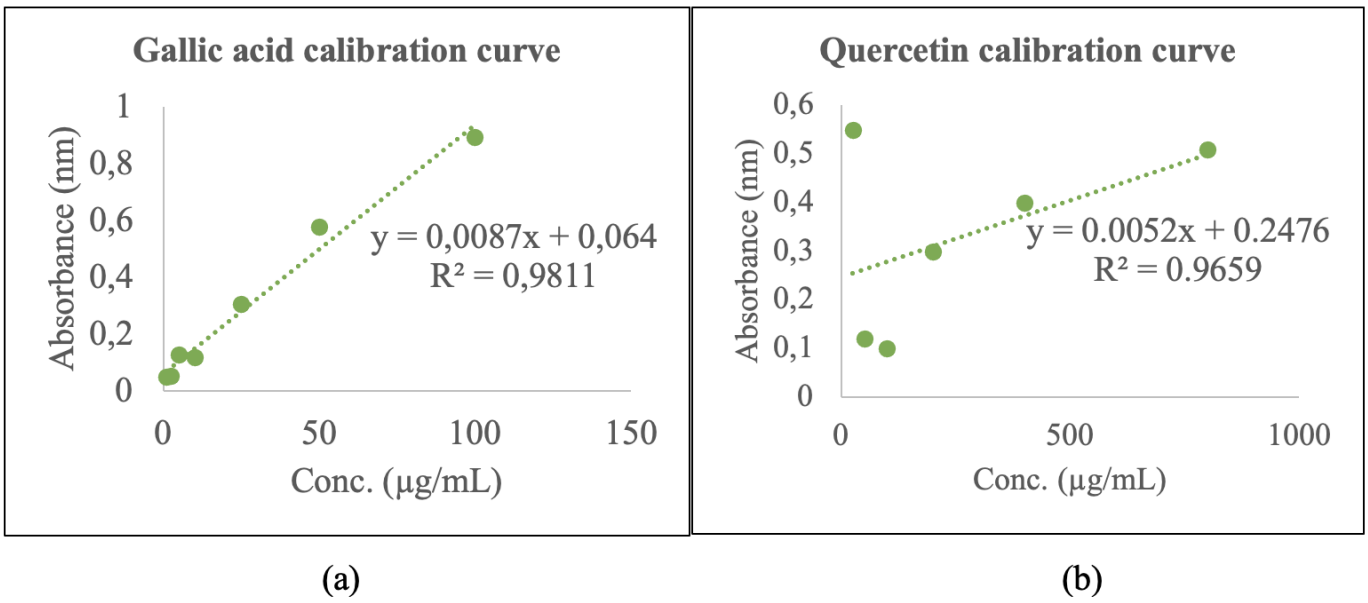

Fig. 2. Standard curves for determination of total phenolic (a) and flavonoid (b) contents of crude leaf extract

There were no abnormal increases in the values of serum electrolytes above the normal ranges after treatment with *C. sinensis* leaf extract in eight weeks. All the animals in groups IV and V recovered from liver injuries caused by high doses of PCM (Table 4).

	Dose (mg/kg)				
Serum Electrolyte (mmol/L)	I	II	III	200 CSE (IV)	400 CSE (V)
Na^+	120.60±0.01	138.17±0.06*	124.90±0.10	126.17±0.06	128.17±0.10*
Cl^-	76.30±0.01	98.40±0.01*	84.10±0.01	86.30±0.01	88.90±0.02*
Ca^{2+}	1.60±0.01	2.83 ± 0.01*	1.89±0.01	1.90±0.01	2.44±0.01*
K^+	4.80±0.01	8.61±0.01*	5.68±0.01	5.88±0.00*	5.91±0.01*
PO^{-4}	1.50±0.01	3.44±0.01*	1.42±0.01	1.20±0.01	1.11±0.01*
Urea	15.10±0.01	25.30±0.01*	15.55±0.01	16.10±0.01	18.00±0.01*
UA	0.05±0.00	0.08±0.01*	0.09±0.01	0.12±0.00*	0.05±0.01*
CRE	0.20±0.01	0.78±0.01*	0.25±0.01	0.28±0.00	0.45±0.05*

Table 4. Effects of *C. sinensis methanol* leaf extract on biochemical parameters of rats.

Results are mean ± SD of six rats per group (n = 6), *p < 0.05 statistically significant (P = 0.000) versus control (one-way ANOVA followed by Dunnett's post hoc), Ser (serum), PO^{-4}(Phosphate), UA (Uric acid), CRE (Creatinine in mg/dL).

Similarly, a dose-dependent activity was witnessed in liver function biomarkers in groups IV and V. These values were statistically significant (p < 0.005; P = 0.001) compared to the control groups (Table 5).

Parameter	Dose (mg/kg)				
	I	II	III	200 CSE (IV)	400 CSE (V)
ALP (U/L)	56.50 ± 0.01	88.30 ± 0.01*	68.50 ± 0.01	74.29 ± 0.01	80.59 ± 0.01*
AST (U/L)	45.30 ± 0.01	65.59 ± 0.01*	48.30 ± 0.01	48.09 ± 0.01	46.44 ± 0.01*
ALT (U/L)	20.09 ± 0.52	30.89 ± 0.01*	23.10 ± 0.00	25.49 ± 0.01	25.79 ± 0.01*
ALB (g/L)	3.79 ± 0.01	5.81 ± 0.01	3.86 ± 0.01	3.92 ± 0.01*	4.51 ± 0.01*
BIL (mg/dL)	0.31 ± 0.01	1.36 ± 0.01*	0.34 ± 0.01	0.32 ± 0.01	0.31 ± 0.01
GLO (g/dL)	1.50 ± 0.01	2.52 ± 0.00	1.88 ± 0.01	1.96 ± 0.01*	2.56 ± 0.01*
TPRN (g/dL)	5.61 ± 0.01	8.61 ± 0.00	5.88 ± 0.01*	5.93 ± 0.01	6.90 ± 0.10
TC (mg/dL)	25.61 ± 0.01	225.61 ± 3.01	101.11 ± 2.01	42.04 ± 0.01	15.01 ± 0.02

Table 5. Effects of *C. sinensis* methanol leaf on liver function biomarkers of rats.

Results are mean ± SD of six rats per group, ALP (alkaline phosphatase), AST (aspartate transaminase), ALT (alanine transaminase), ALB (albumin), BIL (bilirubin), GLO (globulin), TPRN (total protein), TC (total cholesterols) *p< 0.05 statistically significant (one-way ANOVA).

3.4. *Histopathological examinations of the liver of rats*

The photomicrograph of liver sections stained with H and E stains showed a dose-dependent recovery from liver injuries in PCM-induced hepatotoxicity. No vascular congestion and necrosis were observed at 200 and 400 mg/kg extract doses in the animals when compared to group II (Fig. 2 a-f).

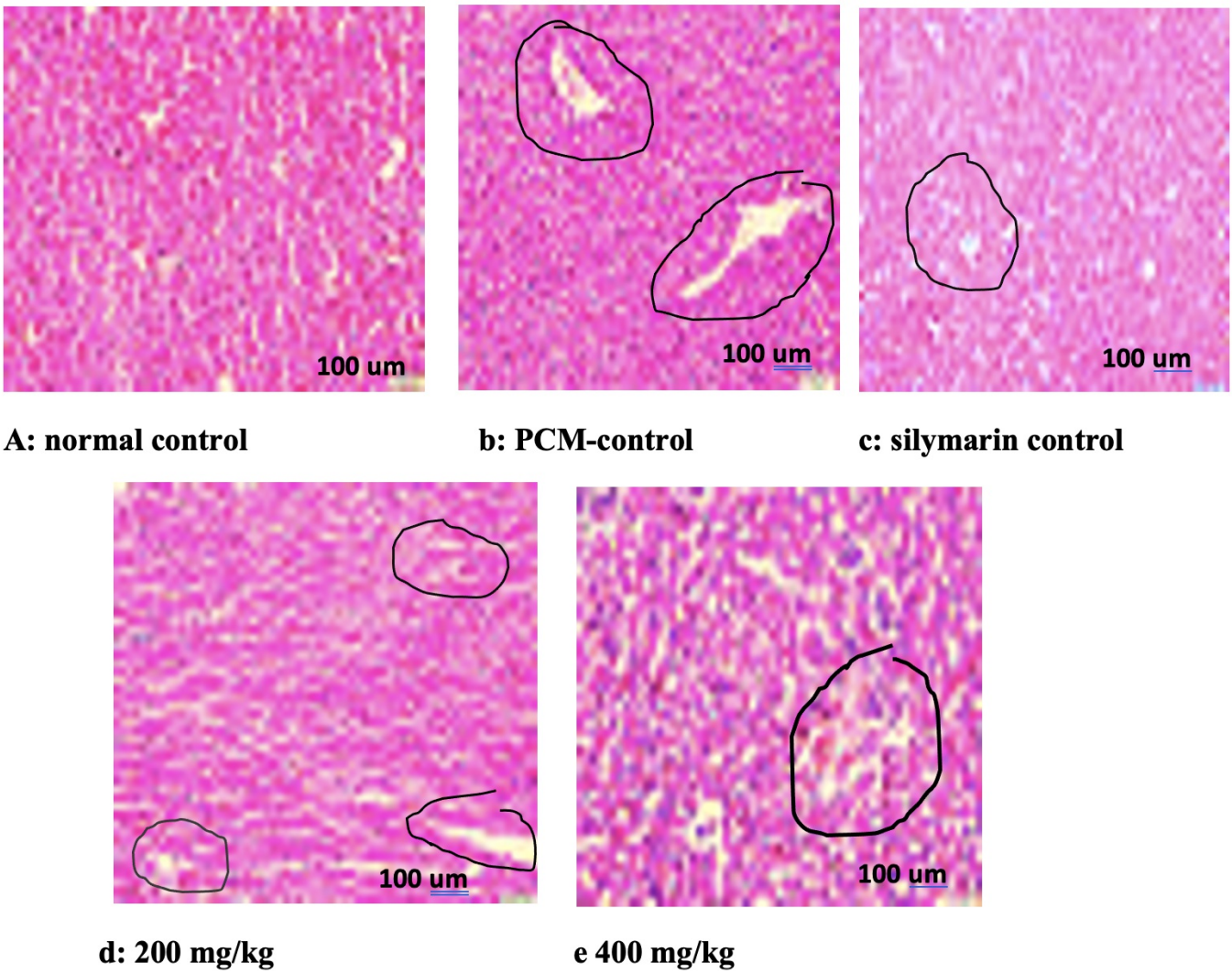

Fig. 3. Photomicrographs of liver sections at various doses 400x.The spherical shapes in the photomicrographs showed normal blood vessels with clear vascular flow.

4. Discussion

Drugs from natural sources provide vital benefits to human healthcare systems. Plants are readily available for use, they are less or non-toxic to the body when used, they are affordable to all persons, and are economically beneficial. They contain bioactive compounds which help in the development of conventional drugs, that is, they serve also raw materials during drug production (Menezes et al., 2011). In a recent survey conducted by the World Health Organization globally, more than 20,000 medicinal plants are being used mainly in pharmaceutical companies or traditional medicines. Interestingly, about 1.4% do possess well-established, widely proven and broadly accepted unequivocally active constituents (Mohammed Golam Rasul, 2018). It has been reported that the usual success rate of discovering new drugs from natural sources is solely based not only on the conception but also on the implementation of ingenious comprehensive strategies which invariably explore and exploit the untapped potential of the natural sources (Jones et al., 2006). There are four ways by which the above objectives may be accomplished reasonably and legitimately, such as isolation of novel compounds from marine and terrestrial ecosystems, genetic engineering: creating novel and altered

compounds, biochemical manipulation of selected metabolic pathways, and supersensitive and specific selection techniques and evaluation for varied bioactivities (Alamgir, 2017). These techniques have led to the screening of many plants for the presence or absence of certain metabolites.

In the current study, phytochemical screening of the methanol leaf extract revealed that it contains alkaloids, flavonoids fats/oils, steroids and triterpenes which agreed with the previous study (Ukwubile et al., 2020). These secondary metabolites detected were responsible for the biological activity of the leaf. For example, flavonoids and alkaloids have played crucial roles as antioxidant, anti-inflammatory and anticancer agents (Salim et al., 2020), thus, these metabolites played similar roles in this present study as seen from their ability to restore normal liver function from paracetamol-induced liver injuries in rats. *Camellia sinensis* has been reported to be a good antioxidant, anticancer and anti-inflammatory medicinal due to the presence of these metabolites (Zhao et al., 2022), which corroborated the findings from this present study. Similarly, gas-chromatography-mass-spectrometry (GC-MS) has been one of the most powerful apparatus for analyzing the phyto-composition of crude extract and pure compounds from plants (Lynch et al., 2023). In this study, the crude methanol leaf extract *C. sinensis* contains 18 bioactive compounds which are mainly fatty acids and fatty acid derivatives as well as carbohydrates among other minor compounds. For instance, compounds such as linoleic and oleic acids have been reported to possess wound healing and anticancer effects (Azarmehr et al., 2019).

Phenolics and flavonoids are very interesting metabolites in plants due to their immense therapeutic uses as antioxidants, wound healing, anticancer, antiageing, anti-inflammatory and antidiabetics (Ayele et al., 2022). Most flavonoids for instance can reduce the scavenging activities of reactive oxygen species and free radicals in the body thereby preventing the body from many diseases (Borquaye et al., 2020). From the study, a higher amount of total phenolic content (1425.22 mg GAE/g) and flavonoid content (802.01 mg QE/g) were obtained. Previous studies had reported far lower values for these metabolites in *C. sinensis* leaf extract (Zhao et al., 2022). The variations in these values may be due to the environmental factors regarding the ecology of the plant used in the current study. The relatively very low temperature in Nguroje where the leaves were collected must have been responsible for the chemical races observed in this study. Various phenolics and flavonoids have been reported to show hepatoprotective effects in paracetamol-induced liver toxicity (Tafere et al., 2020), and this is similar to that of the current study.

Furthermore, the fact there were no elevated levels of biochemical parameters such as Na+, Ca+, Cl-, urea, uric acids, creatinine, etc., and liver function biomarkers such as amino phosphatase (ALP), aspartate amino transaminase (AST), alanine amino transaminase (ALT), bilirubin, and globulins showed the potential of *C. sinensis* leaf extract to reverse the abnormalities caused by paracetamol-induced liver injuries in rats. From the study, the induction of liver toxicities using a higher dose of paracetamol (PCM) in rats for eight weeks resulted in an elevated increase in biochemical and liver function parameters which were later on normalized by extract doses of 200 and 400 mg/kg body weight oral administrations for eight weeks. The results obtained were significant ($p < 0.005$) when compared to the standard drug silymarin. The photomicrographs of liver sections from various groups showed that there was no vascular congestion, necrosis or apoptosis in liver cells of the treated groups unlike the paracetamol-controlled group (disease control). From this histological study, induction of paracetamol at a higher dose to the rats (group II) without any treatment resulted in

vascular congestion, large lumen, necrosis, and extended capillary blockages. These effects were brought to normalcy after administering various doses of *C. sinensis* to the animals. The presence of high amounts of phenolics and flavonoids as well as other metabolites must have been responsible for the observed biological activity in this study.

5. Conclusion

The study showed that *Camellia sinensis* methanol leaf extract contains important phytoconstituents which were responsible for its hepatoprotective effect in rats. The results obtained in this study provide valid scientific backing validating the ethnomedicinal use of *Camellia sinensis* leaf extract for liver disease. Further research on the main bioactive compound responsible for the observed biological activity will form the basis for new drug discovery towards the treatment of liver diseases.

Statements and Declarations

Acknowledgements

The authors are grateful to Mr. Sumaila Mejida of the Central Research Laboratory, University of Lagos, Akoka, for his technical assistance in the GC-MS analytical procedure.

Authors contributions

Dr. Cletus Anes Ukwubile designed the study, drafted the manuscript, was involved in experiments, analyzed the data, and supervised the study; Semen Ibrahim Gangpete was involved in experiments, collection of data, data analysis and review of the manuscript. All authors contributed to the writing, revising and editing of the final manuscript before submission.

Conflict of interest

We have none to declare.

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