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Research article

Alkaloidal extract from *Carica papaya* seeds ameliorates CCl_4-induced hepatocellular carcinoma in rats

Isaac Kyei-Barffour [a,*], Roselind Kyei Baah Kwarkoh [b], Desmond Omane Acheampong [a], Augustine Suurinobah Brah [a], Samuel Addo Akwetey [a], Benjamin Aboagye [c]

[a] Department of Biomedical Sciences, School of Allied Health Sciences, College of Health and Allied Sciences, University of Cape Coast, Ghana
[b] Department of Physician Assistant Studies, School of Allied Health Sciences, College of Health and Allied Sciences, University of Cape Coast, Ghana
[c] Department of Forensic Sciences, School of Biological Sciences, College of Agricultural and Natural Sciences, University of Cape Coast, Ghana

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ABSTRACT

Background: Hepatocellular carcinoma (HCC) is the third cause of cancer-related mortality globally. However, available treatments are expensive and are associated with adverse effects or poor treatment outcomes in advanced disease. Meanwhile, plants like *Carica papaya* have demonstrated various biological activities that further studies may lead to the identification of newer and safer treatment options for HCC.

Aim: To evaluate the anticancer activity of an alkaloidal extract derived from *Carica papaya* seeds using rodent models of HCC.

Experimental procedure: *Carica Papaya* fruits were collected and authenticated. The seeds were isolated and air-dried. Alkaloidal extract was prepared from a 70% ethanol soxhlet crude extract and referred to as *Carica papaya* alkaloidal extract (CPAE). HCC was induced in 68 out of 84 healthy male Sprague Dawley rats by intraperitoneal injection of carbon tetrachloride (CCl_4) for 16 weeks. These rats were put into five groups of 10; *Carica papaya* alkaloidal extract [(CPAE) (50, 100, and 200 mg/kg), Lenvatinib (4 mg/kg)], 1% dimethyl sulphoxide (DMSO), and 2 untreated groups (control and model). A prophylaxis study was performed with 10 rats by co-administration of CPAE (200 mg/kg) and CCl_4 six hours apart for 16 weeks. Rats were sacrificed after a twelve-week treatment program under anesthesia for histological, hematological, and biochemical analyses.

Results and conclusion: CPAE (100 and 200 mg/kg) significantly restored weight loss (48.44 and 51.75% respectively), reduced tumor multiplicity, and dose-dependently reversed liver histomorphological changes induced by CCl_4 compared to the model group. The CPAE (100 and 200 mg/kg) further reduced bleeding time, improved prothrombin time and restored platelet count ($p < 0.01$) compared to the model. The CPAE (200 mg/kg) again significantly ($p < 0.0001$) reduced serum alpha-fetoprotein levels compared to the model group and prevented the establishment of HCC in rats when concerrently administered with CCl_4 in 16 weeks prophylactic study.

1. Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer deaths and the fifth most common cancer type worldwide [1]. HCC is also the commonest gastrointestinal cancer and one of the cancer types with poor prognosis [2]. HCC is usually secondary to chronic viral hepatitis B or C infection, chemical/drug-induced hepatitis, liver cirrhosis (from alcoholism), or non-alcoholic fatty liver disease (NAFLD) [3]. Current therapies such as sorafenib, radiotherapy, adoption of Lenvatinib, and curative resection for HCC are reported to only enhance transient survival in patients [4]. The major setbacks in HCC treatment are drug-induced toxicity, low drug efficacy due to tumor cell resistance, and the high cost of surgical resection or liver transplantation [5]. These setbacks have necessitated the need for alternatively safer and effective therapeutics for HCC [6]. Medicinal plant products are important sources of alternative therapy. These plant products are widely used in ethno-

* Corresponding author.
E-mail address: ikbarffour@stu.ucc.edu.gh (I. Kyei-Barffour).

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medicine and as sources of semi-synthetic drugs in modern medicine [7, 8]. Phytochemicals and plant products have been shown to exhibit anti-tumor effects, by arresting abnormal cell cycle, inhibiting pathways of proteins and enzymes that enhance cancer cell proliferation, and improve DNA repair mechanism [9].

Carica papaya of the family Caricaceae is cultivated in most countries across the world for their fruits. The fruit has been confirmed to have high nutritional value while various parts of the plant are used for ethnomedicinal purposes [10]. *C. papaya* leaves, fruits, seeds, latex, barks, and flowers are confirmed to have rich stores of bio-active constituents [11]. Phytochemical analysis has revealed that *C. papaya* seeds contain phenolic compounds which are known for their potent antioxidant activity, examples of which are benzyl isothiocyanate, glucosinolates, carotenoids, β-cryptoxanthine, oleic acids, and stearic acids [12]. *C. papaya* seeds have also been demonstrated to be rich in alkaloids reported to have chemopreventive properties [13]. In addition, isolated plant alkaloids and their derivatives have been reported to have various biological activities including: anti-bacterial, anti-spasmodic, chemopreventive, anticancer, and analgesic agents [13, 14, 15, 16]. For instance, alkaloids from *Rauwolfia vomitoria* are a major component of some anti-cancer drugs, such as camptothecin and vinblastine [17]. Extracts from *C. papaya* seeds have been shown to possess antioxidant, anti-inflammatory, and anti-ulcerogenic effects [18, 19, 20, 21] indicating a strong potential for use in the treatment of inflammation-related cancers like HCC. Other studies have reported the fertility-enhancing, nephroprotective and hepatoprotective effects of the *C. papaya* seed extract [22, 23].

Ethnomedicinal claims, as well as pharmacological studies, have reported on the therapeutic potentials of *C. papaya* seeds with a few scientific validations. Despite the isolated biological activities of *C. papaya* (anti-inflammatory, anti-oxidant, anti-ulcerative, hepatoprotective, etc.) all of which point to anti-cancer activity towards inflammation-related cancers like HCC, the anti-HCC effects of *Carica papaya* seeds have not been reported in the scientific literature.

Carbon tetrachloride (CCl_4) is an effective hepatotoxin and has been employed in the induction of rodent models of liver injury [15] for the assessment of compounds with hepatoprotective activity. Once administered, CCl_4 is biotransformed by CYP450 enzymes leading to the release of free radicals which subsequently impose oxidative stress and cause various forms of liver damage [15].

The CCl_4 radical ($\bullet CCl_3$) impair fat metabolism through interaction with lipids, proteins, and nucleic acid [24]. Subsequent oxygenation of $\bullet CCl_3$ leads to the formation of trichloromethyl peroxy ($\bullet OOCCl_3$) [25], which increases lipid peroxidation and polyunsaturated fatty acid destruction in the liver [25]. The summation of these effects culminates in liver necrosis, increased liver fat accumulation which stimulates inflammation, cirrhosis, fibrosis, and its progression to HCC [24]. These phases are characterized by changes such as elevated activities (levels) of liver enzymes (AST, ALT) and triglyceride levels as well as a decrease in body weight and serum albumin levels [25]. Thus, subcutaneous administration of CCl_4 is routinely given to rodents for the assessment of compounds and plant products for their hepatoprotective effect. Here, we investigate the anti-HCC effect of an alkaloidal extract derived from the seeds of *Carica papaya* in carbon tetrachloride (CCl_4)-induced HCC in rats.

2. Materials and methods

2.1. Drugs and chemicals

Olive oil was purchased from Borges Mediterranean Group, (China), CCl_4 was purchased from Tianjin Chengyuan Chemical Co, Ltd., (China), Rat Alpha-fetoprotein ELISA Kit was purchased from Cusabio Technology LLC, (Houston, TX 77054, USA), Lenvatinib 4 mg/kg was purchased from Shanghai Bocimed Pharmaceutical Co., Ltd. (China), All other chemicals and reagents used in this study were of analytical grade.

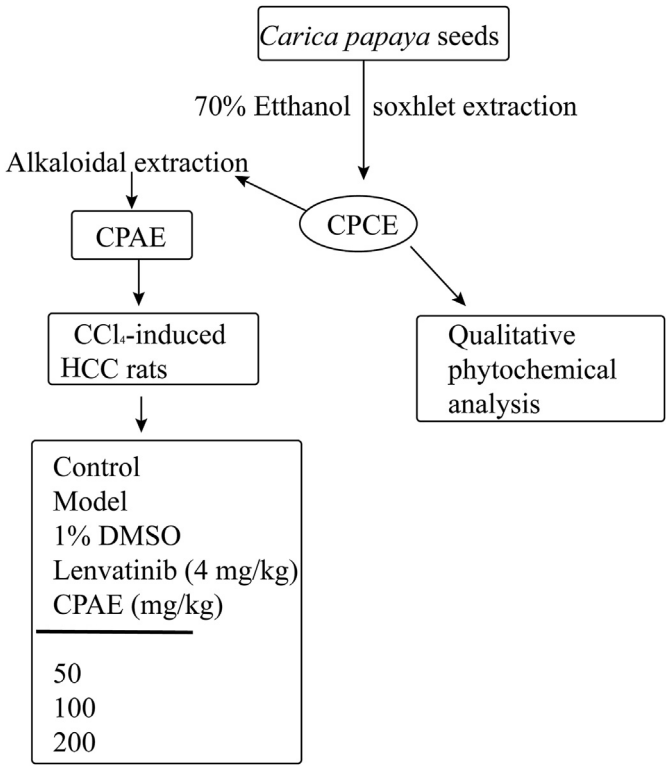

Figure 1. Study Design. CPAE, *Carica papaya* alkaloidal extract; DMSO, Dimethyl sulphoxide CPCE, *Carica papaya* seed crude extract.

2.2. Collection and preparation of plant material

The fruits of *Carica papaya* L. (solo dwarf variety) of the family Caricaceae were collected from Wiamoasi, a town in the Sekeyere South District of the Ashanti Region, Ghana, between 07th November 2016 and 12th December 2016. The sample was identified and authenticated by Mr. Francis Otoo, Curator of the Herbarium Unit, School of Biological Sciences, University of Cape Coast, Ghana, where a voucher specimen number SC/SBS/UCC/32CP013 was deposited. The seeds were extracted from the pulp, washed with tap water, and air-dried for 3 weeks at room temperature. Drying was established when the same weight was recorded through a three days interval weighing of the seeds after 10 days of drying. The dried seeds were reduced into powder using a hammer mill (Polymix Micro Hammer Cutter Mill, Glen Mills Inc, USA) with a 0.3 mm sieve. The powder (3.326 kg) was soxhlet (L3 Soxhlet extractor, Ergotech Soxhlet Apparatus Co, UK) defatted with petroleum spirit (40–60 °C) [26] for 8 h and with 70% ethanol (80 °C) for 12 h (Figure 1). The 70% ethanol extract was condensed in a crucible with a water bath (Premiere HH-4 Digital Water Bath, C & A Scientific Co Inc, USA). The crucible with the extract was transferred to a 40 °C hot air oven (Oven 300 plus series, Gallenkamp, England) for 24 h. The dried extract was weighed (792.42 g) and was referred to as *Carica papaya* crude extract (CPCE).

2.3. Qualitative phytochemical screening of CPCE

The qualitative phytochemical assessment of CPCE was conducted following previously described methods [27]. Briefly, Alkaloids, Tannins, Flavonoids, Glycosides, Lipids, Reducing sugars, Triterpenes, etc. were

screen for using specific phytochemical confirmatory tests. Compounds present were designated as (+) and those absent as (-).

2.4. Preparation of Carica papaya alkaloidal extract (CPAE)

The alkaloidal extraction procedure was carried out as previously described [28]. Briefly, CPCE was acidified with 15 % acetic acid and shaken to convert all free forms of alkaloids into salt form. Chloroform was added to the CPCE-acetic acid mixture, vigorously shaken, and was allowed to stand for 6 h. The aqueous layer from the previous step was basified with 10 % ammonia, thoroughly shaken, and was left idle overnight. The organic layer was extracted the next day with chloroform and concentrated to yield the alkaloidal extract (161.909 g) and was referred to as *Carica papaya* Alkaloidal extract (CPAE). The alkaloidal extract was confirmed with a preliminary phytochemical evaluation. HPLC analysis was performed according to the procedure by Sharma *et al.,* (1993) with slight modifications [Shimadzu LC-20 AD HPLC system, equipped with a model LC- 20 AV pump, UV detector SPD-20AV, Rheodyne fitted with a 5 µl loop, lab solution, auto-injector SIL-20AC, and a hyper CTO-10AS C-18 100Å column (4.6 × 150 nm, 3µm size)].

2.5. Acquisition and husbandry of experimental animals

Matured and healthy male Sprague-Dawley rats (130–180 g) were purchased from Noguchi Memorial Institute of Medical Research, University of Ghana. The rats were kept in the School of Biological Sciences Animal Facility in aluminum cages (12.5 × 16.6 × 7.5 cm) with softwood shavings as bedding at room temperature (26 ± 3 °C) and a 12 h light-dark cycle using a dry bulb to maintain the relative humidity within 40%–60%. Rats were allowed to acclimatize to laboratory conditions for two weeks before all experiments began. Rats were fed with a standard pellet diet (GAFCO, Tema, Ghana) and given water *ad libitum*. Conditions were varied to meet the specific requirements of some experiments. All animal experiments, procedures, and techniques used in this study were conducted in compliance with the National Institute of Health Guidelines for Care and Use of Laboratory Animals (NIH publication No.85-23, revised 1985) as well as specific national and institutional requirements regarding the use of animals in scientific experimentation. Ethical approval (FPPS/PCOL/016/2019) was obtained from the Institutional Review Board, Kwame Nkrumah University of Science and Technology, Ghana.

2.6. Dose selection

The doses of CPAE (50, 100, and 200 mg/kg) used in this study were arrived at following a preliminary acute toxicity dose selection study and doses reported in literature [25, 28, 29, 30]. Briefly, 10 rats that have been fasted overnight were put into 5 groups of 2. Groups 1, 2, 3, 4, and 5 were given CPAE (100, 200, 300, 400, and 500 mg/kg) respectively. rats were closely monitored for the first 5 h after drug administration for piloerection, change in fair color, eye color, tearing, frequency of face rubbing, and body scratching. Feed and water were introduced to their cages 3 h after drug administration and feeding and water intake were monitored up to the fifth hour. Intermittent cage-side observation was conducted to monitor breathing patterns, sniffing, and feed intake for the next 48 h.

2.7. Establishment of CCl_4-induced HCC in rats and experimental design

The establishment of CCl_4-induced HCC in rats was performed using a previously described procedure [25]. Briefly, 80 of the rats were given CCl_4 reconstituted in olive oil (El Gomhorya Co, Cairo, Egypt) thrice a week for 16 weeks (2 ml/kg; 1:1 *v/v*, *ip*). Five rats were randomly selected after 16 weeks of CCl_4 administration and sacrificed under chloroform anesthesia. Liver histology and microscopy and biochemical assays were performed to confirm HCC establishment. The experimental HCC rats were put into 6 groups of 10 rats and one control group as follows (Figure 1):

Control group that received neither CCl_4 nor any treatment,

Model group; received 16 weeks ip injection of CCl_4 but no treatment,

1% DMSO group; received 16 weeks ip injection of CCl_4 and 1% DMSO (vehicle) daily oral treatment for twelve weeks,

Lenvatinib group; received 16 weeks ip injection of CCl_4 and Lenvatinib (4 mg/kg) daily oral treatment for twelve weeks,

CPAE groups; received 16 weeks ip injection of CCl_4 and CPAE (50, 100, and 200 mg/kg) daily oral treatment for twelve weeks.

Prophylactic study was also conducted using 10 rats that were concurrently administered CPAE (200 mg/kg CPAE) and CCl_4 reconstituted in olive oil (1:1 *v/v*) (El Gomhorya Co, Cairo, Egypt; 2 ml/kg; *ip*) thrice a week at 6-hour intervals for 16 weeks. A 72 h washout period was allowed after the last day of treatment. Rats were euthanized and blood aspirated by cardiac puncture for hematological and biochemical analysis. The liver and kidney from each rat were isolated for histological analysis.

2.8. Bodyweight measurement

The baseline rat body weight was measured on day zero of treatment and every other week throughout the treatment period for all rats in each group. The weight gain was calculated and expressed as a percentage (Eq. (1)):

$$\%\ \text{weight gain} = \frac{\text{final weight} - \text{initial weight}}{\text{Initial weight}} \times 100 \quad (1)$$

2.9. Estimation of bleeding time

The mean bleeding time of each group was determined using a previously described procedure with few modifications [31]. The tail vein of each rat was pricked with a sterilized safety pin and the wound was gently blotted with filter paper every 30 s until the bleeding stopped. The procedure was repeated every 4 weeks.

2.10. Determination of prothrombin time

Prothrombin time was estimated using a previously described method with few modifications [32]. Briefly, blood was aspirated by cardiac puncture and transferred into a 10 mL test tube containing citrate ions. The sample was centrifuged at 3000 rpm at 4 °C for 10 min (Yingtai centrifuge TDL5M, Changsha Yingtai Instrument Co., Ltd, Hunan, China) to separate the serum and the plasma. The plasma was aspirated and added to thromboplastin from homogenized brain tissue and incubated on a water bath (Premiere HH-4 Digital Water Bath, C & A Scientific Co Inc, USA) at 37 °C for 3 min. Ionized calcium was added to the sample and timed until the plasma was clotted.

2.11. Estimation of tumor multiplicity

Tumor multiplicity was determined using a previously described method [33]. Briefly, each isolated liver was macroscopically examined by three independent researchers. The number of nodules 3 mm and above in each liver of each group were counted and the mean calculated and designated as the mean tumor multiplicity for each treatment group.

2.12. Measurement of hematological and biochemical parameters

Clotting factors and biochemical parameters were measured by adopting a previously described method [34]. Briefly, blood already dispensed into EDTA tubes when rats were sacrificed was analyzed for hematological indices using an automated hematology analyzer (MAXM Analyzer C23644 – DxH, California, USA). In empty vacutainer tubes, 3 mL of blood was pipetted and centrifuged at 1500 rpm for 8 min (Ynigtai

Table 1. Phytochemical analysis on CPCE.

Constituent	Test	Remarks
Phenols	Ferric Chloride	+
Flavonoids	Alkaline test	+
Steroids	Liebermann-Burchard	+
Saponins	Frothing	+
Tannins	Ferric chloride	+
Glycosides	Keller-Killiani	+
Alkaloids	Mayer's reagent	+
Triterpenoids	Liebermann-Burchard	+
Anthraquinones	Bromine test	-
Lipids	Emulsion	+
Reducing sugars	Benedicts reagent	+

Key: + = present, - = absent.

centrifuge TDL5M, Changsha Yingtai Instrument Co., Ltd, Hunan, China). The serum was retrieved for the estimation of liver biomarkers using an automated biochemical analyzer Hitachi Model 917 (Roche Diagnostics, Indianapolis, IN).

2.13. Measurement of serum alpha-fetoprotein (AFP) levels

The levels of serum AFP were measured using a quantitative enzyme-linked immunosorbent assay kit (Rat Alpha-fetoprotein ELISA Kit, Cusabio Technology LLC, Houston, TX 77054, USA). The assay was performed with strict adherence to the manufacturer's instructions. Briefly, 250 μL of serum diluent or sample (0, 15.6, 31.2, 62.5, 125, 250, and 500, pg/mL of serum matrix AFP) were placed in microtitre wells precoated with 100 μL of the antibody-enzyme conjugate (HRP-labeled anti-mouse AFP). The sample was mixed to form the sandwich complex on the surface of the wells, and were incubated for 2 h at 37 °C. A Hundred microliters (100 μL) of Biotin-antibody was added to each well, covered with a new adhesive strip, and incubated for 1 h at 37 °C. Each well was aspirated, washed and the process repeated two additional times. A 100 μL HRP-avidin was added to each well and incubated for 1 h at 37 °C. The aspiration wash was repeated for 5 times and 90 μL of 3,3′, 5,5′- tetramethylbenzidine (TMB) and $\leq$ 6.0 mM hydrogen peroxide added to each well after which the total mixture was incubated at 37 °C for 15 min. Fifty microlitres (50 μL) of stop solution was added to each well and mixed thoroughly. The optical density was measured at 450 nm with a microplate reader (Victor® Nivo™ multimode microplate reader, PerkinElmer, USA).

2.14. Tissue processing and histology

Organs that had been pre-fixed in 10% formaldehyde solution (formalin) were subjected to the various histological techniques as previously described [28, 35]. Each organ removed from the formalin was immersed into a series of ethanol solutions of increasing concentration to remove all water from the tissue and replaced with alcohol. The dehydrated tissues were cleared with xylene, to enhance the optical resolution. The tissues were waxed into a cassette, microtome, and flamed on a burner, washed under running water, dipped in an alkaline solution, and stained in Eosin Y (1%, for 10 min). The sections were then mounted in mounting media and observed under the light microscope.

2.15. Data analysis

Data were analyzed with GraphPad Prism software, (GraphPad Software, San Diego, California, U.S.A.) v. 7.00. The data were expressed as means ± standard deviation of the mean of each group (mean ± SD). Statistical significance was determined using analysis of variance (ANOVA) and Tukey's multiple comparison tests with $p < 0.05$ as the level of significance.

3. Results

3.1. Qualitative phytochemical profile of CPCE

Qualitative caorimetric analysis of *Carica papaya* crude extract (CPCE) revealed that CPCE contained phenols, alkaloids, tannins, glycosides, flavonoids, steroids, and saponins (Table 1).

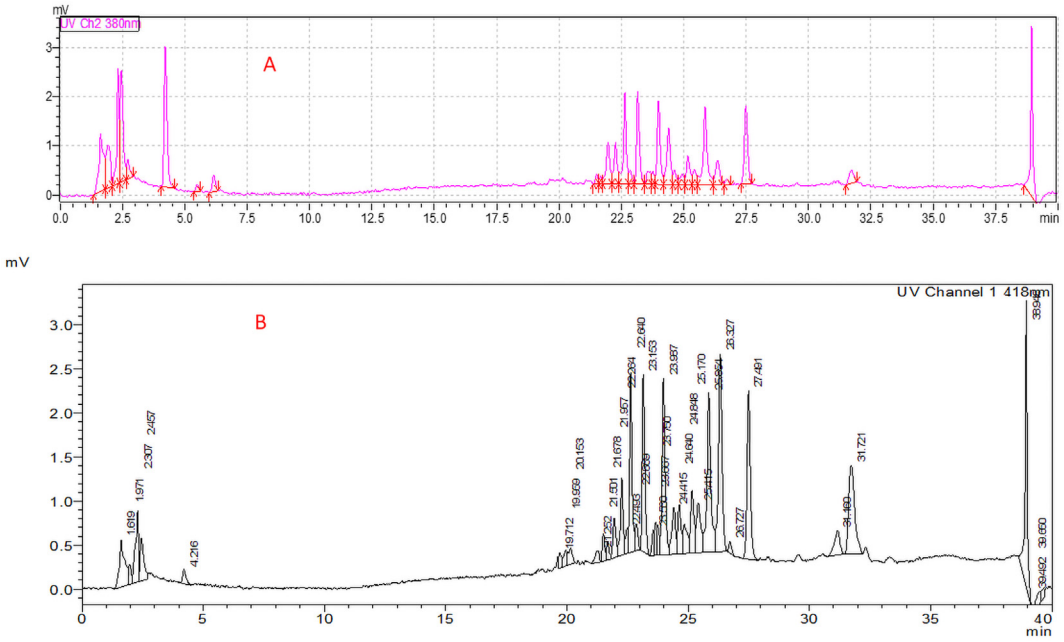

Figure 2. HPLC spectrum of alkaloidal extract from *Carica papaya* seeds. A - HPLC chromatogram of CPAE at 380 nm, B – HPLC chromatogram of CPAE at 410 nm.

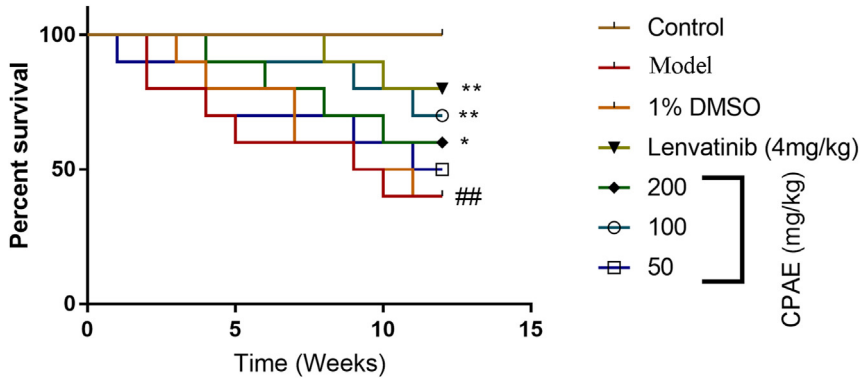

Figure 3. Per centage survival of CCl_4-induced HCC rats. ##, Model vs. control $p < 0.01$; *, Model vs. all treatment groups $p < 0.05$; **, Model vs. all treatment groups $p < 0.01$; CPAE, *Carica papaya* alkaloidal extract; DMSO, Dimethyl sulphoxide.

Table 2. Effect of CPAE on body weight of CCl_4-induced HCC rats.

Treatment groups	Initial mean body weight (g)	Final mean body weight (g)	Change in mean body weight(g)	Change in mean body weight (%)
Control	142.813 ± 9.39	274.428 ± 9.125	131.615 ± 9.002	92.16
Model	151.044 ± 7.335	191.907 ± 9.993	40.863 ± 4.400[a]	27.01
1% DMSO	144.803 ± 7.309	182.002 ± 9.911	37.199 ± 4.624[b]	25.69
Lenvatinib (4 mg/kg)	153.205 ± 5.659	239.334 ± 8.879	86.129 ± 7.411[b]	56.22
CPAE (mg/kg)				
50	149.466 ± 7.006	198.613 ± 9.347	49.147 ± 5.965[c]	32.88
100	153.127 ± 3.58	227.309 ± 5.271	74.182 ± 3.862[bcd]	48.44
200	154.477 ± 3.331	234.419 ± 6.388	79.942 ± 5.686[bd]	51.75

a = control vs model ($p \leq 0.05$), b = model vs all treatment groups ($p \leq 0.05$), c = Lenvatinib vs CPAE ($p \leq 0.05$), d = CPAE (100 and 200 mg/kg) vs CPAE (50 mg/kg) ($p \leq 0.05$). CPAE – *Carica papaya* alkaloidal extract.

3.2. HPLC analysis of CPAE

HPLC analysis of CPAE revealed the wide variability of alkaloid content in *Carica papaya* seeds (Figure 2). In all, about twenty-seven (27) alkaloids were visualized in the form of peaks (Figure 2). There were nine (9) prominent peaks with the retention times: 21.957, 22.640, 23.153, 23.987, 25.170, 25.854, 26.327, 27.491, and 31.740 min identified at a wavelength of 410 nm. The compound with the retention time of 26.327 min had the highest sharp peak with 2.7 mV intensity. Compound with

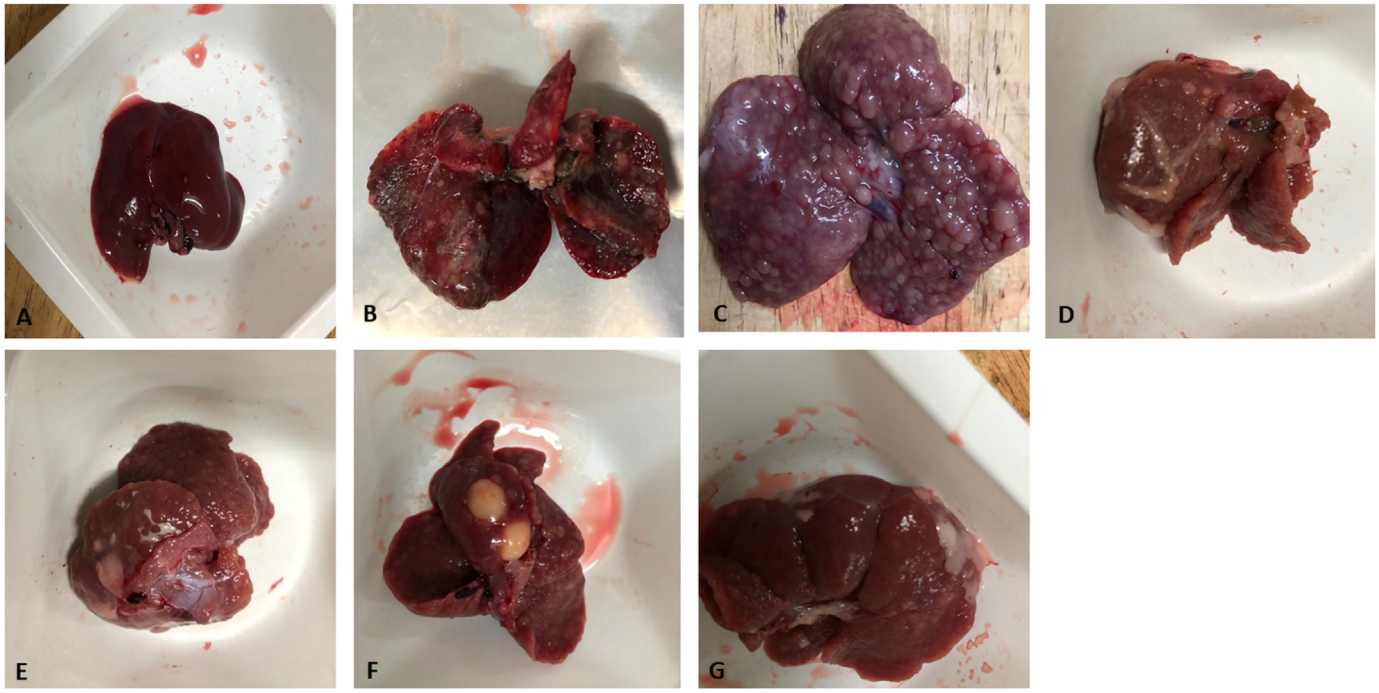

Figure 4. Effect of CPAE on tumor multiplicity of CCl_4-induced HCC rats. A, control group; B, Model group; C, 1% DMSO group; D, Lenvatinib (4 mg/kg) group; E, CPAE (50 mg/kg group; F, CPAE (100 mg/kg) group; G, CPAE (200 mg/kg) group; CPAE, *Carica papaya* alkaloidal extract; DMSO, Dimethyl sulphoxide.

Table 3. Effect of CPAE on tumor incidence of CCl_4/-induced HCC in rats.

Treatment groups	Tumor incidence α
Control	0 to 5 ± 0.0
Model	˃ 20 ± 4.3[a]
1% DMSO	˃ 20 ± 2.2[b]
Lenvatinib (4 mg/kg)	6 to 10 ± 3.3[b]
CPAE (mg/kg)	
50	15 to 20 ± 2.3[bc]
100	10 to 15 ± 1.8[b]
200	6 to 10 ± 1.7[b]

a = control vs. model ($p \leq 0.05$), b = model vs all treatment groups ($p \leq 0.05$), ns = model vs treatment groups ($p > 0.05$), c = Lenvatinib vs CPAE ($p \leq 0.05$). 'α' was determined by finding the average of tumor counted by three independent researchers for each treatment group.

retention time 22.64 min had the second highest peak with 2.5 mV intensity (Figure 2).

3.3. CPAE increased the survival rate in CCl_4-induced HCC rats

As expected, the control group recorded no fatality (0%). However, there was a 60% fatality rate in the model group, which was signoificantly higher ($P < 0.01$) compared to the control group. Lenvatinib (4 mg/kg) and CPAE (100 mg/kg) groups both recorded a 20 % mortality rate and this was significantly lower ($P < 0.01$) compared to the model. It was surprising that the highest dose of CPAE (200 mg/kg) group recorded a 20% higher mortality rate than the CPAE (100 mg/kg) (Figure 3).

3.4. CPAE restored weight loss in CCl_4-induced HCC in rats

By the end of the 12 weeks treatment period, the weight of the control group had nearly doubled (92.16%) and was significantly higher ($P < 0.001$) compared to the model which increased by only 27.01% (Table 2). CPAE showed dose-dependent weight-gain management in rats in the CPAE (200 mg/kg) recording a 51.75 % increase in body weight.

3.5. CPAE reduced tumour incidence in CCl_4-induced HCC in rats

There were no visible anatomical deformities seen in livers isolated from the control group, however, the model group and the 1% DMSO group had diffused tumors in their entire liver tissue (Figure 4) and a corresponding high tumor incidence (>20 ± 4.3 and >20 ± 2.2) for the model and the 1% DMSO group respectively (Table 3). In the CPAE (100 and 200 mg/kg) groups livers however, nodules were relatively samller. CPAE also showed a dose-dependent reduction in tumor incidence with the CPAE (200 mg/kg) having 6 to 10 tumor incidence range ($P < 0.01$) compared to the model group.

3.6. CPAE improved hematological parameters in CCl_4-induced HCC in rats

Even though the difference recorded for ionized calcium was not significant between any two groups, the control group had a significant high platelet count and a shorter prothrombin time compared to the model and all treatment groups (Table 4) correlating with a uniform and shorter bleeding time that deviates only slightly from its mean (Figure 5). Treatment with CPAE (100 and 200 mg/kg) and Lenvatinib (4 mg/kg) recorded significantly higher platelet count and a relatively shorter prothrombin time which kept improving with the duration of treatment (Figure 5). For instance, there was a significant improvement in the bleeding time ($p < 0.05$) of all treatment groups by the 8th week of treatment while that of the modelkept deteriorating (Figure 5).

3.7. CPAE regulated liver enzymes in CCl_4-induced HCC in rats

Mean serum GGT, AST, ALP, total protein, albumin, and direct bilirubin were within the normal range for the control group. Mean serum levels of all liver enzymes were high in the model group compared to the control. In particular, the mean level of GGT in the model was 27.25 ± 4.27 compared to the CPAE (200 mg/kg) which was 4.98 ± 1.14 ($P < 0.001$). Interestingly, the CPAE (200 mg/kg) group had serum GGT levels almost parallel with the control group. Even though all treatment groups were exposed to the same relative bodyweight concentration of CCl_4, mean serum levels of AST of the model was significantly higher 84.24 ± 4.32 compared to CPAE treated (62.32 ± 6.44, 32.5 ± 5.16, and 34.78 ± 6.48) for the 50, 100 and 200 mg/kg doses respectively (Table 5). It is worthy of note that the mean serum liver biomarkers of the CPAE (200 mg/kg) were in most cases, parallel with that of the control group, and in cases that they were different, the difference was not statistically significant.

3.8. CPAE reduced serum alpha-fetoprotein (AFP) in CCl_4-induced HCC in rats

The mean AFP level of the model was significantly higher ($P < 0.0001$) compared to the control group. Treatment of HCC with [CPAE (100 and 200 mg/kg) and Lenvatinib (4 mg/kg) significantly reduced mean serum AFP levels ($P < 0.001$) compared to the model group (Figure 6).

3.9. CPAE improved liver histoarchitecture in CCl_4-induced HCC in rats

The control group showed normal liver histology with hepatocytes having rounded nucleus and fewer infiltration of inflammatory cells. The model group and the 1% DMSO group showed highly differentiated and multiple tumors and infiltration of inflammatory cells. The model group also showed tumors with distinct margins and high fatty droplets. Treatment with CPAE (100 and 200 mg/kg) and Lenvatinib (4 mg/kg) reduced the tumor incidence and tumor differentiation. Tumor sizes were

Table 4. Effect of CPAE on clotting factors of CCl_4-induced HCC rats.

Treatment groups	Platelet count ($\times 10^4/\mu L$)	Ionized calcium (mg/dL)	Prothrombin time (sec)
Control	1.3 ± 0.004	4.91 ± 0.771	9.3 ± 0.65
Model	0.06 ± 0.001[a]	4.502 ± 1.802	23.5 ± 1.66[a]
1% DMSO	0.05 ± 0.009	4.201 ± 0.074	21.7 ± 1.35
Lenvatinib (4 mg/kg)	0.95 ± 0.002	4.33 ± 0.433	15.4 ± 0.36[b]
CPAE (mg/kg)			
50	0.74 ± 0.006[bc]	4.095 ± 0.083	19.7 ± 0.47[c]
100	1.09 ± 0.020[b]	4.109 ± 0.116	16.5 ± 0.92[bc]
200	1.4 ± 0.008[b]	4.62 ± 0.061	10.4 ± 0.201[bd]

a = control vs model ($p \leq 0.05$), b = model vs all treatment groups ($p \leq 0.05$), c = Lenvatinib vs CPAE ($p \leq 0.05$), d = CPAE (200 mg/kg) vs CPAE (50 and 100 mg/kg) ($p \leq 0.05$). CPAE – *Carica papaya* alkaloidal extract.

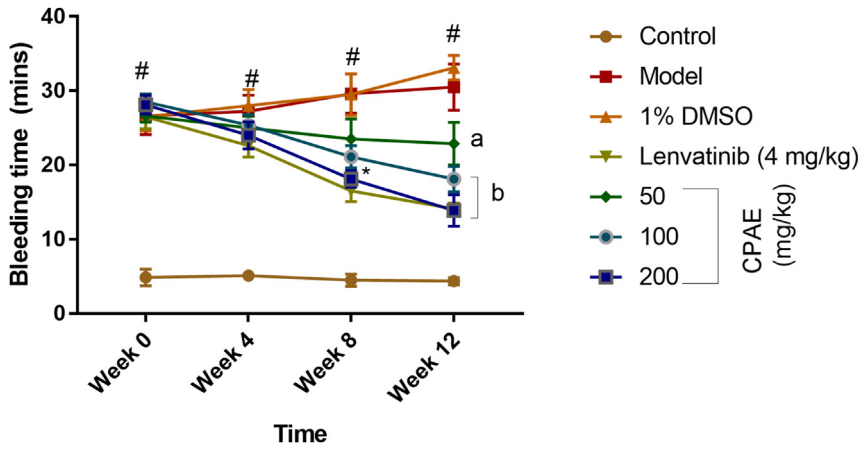

Figure 5. Effect of CPAE on bleeding time curve of CCl_4-induced HCC rats. #, Model vs. control $p < 0.0001$ CPAE; *, Model Vs. all treatment groups $p < 0.01$; b, Model vs. all treatment groups $p < 0.001$; *Carica papaya* alkaloidal extract; DMSO, Dimethyl sulphoxide.

Table 5. Effect of CPAE on liver biomarkers of CCl_4-induced HCC in rats.

Treatment groups	Direct bilirubin (μmol/L)	Total protein (μmol/L)	Albumin (g/L)	ALP (IU/L)	AST (IU/L)	ALT (IU/L)	GGT (IU/L)
Control	2.046 ± 0.401	72.48 ± 2.24	44.7 ± 4.524	25.20 ± 3.26	19.75 ± 6.6	25.066 ± 3.266	4.75 ± 1.5
Model	5.025 ± 1.018^{a}	124.91 ± 10.94^{a}	61.163 ± 4.418^{a}	63.5 ± 9.07^{a}	84.24 ± 4.32^{a}	63.5 ± 4.22^{a}	27.25 ± 4.27^{a}
1% DMSO	5.608 ± 1.306	118. 22 ± 12.61	67.07 ± 4.454	68.7 ± 11.46	72.63 ± 5.42	68.75 ± 7.46	26.85 ± 6.21
Lenvatinib (4 mg/kg)	3.002 ± 0.493^{b}	88.36 ± 7.54^{b}	48.807 ± 1.615^{b}	36.6 ± 9.22^{b}	38.44 ± 3.65^{b}	36.5 ± 4.77^{b}	6.44 ± 0.82^{b}
CPAE (mg/kg)							
50	4.004 ± 0.183^{c}	136.96 ± 9.83	54.396 ± 5.345^{c}	59. 3 ± 4.86^{c}	62.32 ± 6.44^{bc}	50.2 ± 11.36^{bc}	9.5 ± 3.01^{bc}
100	3.241 ± 0.048^{bc}	85.49 ± 11.11^{ae}	42.95 ± 3.664^{bf}	43.6 ± 6.11^{b}	32.5 ± 5.16^{be}	43.2 ± 3.86^{be}	5.2 ± 2.96^{be}
200	2. 886 ± 0.528^{bd}	93.85 ± 1.42^{be}	49.54 ± 2.308^{b}	38.7 ± 7.6^{b}	34.78 ± 6.48^{be}	38 75 ± 5.20^{be}	4.98 ± 1.14^{be}

a = control vs model ($p \leq 0.05$), b = model vs all treatment groups ($p \leq 0.05$), c = Lenvatinib vs CPAE ($p \leq 0.05$), d = CPAE [(200 mg/kg) vs CPAE (50 mg/kg) ($p \leq 0.05$)] e = CPAE [(100 and 200 mg/kg) vs CPAE (50 mg/kg) ($p \leq 0.05$)], f = CPAE (100 mg/kg) vs CPAE (50 mg/kg) ($p \leq 0.05$). CPAE – *Carica papaya* alkaloidal extract.

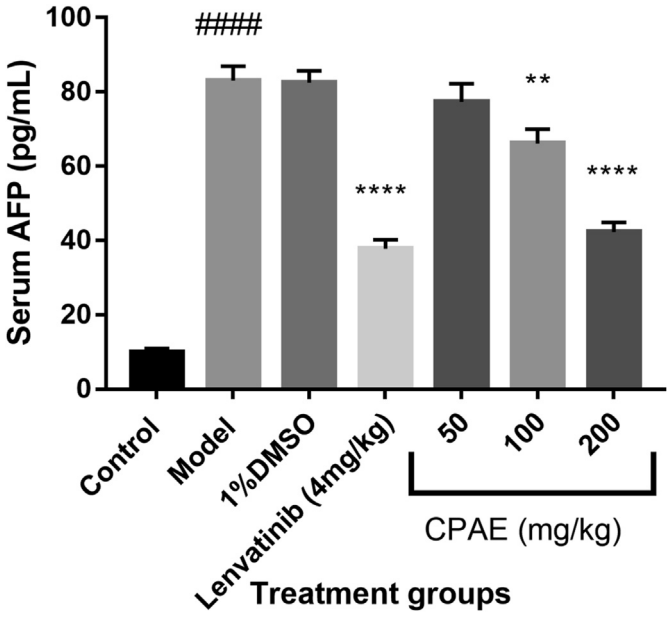

Figure 6. Effect of CPAE on serum alpha-fetoprotein levels of CCl_4-induced HCC rats. ####, Model vs. control $p < 0.0001$; **, Model vs. CPAE (100 mg/kg) groups $p < 0.01$; ****, Model vs. Lenvatinib (4 mg/kg) and CPAE (100 mg/kg) groups $p < 0.0001$; *Carica papaya* alkaloidal extract; DMSO, Dimethyl sulphoxide.

also significantly smaller compared to the model and the 1% DMSO groups (Figure 7).

3.10. CPAE showed prophylactic activity against CCl_4-induced HCC in rats

The control group recorded a significant increase in mean body weight compared to the control ($P < 0.05$) group. The difference in body weight improvement between the model and the prophylaxis group was also significantly higher ($P < 0.05$). These differences in body weight was correlated with differences in the mean liver weight to body weight ratio recorded for the control and model ($P < 0.01$), and the prophylaxis group and the model group ($p < 0.05$). In the prophylaxis group where CPAE (200 mg/kg) and CCl_4 were concurrently administered for 16 weeks, there was the establishment of liver fibrosis but could not progress to HCC as was seen in the model group that was administered only CCl_4 for the same duration (Table 6, Figure 8).

The mean serum liver enzymes and proteins; AST, ALT, ALP, GGT, direct bilirubin, total protein, albumin, and AFP were significantly ($P < 0.05$) higher in the model group than in the control group (Table 7). The mean serum levels of these liver biomarkers were significantly reduced in the prophylaxis group when compared to the model group. In the prophylaxis group, the platelet count and prothrombin time were significantly ($P > 0.05$) different from the model group, however, mean serum ionized calcium levels were not statistically different between the prophylaxis group and the model group (Table 8).

4. Discussion

This study found that *Carica papaya* alkaloidal extract (CPAE) prevented weight loss, reduced serum alpha-fetoprotein level, improves

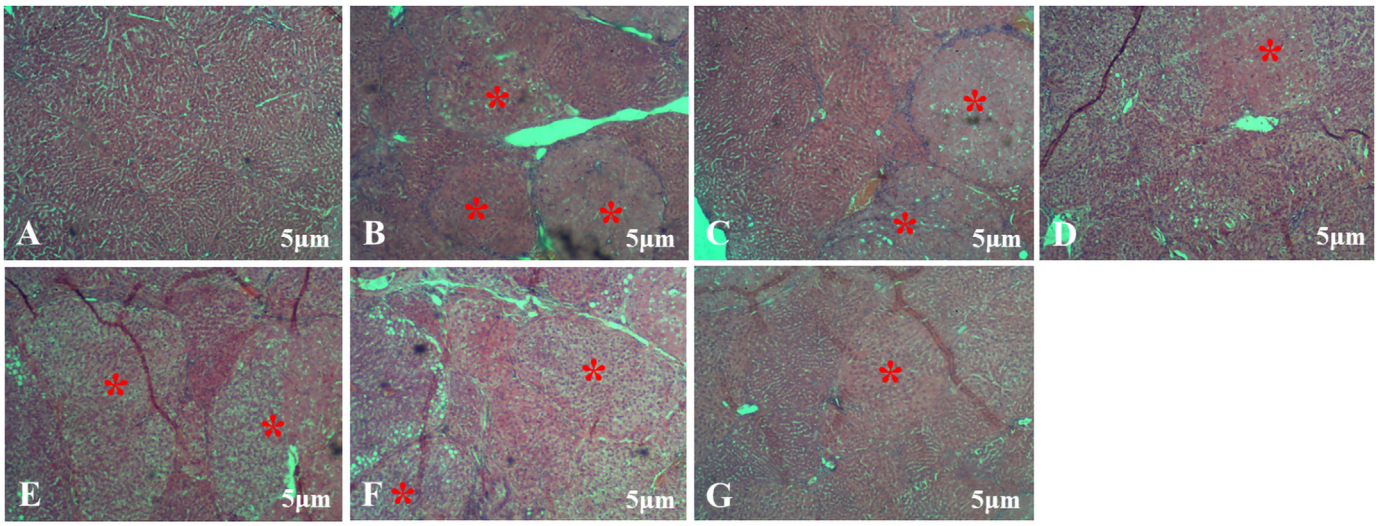

Figure 7. Effect of CPAE on liver histology of CCl_4-induced HCC rats. A, (control group, ×100) showed normal liver histomorphology and tightly packed hepatocytes; B, (Model group ×100) showed well-differentiated tumors bordered by distinct tumor margins and fatty changes; C, (1% DMSO group ×100) also showed well-differentiated tumors with thin trabecular, fatty changes and distinct tumor margins; D, (Lenvatinib (4 mg/kg) group ×100) showed indistinct tumors, without margins with largely tightly packed hepatocytes. Tumor size is small and less frequent; E, (CPAE (50 mg/kg group ×100) with intracytoplasmic fat and Mallory hyaline with loosely packed hepatocytes; F, (CPAE (100 mg/kg) group ×100) also showed intracytoplasmic fat and Mallory hyaline with loosely packed hepatocytes and fat deposits; G, (CPAE (200 mg/kg) group ×100) showed smaller tumor sizes with dissolved margins, hepatocytes are tightly packed with fewer fat depositions. CPAE, *Carica papaya* alkaloidal extract; DMSO, Dimethyl sulphoxide. *, tumors.

Table 6. Effect of CPAE on bodyweight and tumor multiplicity of the prophylaxis group.

Treatment groups	Change in mean body weight (g)	Mean liver weight (g)	Tumor multiplicity	Survival rate (%)
Control	91.60 ± 9.002	6.967 ± 0.412	0 to 5 ± 0.0	100
model	18.250 ± 4.400[a]	13.74 ± 1.118[a]	˃ 20 ± 3.5[a]	40[a]
Prophylaxis*	73.299 ± 1.567[b]	9.453 ± 0.717[b]	0 to 5 ± 0.0[b]	100[b]

a = model vs control ($p \leq 0.001$) b = model vs prophylaxis ($p \leq 0.001$).

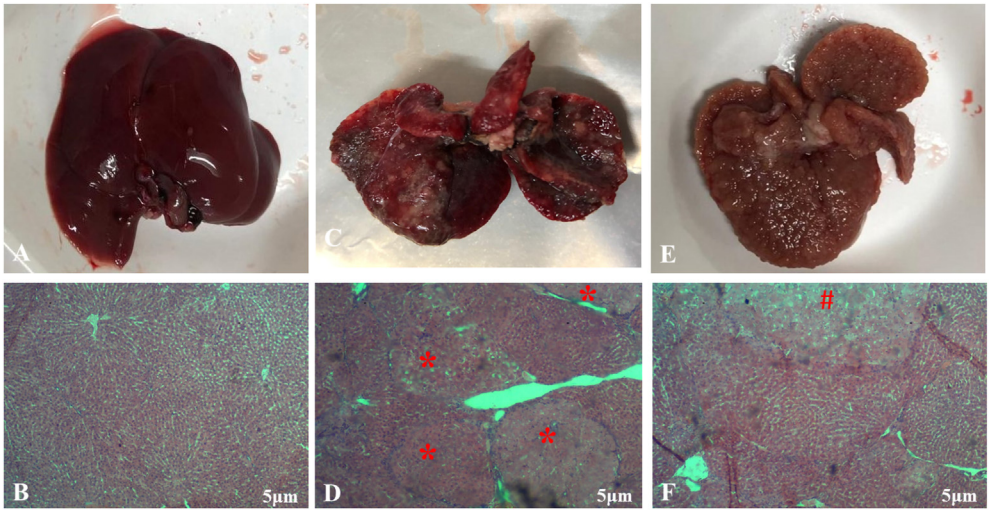

Figure 8. Prophylactic Effect of CPAE on CCl_4-induced HCC in rats. A, (control group liver macro-morphology), B (Control group liver histology ×100) showed tightly packed hepatocytes; C (model group liver macro-morphology), D, (Model group ×100) showed well-differentiated tumors bordered by distinct tumor margins and fatty changes; E, (Prophylaxis group liver macro-morphology) F, (prophylaxis group liver histology ×100) showed extreme fatty changes (hash) but no tumors. *, tumors.

clotting factors and bleeding time, and improved liver histoarchitecture in CCl_4-induced HCC rats. Further, we report that treatment of CCl_4-induced HCC rats with CPAE and Lenvatinib significantly improves weight gain (Table 2), increased the survival rate (Figure 3), and improved serum liver biomarkers (AST, ALP, ALT, bilirubin, etc.) (Table 5).

Though not specific, AFP is a key biomarker that is routinely measured to determine HCC prognosis and to also inform treatment options [36, 37]. Thus, other parameters such as serum ionized calcium, prothrombin time, and platelet count are commonly measured in addition to AFP for the assessment of liver health and function [38, 39]. In addition, platelets have been implicated in the progression and metastasis in HCC patients [40]. Reduced levels of platelets have been associated with the progression of liver cirrhosis and HCC [15]. We report that CPAE significantly reduced serum AFP (Figure 6) and upregulated mean platelet count (Table 4) suggesting an improvement in liver

Table 7. Effect of CPAE o liver enzymes on the prophylactic group.

Biomarker	Control	Model	prophylaxis
AST (IU/L)	17.25 ± 4.46	72.22 ± 7.263[a]	35.007 ± 9.421[b]
ALP (IU/L)	28 ± 6.268	68.35 ± 9.17[a]	32.05 ± 5.226[b]
ALT (IU/L)	33.75 ± 6.935	84.44 ± 7.27[a]	41.57 ± 7.44[b]
Direct bilirubin (μmol/L)	2.663 ± 0.931	5.22 ± 1.443[a]	2.208 ± 0.419[b]
Total bilirubin (μmol/L)	7.003 ± 1.476	21.34 ± 7.972[a]	13.263 ± 1.438[b]
Albumin (g/L)	49.02 ± 3.524	59.42 ± 4.33[b]	44.34 ± 1.665[b]
GGT (IU/L)	4.21 ± 1.4	25.77 ± 5.285[a]	7.346 ± 0.866
Total protein (g/L)	72.48 ± 4.247	104.9 ± 10.95[a]	81.17 ± 3.666[b]
AFP (pg/mL)	17.975 ± 0.995	83.48 ± 3.948[a]	54.05 ± 4.923[b]

a = control vs model ($p \leq 0.05$) b = model vs all prophylaxis ($p \leq 0.05$).

Table 8. Effect of CPAE on blood clotting profile of the prophylactic group.

Clotting factor	Control	Model	prophylaxis
Platelet count ($\times 10^4/\mu L$)	1.3 ± 0.04	0.06 ± 0.001[a]	0.13 ± 0.033[b]
Ionized calcium (mg/dL)	4.91 ± 0.771	4.502 ± 1.802	5.199 ± 1.469
Prothrombin time (sec)	9.3 ± 0.65	23.5 ± 1.66[a]	15.35 ± 0.3[b]

a = control vs model ($p \leq 0.05$) b = model vs all prophylaxis ($p \leq 0.05$).

functional health. Prothrombin time which is used to assess the prognosis of liver cirrhosis is mainly controlled by clotting factor (VII), the level of which is regulated by liver functional mass [41]. In this study, CPAE (100 and 200 mg/kg) and Lenvatinib (4 mg/kg) significantly reduced the prothrombin time (Table 4) which led to an improvement in bleeding time (Figure 5). These findings were corroborated with a significant improvement in liver histoarchitecture in the CPAE and the Lenvatinib treatment groups compared to the model (Figure 7) and are consistent with findings reported elsewhere [42].

In CCl_4 toxicity, the production of trichloromethyl peroxy ($\bullet OOCCl_3$) reduces oxygen partial pressure causing oxidative stress and increase the production of DAG-O(O)H [43]. The DAG-O(O)H stimulates PKCα–NF–kB mediated inflammation, fatty degeneration, and fibrosis in the liver which progresses to HCC [44]. Among the primary functions of the liver is to process absorbed nutrients and the synthesis of aromatic and branched amino acids [45]. Thus, impaired liver functional health causes a significant reduction in muscle mass leading to a reduction in the overall weight of liver-diseased patients [46]. A similar reduction in bodyweight post CCl_4 exposure in rats has been reported elsewhere [25, 47]. In this study, chronic exposure to CCl_4 led to a redction in bodyweight which was restored following treatment with CPAE. CPAE also reduced tumor multiplicity (Table 3 and Figure 4) and improve the general liver health in CCl_4-induced HCC in rats.

Interestingly, concurrent administration of CPAE (200 mg/kg) and CCl_4 for 16 weeks prevented the establishment of an HCC-like phenotype in rats. The prophylaxis group also had relatively lower serum AFP, AST. ALP, ALT, total protein, and total bilirubin compared to the model (Table 7). Platelet count, prothrombin time, and liver histomorphology were also relatively better compared to the model group (Figure 8 and Table 8). Following this revelation, we are hypothesizing that though CPAE may be working through multiple mechanisms to mediate its anti-HCC effect, interference with the PKCα–NF–kB mediated inflammation is likely to be a significant component of the mechanism of action for the observed anti-HCC activity.

Generally, the diversity of phytocompounds in a plant can be used to predict biological activities and/or ethnopharmacological application. Flavonoid-rich [16] and methanolic alkaloidal extract [13] from *Carica papaya* seeds have been shown to have chemopreventive properties. Fractionation alkaloidal extract (CPAE) from CPCE was subjected to HPLC analysis which revealed 27 peaks (Figure 2) most of which were clustered within a close time margin. Indeed, while we could have isolated and characterized each of the 27 individual alkaloids, we sought to establish the biological activity of CPAE and provide an evidence-based argument for the isolation and characterization of these individual alkaloids in future studies. Thus, the current results have provided the rationale to isolate prominent peaks of the CPAE fractions to obtain pure individual isolates that further studies can be conducted to advance frontiers in the development of new treatment options for HCC.

5. Conclusion

CPAE has demonstrated a relatively potent anti-HCC effect by improving liver histoarchitecture, restoring weight loss, reducing serum alpha-fetoprotein levels, and improving hematological indices in CCl_4-induced HCC rats. These findings call for further studies on this potential anti-HCC agent to be developed as an alternative therapeutic agent for HCC treatment, but first, elucidation of the mechanism of action.

Declarations

Author contribution statement

Isaac Kyei-Barffour: Conceived and designed the experiments; Performed the experiment; Analyzed and interpreted the data; Contributed reagents, tools or data; Wrote the paper.

Roselind Kyei Baah Kwarkoh; Augustine Suurinobah Brah; Samuel Addo Akwetey: Performed the experiment; Analyzed and interpreted the data.

Desmond Omane Acheampong; Benjamin Aboagye: Performed the experiment, Analyzed and interpreted the data; Contributed reagents, tools or data; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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