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IN VIVO CARBON TETRACHLORIDE-INDUCED HEPATOPROTECTIVE AND *IN VITRO* CYTOTOXIC ACTIVITIES OF *GARCINIA HOMBRONIANA* (SEASHORE MANGOSTEEN)

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Abstract

Background: *Garcinia hombroniana,* known as "manggis hutan" (jungle mangosteen) in Malaysia, is distributed in tropical Asia, Borneo, Thailand, Andaman, Nicobar Islands, Vietnam and India. In Malaysia, its ripened crimson sour fruit rind is used as a seasoning agent in curries and culinary dishes. Its roots and leaves decoction is used against skin infections and after child birth. This study aimed to evaluate *in vivo* hepatoprotective and *in vitro* cytotoxic activities of 20% methanolic ethyl acetate (MEA) *G. hombroniana* bark extract.
Materials and Methods: In hepatoprotective activity, liver damage was induced by treating rats with 1.0 mL carbon tetrachloride (CCl_4)/kg and MEA extract was administered at a dose of 50, 250 and 500 mg/kg 24 h before intoxication with CCl_4. Cytotoxicity study was performed on MCF-7 (human breast cancer), DBTRG (human glioblastoma), PC-3 (human prostate cancer) and U2OS (human osteosarcoma) cell lines. ^{1}H, ^{13}C-NMR (nuclear magnetic resonance), and IR (infrared) spectral analyses were also conducted for MEA extract.
Results: In hepatoprotective activity evaluation, MEA extract at a higher dose level of 500 mg/kg showed significant ($p<0.05$) potency. In cytotoxicity study, MEA extract was more toxic towards MCF-7 and DBTRG cell lines causing 78.7% and 64.3% cell death, respectively. MEA extract in ^{1}H, ^{13}C-NMR, and IR spectra exhibited bands, signals and *J* (coupling constant) values representing aromatic/phenolic constituents.
Conclusions: From the results, it could be concluded that MEA extract has potency to inhibit hepatotoxicity and MCF-7 and DBTRG cancer cell lines which might be due to the phenolic compounds depicted from NMR and IR spectra.

Keywords: Carbon tetrachloride; *Garcinia hombroniana*; Hepatoprotective activity; Cytotoxicity; NMR

Introduction

Living cells have the natural ability to scavenge excess free radicals which are produced as a result of natural cell metabolism and external environmental factors. These abilities are due to the presence of endogenous enzymatic antioxidants including catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione reductase (GR), and non-enzymatic antioxidants such as reduced glutathione (GSH). However, due to the overwhelming production and external stresses, there is an imbalance between prooxidant and antioxidant homeostasis and thus, natural process of inhibiting free radicals is not 100% effective. These excessive free radicals lead to membrane lipid peroxidation, cell apoptosis, necrosis, hepatotoxicity, neurodegenerative disorders, cardiovascular diseases, immune system decline, diabetes mellitus and cancer (Jothy *et al*., 2011).

Natural products have been proved to be promising source of bioactive compounds in drug discovery and development (Kingston, 2010). For example, some plants such as *Picrorhiza kurroa* (Schuppan *et al*., 1999) and *Phyllanthus niruri* (Bhattacharjee & Sil, 2007) have been traditionally used for the ailments of liver complications. Silymarin and silybin, the most effective hepatoprotective and clinically used agents in hepatic diseases were isolated from *Silybum marianum* (family Asteraceae) (Fernandes *et al*., 1995). Likewise, the use of natural products as cytotoxic agents such as vinblastine and vincristine, etoposide and teniposide, taxanes, irinotecan and topotecan used in cancer chemotherapy, are either isolated from plant species or derived from a natural prototype (Cragg *et al*., 1994).

Garcinia, a genus of Guttiferae (Clusiaceae) family, is enriched with potential bioactive compounds such as prenylated xanthones (*α*- and *β*-mangostin), benzophenones (guttiferones, xanthochymol), flavonoids and biflavonoids (kolaviron, morelloflavone), and triterpenes (garchombronanes) with significant anti-inflammatory, anti-HIV, cytotoxic, hepatoprotective, antimicrobial and anticholinesterase activities (Gustafson *et al*., 1992; Masullo *et al.*, 2008; Mackeen *et al*., 2000; Rukachaisirikul *et al*., 2006; Zhang *et al*., 2010; Jamila *et al.*, 2015a & b). Previously, several

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investigations carried out on the pharmacological effects of chemical constituents isolated from *Garcinia* species, have reported their anti-hepatotoxic and cytotoxic activities. For example, kolaviron (a mixture of GB1, GB2 and kolaflavonone) isolated from *G. kola* seeds (Kapadia *et al.*, 1994) is reported to have hepatoprotective properties (Nwokocha *et al*., 2011). Similaly, Panda *et al*. (2013), and Mahendran and Devi (2001) in their invsitgation on *G. indica* and *G. cambogia*, reported their antihepatotoxic activities against D-galactosamine-, antitubercular drug-, and ethanol-induced toxicities. However, due to the limited number of hepatoprotective agents, and easy accessibility of plants, the demand of herbal medicines in the management and treatment of hepatic complications is increasing (Mukherjee *et al*., 2006).

Concerning the cytotoxic activities of *Garcinia* species, guttiferones A and K isolated from *G. cambogia* have shown significant cytotoxic effects on HCT-11, HT-29 and SW-480 human colon cancer cell lines with IC_{50} ranging from 5 to 25 µM (Yang *et al*., 2010). In addition, garciyunnanins A and B, and a triterpene (3*β*-hydroxy-5-glutinen-28-oic acid) were respectively found active against HeLa-C3 sensor and MRC-5 cells in the previous studies (Xu *et al*., 2008; Elfita *et al*., 2009).

Garcinia hombroniana, which is distributed in the mountainous forests of Malaysia (Nazre, 2010), has yielded different classes of bioactive compounds including xanthones, benzophenones, flavonoids, biflavonoids, and triterpenes with antioxidant, antiplatelet aggregation, antimicrobial, cytotoxic and anticholinesterase activities (Klaiklay *et al*., 2013; Saputri & Jantan 2012; Jamila *et al*., 2014a; Jamila *et al*., 2014b; Jamila *et al*., 2014c; Jamila *et al*., 2015a; Jamila *et al*., 2015b; Jamila *et al*., 2016). Regarding the hepatoprotective and cytotoxic effects of *G. hombroniana* crude extracts, there has been no previously published report. Hence, taking this, and the hepatoprotective and cytotoxic background of *Garcinia* species into account, the objective of the present study was to evaluate the hepatoprotective and cytotoxic activities of MEA *G. hombroniana* bark extract obtained by Soxhlet extraction.

Materials and Methods

Plant materials

The analyzed plant material (*G. hombroniana*) was collected from Penang Botanical Garden, Malaysia. A voucher specimen; PBGK12 has been deposited at the herbarium of this garden for future reference.

Chemicals and instruments

The solvents; methanol and ethyl acetate of analytical reagent grade used for extraction were purchased from QRëC, Malaysia. Carbon tetrachloride; CCl_4 (toxicant) and silymarin (reference hepatoprotective agent), and solvent; DMSO-d_6 for the experiment of ^1H and ^{13}C NMR spectral analysis were purchased from Sigma-Aldrich Co., USA. Silymarin (200 mg/kg) was dissolved in distilled water. The serum activities of alanine aminotranferase (ALT), aspartate aminotransferase (AST) and alkaline phosphate (ALP) were measured by using Hitachi 902 Automatic Chemical Analyser (Japan). The chemicals of cytotoxic activity; DBTRG (glioblastoma), MCF-7 (human breast cancer), U2OS (osteosarcoma) and PC-3 (prostate) cell lines were purchased from Sigma-Aldrich (USA). IR spectra were recorded by KBr using Perkin Elmer (USA) 2000 FT-IR spectrophotometer. The ^1H and ^{13}C NMR experiments were performed at room temperature using a Bruker Ascend 500 MHz (^1H) and 125 MHz (^{13}C) spectrometer (Bruker Biospin, Switzerland).

Extraction and isolation

The air dried ground defatted bark of *G. hombroniana* was extracted by Soxhlet extractor with a mixture of polar solvents of 20% methanol in ethyl acetate (1:4). The filtered extract was evaporated and concentrated using a rotary evaporator at 40 °C and the obtained concentrated extract then dried by passing oxygen free nitrogen gas. A shiny brown dried 20% methanolic ethyl acetate (MEA) extract was obtained and screened for hepatoprotective and cytotoxic activities.

Hepatoprotective activity

Experimental animals

The experimental animals including male Sprague-Dawley rats weighed 180 to 220 g at the time of drug treatment, were obtained from the Veterinary Animal Unit, Universiti Putra Malaysia (UPM). They were housed at the animal house, Faculty of Medicine and Health Sciences, UPM. The animals were acclimatized in polypropylene cages, and standard food pellet and water were provided ad libitum. They were maintained in a 12 h light/dark cycle at 27±2 °C before the commencement of treatment with drugs. They were cared in accordance to the current UPM principles and ethical guidelines which are established for the care and use of laboratory animals.

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CCl_4-induced toxicity

A slightly modified method of Zakaria *et al*., (2011) for CCl_4 toxicity assay was used. The rats were randomly divided into six groups each consisting of six rats (n = 6). Group I which served as normal control only received 10% DMSO. Group II served as negative control received 10% DMSO and was intoxicated by induction of 1.0 mL CCl_4/kg body weight diluted in olive oil with a ratio 1:1 intraperitoneally (i.p). Group III served as positive control group was treated with 200 mg/kg silymarin, followed by induction of toxicity with CCl_4. Other three groups; IV, V and VI were administered orally with MEA extract of 50, 250 and 500 mg/kg, respectively and toxicity in them was induced by CCl_4. All the samples and drugs to all the groups were administered orally. Prior to treatment, all the animals were fasted for 48 h under standard laboratory conditions. Each group received respective oral dose of test solution for seven consecutive days. After 3 hours of the solution administration on seventh day, CCl_4 was induced i.p. At the end of induction for 48 h, the rats were fasted again and anesthetized with the help of diethyl ether. For hepatoprotectivity test of the MEA extract, the blood was collected for biochemical assay, and then the animals were sacrificed by cervical dislocation. The livers were removed and preceded for histopathological study.

Biochemical evaluation

Liver damage was assessed by evaluation of serum activities of ALT, AST ALP and measured by Hitachi 902 Automatic Chemical Analyser using standard methods. A 3 mL of the blood was collected by cardiac puncture with disposable sterile syringe and kept at room temperature for 45 min to clot. To separate the serum, it was centrifuged at 2500 rpm at 30 °C for 15 min, and used for the evaluation of ALP, ALT and ASP. Then, the rats were sacrificed, their livers were removed and separated from the body immediately, its weight per 100 g body weight was measured, and fixed in 10% formalin for histopathology studies.

Histopathological studies

The liver was fixed in 10% formalin after dissected out, dehydrated in gradual ethanol (50–100%), cleared in xylene, and then embedded in paraffin wax. The sections were trimmed to approximately 4-5 µm thickness and prepared for staining with hematoxylin and eosin dye for microscopic evaluation. Pathological changes (normal hepatocyte, steatosis, coagulative necrosis, haemorrhage and inflammation) of the liver structure were scored according to the severity of the hepatic injury using modified El-Beshbishy *et al*., (2010) method.

Cytotoxic Activity

Cytotoxicity assay was conducted according to the modified method of Mosmann (1983). Briefly, a stock solution containing 500 µg/mL of MEA extract was prepared by dissolving 0.5 mg of extract in 1 mL of dimethyl sulfoxide (DMSO). Working solutions of 10, 25, 50, 75 and 100 µg/mL in triplicates were prepared from the stock solution. MCF-7 and DBTRG cell lines were grown in the Roselle's Park Memorial Institute (RPMI) 1640 medium, U2OS cells in the Dulbecco's Modified Eagle's Medium (DMEM), whereas PC-3 were grown in the Ham F12 K medium. These all cells were then supplemented with 10% of fetal bovine serum and 100 units/mL penicillin. They were kept at 37 °C in a humidified condition, 5% of CO_2 in air and passage 2 to 3 times per week by light trypsinization. Control cells received the vehicle alone (<0.1%). A cytotoxicity detection kit was used to determine the cytotoxic effect of MEA extract on the aforementioned cell lines. The amount of lactate dehydrogenase (LDH) was measured by using a test kit. Cells were seeded in 24-well plates at 50,000 cells per mL, incubated for 24 h and allowed to attach overnight. Prior to expose cells to MEA extract (10, 25, 50, 75 and 100 µg/mL), the medium was replaced with fresh one containing 2% of fetal bovine serum for up to 72 h. The change in colour generated and its absorbance was determined spectrophotometrically at 490 and 620 nm referenced wavelengths using a microplate reader. The amount of LDH released was calculated according to the formula given in the manufacturer's protocol. As a control for maximum releasable LDH activity, cells were treated with a lysis reagent (1% of triton X-100) for 10 min, whereas spontaneous LDH released in the supernatant of untreated cultures was measured. Additional cell-free wells containing assay medium alone were prepared which were used as a blank. The results were expressed as mean ± standard deviation.

Statistical analyses

All the numerical data presented as mean ± SEM were analyzed using one way analysis of variance (ANOVA) followed by Dunnett's post hoc test ($p < 0.05$) with SPSS software, version 19.0 (SPSS Inc., Chicago, USA).

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Results and Discussion

Hepatoprotective activity

Biochemical study

Liver is the vital organ of living organisms, performing chemical processes of metabolism, secretion, detoxification, protein synthesis, hormone production, and production of biochemicals necessary for digestion (Adaramoye & Adeyemi, 2006). Carbon tetrachloride (CCl_4) when taken is accumulated in the hepatic parenchyma cells where it is activated by cytochrome P450 to form trichlormethyl radicals. These radicals oxidize fatty acids and cellular proteins to produce lipid peroxides which lead to liver damage. Damage to liver structural integrity can be evaluated by the increased level of hepato-specific enzymes such as ALP, ALT and AST in the serum. These enzymes are present in cytoplasm which release into the serum after cellular damage (Suja *et al*., 2004). In the present study, the anti-hepatotoxicity of MEA extract was evaluated by its ability to reduce the elevated levels of ALP, ALT and AST in blood of rats treated with doses of CCl_4 (hepatotoxin). MEA extract administered to rats at a dose of 50, 250 and 500 mg/kg i.p. 24 h before intoxication with CCl_4, reduced the elevation of serum enzymes measured as indices of liver function and hepatotoxicity in rats. Pre-treatment study (prophylactic) of MEA extract prevented progression of CCl_4 induced acute liver injury. The administration of CCl_4 caused significant increase in the ALP, ALT and AST serum marker level in group pre-treated with 10% DMSO as compared to the normal 10% DMSO-pre-treated non-CCl_4-induced group. The results of effect of MEA extract on serum liver enzyme activities in CCl_4 intoxicated rats is given in Table 1. From the results, it was found that the high dose (500 mg/kg) oral administration of MEA extract and silymarin of 200 mg/kg were able to lessen the hepatotoxic effects by decreasing the level of the ALP, ALT and AST enzymes in the liver of rats intoxicated with CCl_4.

Table 1: Effect of CCl_4 and protective treatments of MEA (20% methanolic ethyl acetate) extract of *G. hombroniana* at ALT, AST and ALP (U/L)

Treatment	Dose(mg/kg)	ALT (U/L)	AST (U/L)	ALP (U/L)
Control (10% DMSO)	-	13.11 ± 1.1*	89.6 ± 2.5*	110.9 ± 6.4*
10% DMSO+CCl_4	-	4691.0 ± 650.4	5580.0 ± 1074.0	430.0 ± 48.4
Silymarin+CCl_4	200	441.6 ± 194.5*	534.2 ± 248.2*	189.4 ± 21.9*
MEA extract + CCl_4	50	3430.0 ± 513.8	3964.0 ± 785.7	450.0 ± 40.1
	250	2733.0 ± 170.4*	3224.0 ± 505.6*	552.2 ± 45.8
	500	2048.0 ± 363.8*	2187.0 ± 297.1*	324.3 ± 35.9*

* Data differed significantly ($p<0.05$) when compared to 10% DMSO+CCl_4-treated group in respective column
Values are expressed as means ± SEM of six replicates

Histopathological study

From Figure 1a, it can be seen that the histology of liver of normal control animals exhibited normal architecture of hepatic cells having well-defined cytoplasm and sinusoid, prominent nucleus, and central vein. However, section of liver tissue of a group of rats treated with 1.0 g/kg CCl_4-treated group (i.p) given in Figure 1b, showed massive coagulative necrosis, haemorrhage and infiltration of inflammatory cells. Section of liver tissue of another group serving as positive control, where the liver was treated with 200 mg/kg of silymarin followed by CCl_4 induction showed preservation of normal hepatocytes (Figure 1c). Rats treated with 50 mg/kg body weight of MEA extract along with CCl_4 intoxication exhibited both necrosis and inflammation (Figure 1d), whereas those treated with moderate dose of 250 mg/kg body weight of MEA extract showed less marked damage and vacuole formation, and the coagulative necrosis was less (Figure 1e). Furthermore, in rats which were treated with a dose of 500 mg/kg body weight, the liver appeared was normal but with mild inflammation, of similar texture and cell arrangement observed for the liver of rats which were treated with 200 mg/kg of silymarin (Figure 1f). So, MEA extract inhibited the CCl_4 induced hepatotoxicity in rats at a dose equal to the half of the standard drug; silymarin. The results of histopathological scoring of the liver tissues pre-treated with the respective test solutions are given in Table 2. These results provided evidence to support biochemical analysis which further suggested that MEA extract has the ability to regenerate the parenchyma cells, to protect the membrane fragility and to decrease the enzymes release into circulation. In histopathological study, the presence of marked necrosis, inflammation, and haemorrhage in CCl_4 intoxicated rats (shown by negative control group) was reduced remarkably when pre-treated either with MEA extract or silymarin. These changes in the liver histology were consistent with the corresponding changes in the enzyme levels, confirming the anti-hepatotoxic effects and inhibition of hepatocellular necrosis by the tested extract.

Cytotoxic Activity

The results of cytotoxicity test of the MEA extract at a concentrations range of 10–100 µg/mL carried out against MCF-7, DBTRG, PC-3 and U2OS cells, showed that the tested extract is more toxic towards MCF-7 and

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DBTRG cells causing maximum cells death with 78.7% and 64.3% after 24 h, respectively. The MEA extract showed a significant dose-dependent activity against MCF-7 and DBTRG cancer cells which is also depicted from Figure 2.

However, the inhibition did not increase anymore with more time and no further increase up to 72 h was noted. The cell concentration decreased with increasing concentration of the extract. The inhibition of the cancer cell lines might be due to the presence of phenolic compounds such as anthocyanins, benzophenones, flavonoids and xanthones which have been previously reported to be the main constituents of *Garcinia* species (Gustafson *et al*., 1992; Kapadia *et al.*, 1994; Masullo *et al.*, 2008; Mackeen *et al*., 2000; Rukachaisirikul *et al*., 2006; Zhang *et al*., 2010; Jamila *et al.*, 2015a; Jamila *et al.*, 2015b).

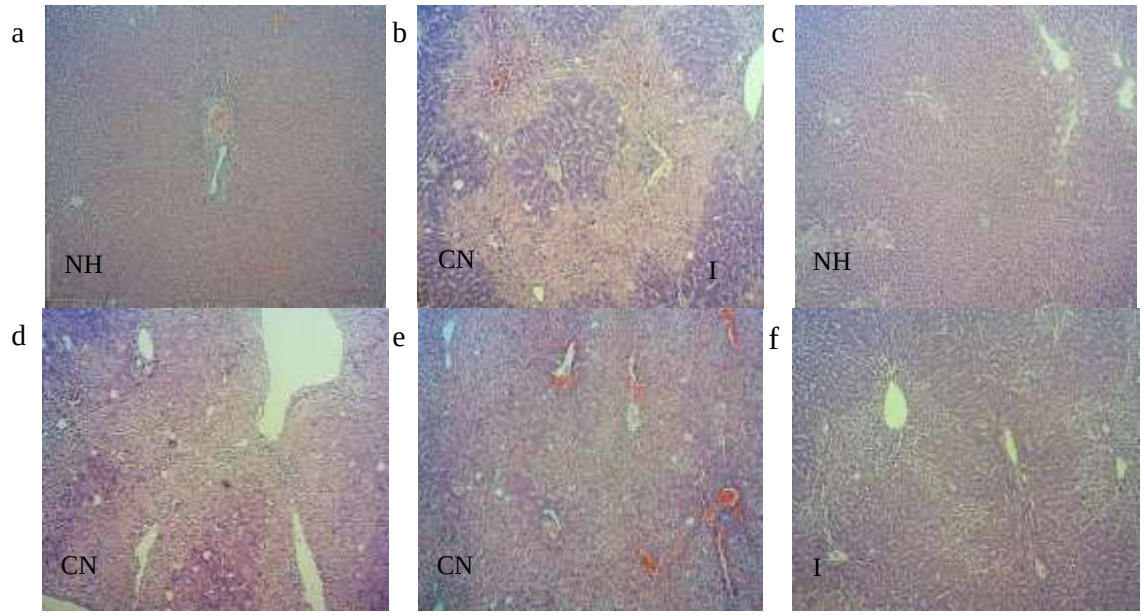

Figure1: a-f. Hepatoprotective activity of the MEA (20% methanolic ethyl acetate) bark extract of *Garcinia hombroniana* NH: normal hepatocyte, CN: coagulative necrosis, I: inflammation

Table 2: Histopathological evaluation of the effect of various doses of MEA (20% methanolic ethyl acetate) extract of *G. hombroniana* against CCl_4-induced hepatic injury in rats

Treatment	Dose(mg/kg)	Steatosis	Necrosis	Inflammation	Haemorrhage
Normal	-	-	-	-	-
10% DMSO	-	-	+++	++	++
Silymarin	200	-	+	+	-
	50	-	++	+	-
MEA extract	250	-	++	+	-
	500	-	+	+	-

The severity of various features of hepatic injury was evaluated based on the following scoring scheme:
- normal, + mild effect, ++ moderate effct, +++ severe effect.

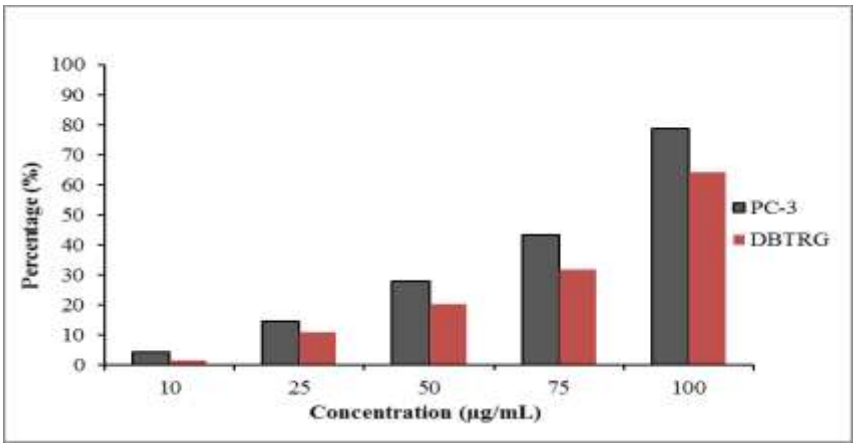

Figure 2: Cytotoxic activity of MEA (20% methanolic ethyl acetate) bark extract of *Garcinia hombroniana* against PC3 and DBTRG cancer cell lines

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^{1}H, ^{13}C-NMR and IR spectral analysis

The tested extract was also analyzed for ^{1}H and ^{13}C NMR, and IR experiments. The ^{1}H and ^{13}C NMR spectra (Figures 3 and 4) displayed signals of the regions representing the glycosylated aromatic/phenolic compounds, most probably xanthones, benzophenones, flavonoids, biflavonoids and their glycosides. IR spectrum (Figure 5) exhibited absorption bands at 3255 cm^{-1} for O—H, 1646 cm^{-1} for C=O and 1519 cm^{-1} for C=C stretching, confirming the presence of phenolic compounds with carbonyl group. Plants which are used for the protection of liver contain an array of bioactive compounds particularly, phenolics such as flavonoids, benzophenones, coumarins, organic acids and xanthones (Kumar, 2012). Xanthones, benzophenones and biflavonoids are the important classes of compounds of *Garcinia* species which are reported to possess antioxidant activities due to the reducing abilities of their hydroxyl groups by donating hydrogen to free radicals, and thus, prevent the free radical chain reactions and consequently the complications arose from these radicals (Riaz *et al*., 2011). Adaramoye and Adeyemi (2006), and Adaramoye *et al*., (2008) in their study on *G. kola* found that "kolaviron", a mixture of three biflavonoids (GB1, GB2 and kolaflavanone) has potent hepatoprotecive effects. From the NMR and IR spectral analysis results of MEA extract of *G. hombroniana*, it could be deduced that its hepatoprotective and cytotoxic activities might be due to the phenolic compounds present in it.

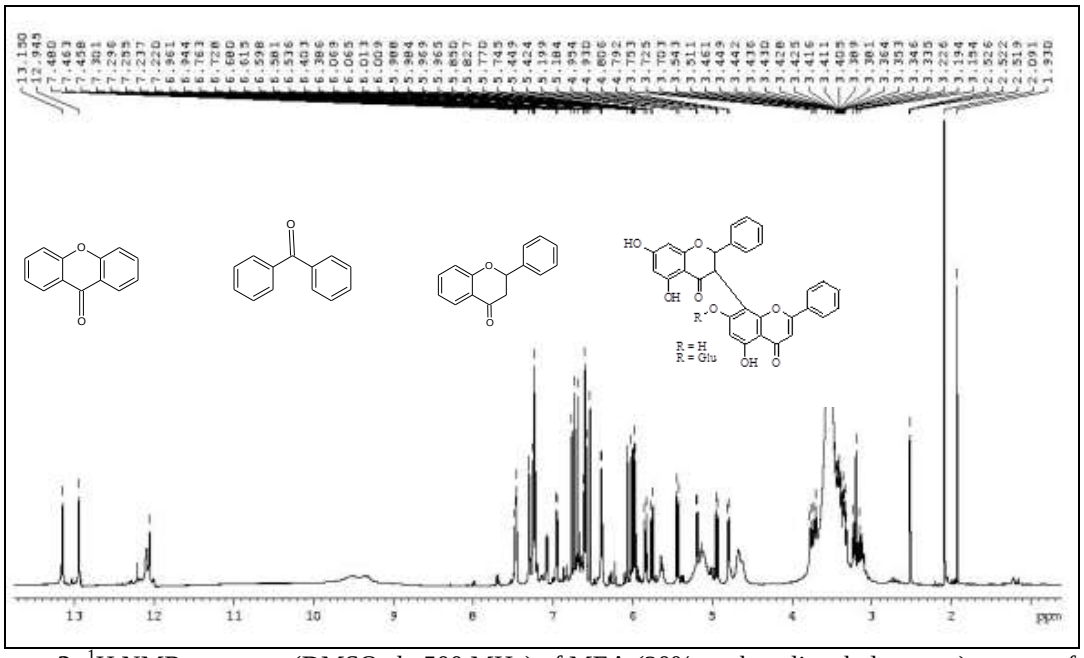

Figure 3: ^{1}H NMR spectrum (DMSO-d_6, 500 MHz) of MEA (20% methanolic ethyl acetate) extract of *G. hombroniana*

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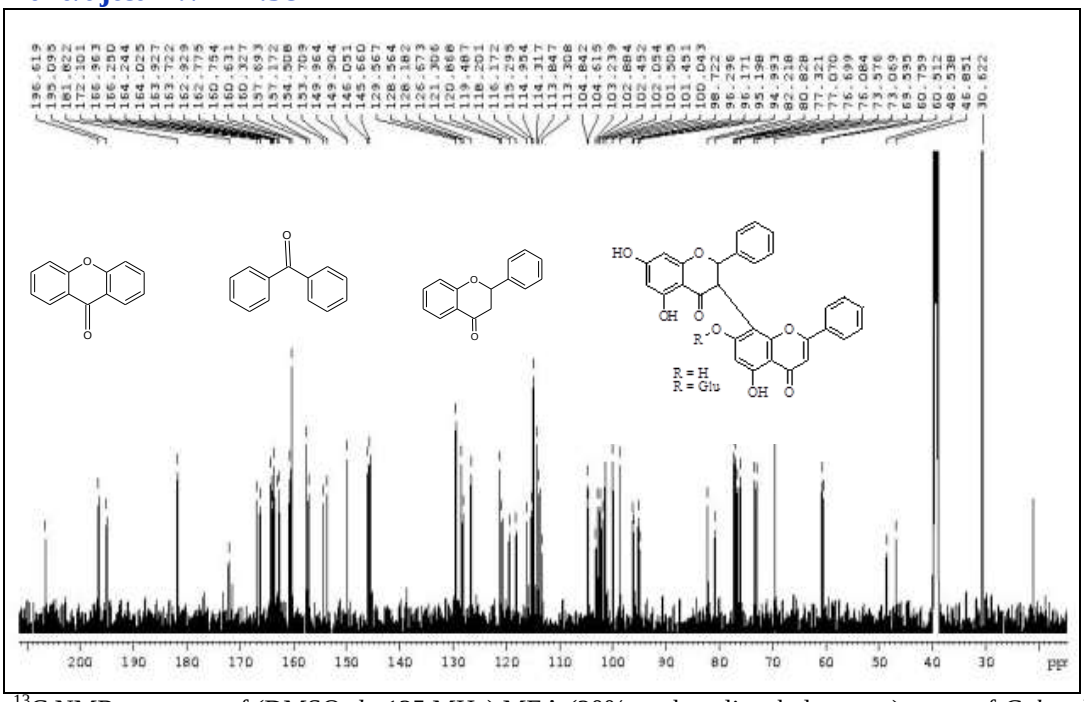

Figure 4: ^{13}C NMR spectrum of (DMSO-d_6, 125 MHz) MEA (20% methanolic ethyl acetate) extract f *G. hombroniana*

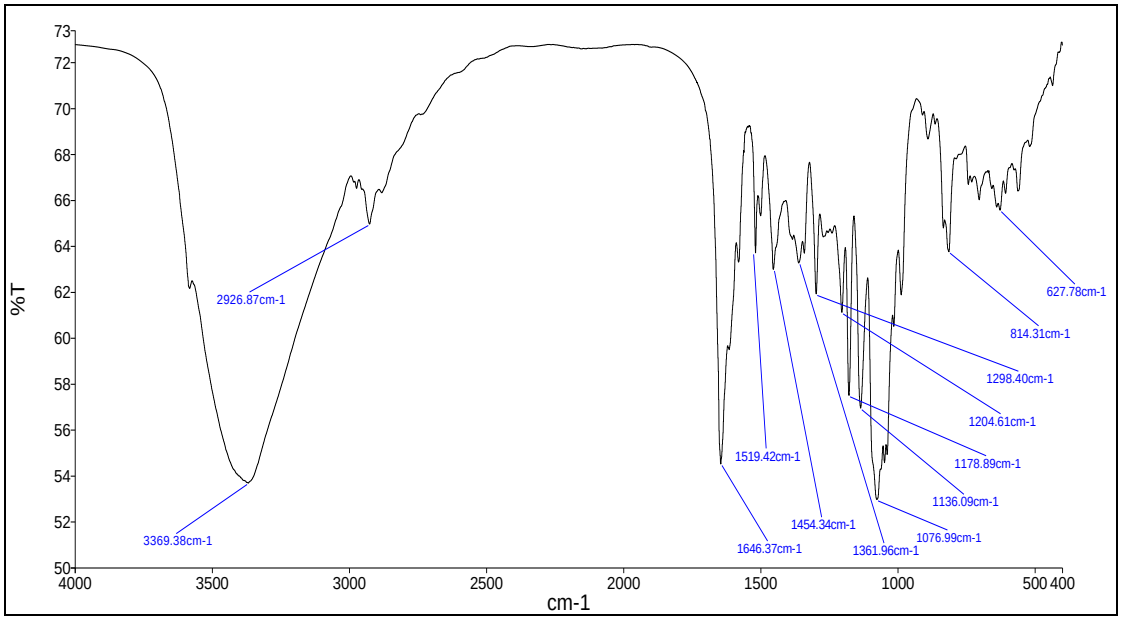

Figure 5:IR spectrum of MEA (20% methanolic ethyl acetate) extract of *G. hombroniana*

Conclusions

MEA bark extract of *G. hombroniana* offers a good protection against CCl_4-induced liver toxicity and cytotoxic activity against MCF-7 (human breast cancer) and DBTRG (human glioblastoma) cell lines. From the results of hepatoprotective activity obtained in this study, it can be conclusively say that the MEA extract of *G. hombroniana* at a concentration level of 500 mg/kg significantly attenuated the increased activity of the blood enzymes. Furthermore, the ^{1}H and ^{13}C NMR, and IR spectra of the analyzed extract showed characteristic signals of phenolic compounds to which the hepatoprotective and cytotoxic activities might be linked.

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