Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/jff

Cytotoxic, caspase-3 induction and *in vivo* hepatoprotective effects of phyllanthin, a major constituent of *Phyllanthus niruri*

Kheng Leong Ooi [a], *Suh In Loh* [a], *Munavvar Abdul Sattar* [b], *Tengku Sifzizul Tengku Muhammad* [c], *Shaida Fariza Sulaiman* [a,*]

[a] *School of Biological Sciences, Universiti Sains Malaysia, 11800 USM, Pulau Pinang, Malaysia*
[b] *School of Pharmaceutical Sciences, Universiti Sains Malaysia, 11800 USM, Pulau Pinang, Malaysia*
[c] *Institute of Marine Biotechnology, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Terengganu, Malaysia*

ARTICLE INFO

Article history:
Received 25 June 2014
Received in revised form 13 January 2015
Accepted 22 January 2015
Available online 16 February 2015

Keywords:
Phyllanthin
Phyllanthus niruri
Antiproliferation
HepG2
Apoptosis
Hepatoprotective effect

ABSTRACT

The antiproliferative activity of phyllanthin (the major constituent in the decoction of *Phyllanthus niruri* L.) against human liver carcinoma HepG2 cells, the mechanism of cell death and the protective effect against carbon tetrachloride (CCl_4)-induced hepatotoxicity in mice were investigated. Phyllanthin showed dose- and time-dependent growth inhibitions of HepG2 cells with the lowest EC_{50} value (10.16 ± 0.21 μg/ml) at 72 hours of treatment. Acute exposure to this compound exerted a significant caspase-3 expression that reached its peak after 8 hours of treatment ($P < 0.001$). Oral pre-treatment of phyllanthin at a dose of 120 mg/kg prior to CCl_4-treatment effectively prevented the hepatic damage by reducing alanine aminotransferase (ALT) and aspartate aminotransferase (AST). These are the indications that phyllanthin is able to act independently to inhibit HepG2 cell proliferation *via* apoptotic cell death and to protect from hepatic injuries. Therefore, the effects of *P. niruri* extracts in the treatment of liver diseases can be related to the presence of phyllanthin.

1. Introduction

Liver diseases are commonly caused by viral infections, drug overdose, reactions to prescribed drugs and medications, prolonged or excessive exposure to environmental toxins, and long term effects of alcohol consumption (Kew, 2010; Kim, Hattori, & Phongsamran, 2010; Qua & Goh, 2011). Chronic liver diseases may also lead to the development of cancers (McClune & Tong, 2010). Although extensive studies have been conducted to understand the pathogenesis of these diseases, their effective therapies are still lacking (Song et al., 2006). Thus, these diseases should be prevented by avoiding the risk factors, maintaining a healthy balanced diet (that includes foods to strengthen the liver and improve its functions), and considering functional food supplements with hepatoprotective activity.

The decoction of *Phyllanthus niruri* L. (Euphorbiaceae; Synonym: *Phyllanthus amarus* Schumach. & Thonn.) was clinically proven as a functional food to protect the liver (Patel, Patel,

* *Corresponding author.* School of Biological Sciences, Universiti Sains Malaysia, 11800 USM, Pulau Pinang, Malaysia. Tel.: +60 4 6534095; fax: +60 4 6565125.
E-mail address: shaida@usm.my (S.F. Sulaiman).
http://dx.doi.org/10.1016/j.jff.2015.01.032
1756-4646/

Patel, Seth, & Patel, 2010). It is also consumed to cleanse and detoxify the liver, and to treat liver disorders such as hepatitis and hepatocarcinoma. All these bioactivities have been proven scientifically (Patel, Tripathi, Sharma, Chauhan, & Dixit, 2011). The hepatoprotective activity was found to be associated with the major content of phyllanthin that may act synergistically with other compounds in the decoction (Krithika, Verma, Shrivastav, & Suguna, 2011). To the best of our knowledge, there were only three earlier studies that had determined the anti-hepatotoxic activity of phyllanthin. Krithika et al. (2009) and Syamasundar et al. (1985) had reported the *in vitro* activities on CCl_4-induced toxicity in human liver carcinoma HepG2 cells, and on CCl_4- and galactosamine-induced toxicity in primary cultured rat hepatocytes, respectively. In addition, a study by Krithika and Verma (2014) recently reported the *in vivo* activity on CCl_4-induced hepatic damage in female mice (*Mus musculus*). However, not all compounds or extracts with anti-hepatotoxic activity may also have hepatoprotective activity. Thus far, there has only been one report on the hepatoprotective activity of phyllanthin that was determined using an *in vitro* system on ethanol-induced toxicity in rat hepatocytes (Chirdchupunseree & Pramyothin, 2010). Therefore, this study is the first to determine the *in vivo* hepatoprotective activity of phyllanthin.

Among the compounds isolated from foods that were reported to have hepatoprotective activity are lycopene from *Solanum lycopersicum* (tomato) fruit (Pinto, Rodrıguez-Galdón, Cestero, & Macıas, 2013) and hesperidin from *Citrus sinensis* (sweet orange) peel (Chen et al., 2013). Lycopene and hesperidin were also reported to reduce proliferation (Burgess et al., 2008), and to induce paraptosis-like cell death (Yumnam et al., 2014) of HepG2 cells, respectively. To date, the antiproliferative activity of phyllanthin was only reported by Islam, Selvan, Mazumder, Gupta, and Ghosal (2008) against Ehrlich ascites carcinoma in Swiss albino mice.

The induction of apoptosis has been well recognized as a major hindrance to cancer growth and metastasis that involves the elimination of cancer cells without injuring non-cancerous cells. The apoptosis mechanism is mainly regulated by the intracellular protease, especially caspase-3. Caspase-3 was identified as the main executor for morphological and biochemical alterations of apoptotic cells in both extrinsic (death receptor) and intrinsic (mitochondrial) apoptosis signaling pathways (Olsson & Zhivotovsky, 2011). The deficiency of this gene was also found to cause chemotherapeutic resistance in human cancer cells (Rebucci & Michiels, 2013). Hence, targeting only caspase-3 (the terminal protease in the apoptotic cascade) and not other upstream regulators of apoptosis could lead to a more effective therapy of cancers (MacKenzie, Schipper, & Clark, 2010). Many studies have revealed the potential of *P. niruri* extracts to induce caspase-3 in human breast (MCF-7), lung (A549) and liver (HepG2 and Huh-7) carcinoma cells (de Araújo Júnior et al., 2012; Lee, Jaganath, Wang, & Sekaran, 2011). However, no report on the involvement of phyllanthin in inducing caspase-3 is available.

This study was carried out (i) to assess the antiproliferative activity and induction of caspase-3 by phyllanthin on HepG2 cells, and (ii) to determine the hepatoprotective activity of the said compound against CCl_4-induced hepatotoxicity in mice.

2. Materials and methods

2.1. Chemicals

Dimethylsulfoxide (DMSO), ethidium bromide, methylene blue, Tween 80 and vincristine sulfate were obtained from Sigma Aldrich, St. Louis, MO, USA. All culture media and additives (such as Earle's balanced salt solution (EBSS), Eagle's minimum essential medium (MEM), fetal calf serum (FCS), L-glutamine, penicillin, and streptomycin) were purchased from GE Healthcare HyClone, South Logan, UT, USA. Agarose gel, glutaraldehyde, phosphate-buffered saline (PBS) and sodium chloride (NaCl) were obtained from Amresco, Solon, OH, USA. Carbon tetrachloride (CCl_4) and silymarin were purchased from Merck, Darmstadt, Germany and Simepar, Acino, Switzerland, respectively. Assay kits for serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were purchased from DiaSys Diagnostic Systems GmbH, Holzheim, Germany. Acetic acid, acetone, acetonitrile, chloroform, ethanol, ethyl acetate, hydrochloric acid (HCl), *n*-hexane, methanol, and sulfuric acid were purchased from Fisher Scientific, Selangor, Malaysia.

2.2. Extraction, fractionation and isolation of phyllanthin

The leaves of *P. niruri* (1 kg) were dried to a constant weight at 60 °C in a vacuum oven (Memmert, Frankfurt, Germany). The dried sample was ground for 1 min using a grinder to yield a fine powder (80 mesh of particle size). The sample was extracted using chloroform in a Soxhlet apparatus. The dried extract was separated by silica gel column chromatography (60–120 mesh) using a gradient of *n*-hexane and ethyl acetate (from ratio 9:1 (v/v) to 1:9 (v/v)) as described by Tripathi, Verma, Gupta, Gupta, and Khanuja (2006). Phyllanthin was purified by crystallization (3 times) in petroleum ether (Fig. 1). The identification of phyllanthin was further confirmed by ultra violet (UV), infra red (IR) and ultra performance liquid chromatography (UPLC) analyses in comparison with the standard of phyllanthin (ChromaDex, Irvine, CA, USA). The UV and IR spectra were detected by a CARY 50 Conc-UV visible spectrometer (Santa Clara, CA, USA) and a Perkin Elmer system 2000 FT-IR spectrometer with KBr discs (Waltham, MA, USA), respectively. The UPLC analysis was carried out using the method described by Murugaiyah and Chan (2007) with some modifications using an Acquity UPLC system (Waters, Milford, MA, USA). The compound was dissolved in 99.9% (v/v) DMSO to prepare a stock solution at a concentration of 10 mg/ml. The

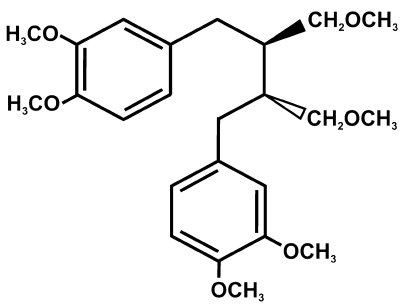

Fig. 1 – Chemical structure of phyllanthin.

stock solution was serially diluted to eight different concentrations (10–0.078 mg/ml) for the antiproliferative test.

2.3. Cell culture

The HepG2 (ATCC, HB-8065) cells were obtained from American Type Cell Culture (ATCC) and grown in MEM/EBSS medium.

2.4. Antiproliferative analysis

The methylene blue assay was carried out as previously described (Ooi, Muhammad, Lam, & Sulaiman, 2014). Briefly, 100 µl of cells were plated at a density of approximately 6000 cells/well into a 96-well plate. The cells were treated with different concentrations of phyllanthin by adding 1 µl of the serially diluted compound into each well. Positive control cells were treated with vincristine sulfate and negative control cells were cultured in 0.5% (v/v) FCS-containing medium alone. The final concentration of DMSO in all wells was kept less than 1% (v/v). After 24, 48 and 72 hours of incubation with the compound, the surviving cells were fixed with 2.5% (v/v) glutaraldehyde for 15 min. One hundred microliters of 0.05% (w/v) methylene blue solution was added to stain the fixed cells for 15 min. The absorbance was read at 650 nm using a Vmax Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). The percentage of growth inhibition was calculated according to the following equation:

$$\text{Growth inhibition (\%)} = ((\text{absorbance of negative control} - \text{absorbance of sample})/\text{absorbance of negative control}) \times 100\%.$$

The EC_{50} values for growth inhibition were derived from a nonlinear model (curvefit) based on a sigmoidal dose–response curve (variable) and computed using GraphPadPrism, San Diego, CA, USA. All experiments were performed in six replicates.

2.5. Determination of caspase-3 gene expression

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed as previously described by Ooi et al. (2014) to determine the caspase-3 expression level. The cells were then treated at different incubation periods with phyllanthin using the concentration of EC_{50} at 72 hours (10.16 ± 0.21 µg/ml). After the treatment, total cellular RNA was isolated using Tri-Reagent LS according to the manufacturer's instructions (Molecular Research Center, Cincinnati, OH, USA). The amplification products were separated on 1.2% (w/v) agarose gel and stained with ethidium bromide. Gene expressions signaling at each time interval were quantified using GeneTools analysis software on a GENEGENIUS gel documentation system (Syngene, Cambridge, UK). The signals of caspase-3 were normalized against β-actin and the ratio in untreated cells (negative control) was assigned as 1.

2.6. Experimental animals

Male *Mus domesticus* (21–32 g) were obtained from the Animal House Facility, Universiti Sains Malaysia and used throughout the study. They were housed in a 12 hours light–dark cycle at 25 ± 2 °C with a relative humidity of 30–60%, and were fed standard mice pellets and water *ad libitum*. The animals were allowed to acclimatize to laboratory conditions for 7 days before the commencement of experiments. The experiments were conducted in accordance with the Animal Ethics Committee Guidelines of Universiti Sains Malaysia (Protocol acceptance number: USM/PPSF/50(091)JLD2).

2.7. Hepatoprotective analysis

For this analysis, the compound was reconstituted with Tween-80. In order to ensure that there are no toxic effects of the compound, the doses used in this study were selected according to our preliminary toxicity evaluation. The mice were randomly divided into six groups consisting of six animals in each group. Group I received Tween-80 once a day orally for 6 days and served as the normal control. Group II received CCl_4 at a dose of 12.8 µl/kg body weight (b.wt.) by gastric gavages only on days 5 and 6 to induce hepatotoxicity. Mice in Groups III–V were pre-treated with phyllanthin for 4 days, at doses of 60, 120, 180 mg/kg b.wt., respectively. On days 5 and 6, the mice were still treated with phyllanthin and after an hour of treatment, they were administrated with CCl_4 (12.8 µl/kg b.wt.). Group VI (positive control) received silymarin (35 mg/kg b.wt.) for 6 days and were administrated with the CCl_4 on days 5 and 6. The mice were anesthetized with diethyl ether, 24 hours after the last oral dose of each experiment. Blood and liver samples were collected for biochemical and histopathological examinations. The serum was separated by centrifugation at 1000 rpm for 5 min and used for the measurement of biochemical markers namely, ALT and AST activities using commercially available assay kits (DiaSys Diagnostic Systems GmbH, Holzheim, Germany). The absorbance of reaction was determined at 340 nm. For the histopathological studies, the livers were quickly removed after autopsy, blotted with filter paper, weighed and fixed in 10% neutral buffered formalin. The paraffin sections were prepared and stained with hematoxylin and eosin (H&E) dye and examined using light microscopy (Olympus BH2, Shinjuku-ku, Tokyo, Japan).

2.8. Statistical analysis

The results were expressed as mean ± standard deviation (SD) using GraphPadPrism. Analysis of variance was determined by one-way ANOVA (GraphPadPrism). Significant differences between the means were calculated according to the Duncan's multiple range test. Differences at $P < 0.05$ and $P < 0.001$ were considered statistically significant.

3. Results

3.1. Antiproliferative effects of phyllanthin against HepG2 cells

In the present study, the antiproliferative effects of the phyllanthin against HepG2 cells were evaluated at incubation periods of 24, 48 and 72 hours. As indicated in Fig. 2, more

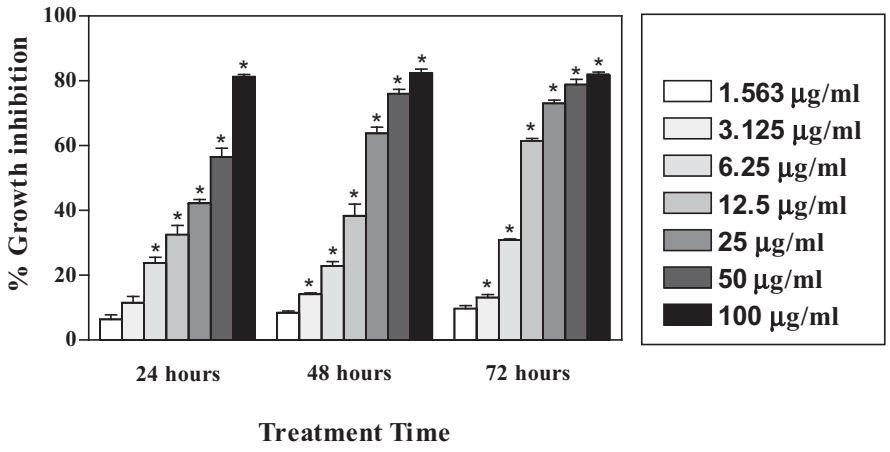

Fig. 2 – Effects of phyllanthin on the proliferation of HepG2 cells at 24, 48 and 72 hours of incubation. Each value represents the mean ± SD of six replicates (n = 6). *P < 0.001 (compared to control).

than 55% of the growth inhibition of HepG2 cells was observed at higher concentrations (50–100 µg/ml) for all the incubation periods. A time dependent inhibition of HepG2 cells was also observed. The range of concentrations with significant growth inhibition was increased from 6.25–100 µg/ml at 24 hours to 3.125–100 µg/ml at 48 and 72 hours ($P < 0.05$). Thus, the EC_{50} values were decreased as the incubation periods were increased from 24 (39.53 ± 6.47 µg/ml) to 48 (18.04 ± 2.44 µg/ml) and 72 (10.16 ± 0.21 µg/ml) hours (Table 1). The lowest EC_{50} value was obtained after 72 hours of treatment. Thus, this concentration was used to determine the mechanism of cell death.

3.2. *Induction of caspase-3 gene expression in HepG2 cells by phyllanthin*

The semi-quantitative RT-PCR showed that the mRNA expression of caspase-3 in HepG2 cells was significantly induced as early as 4 hours of treatment (nearly 6.0-fold increase) ($P < 0.001$) (Fig. 3) with phyllanthin using the concentration of EC_{50} at 72 hours (10.16 ± 0.21 µg/ml). The gene expression was then elevated continuously and reached its peak after 8 hours of treatment (approximately 7.0-fold increase). This was followed by a gradual decrease at 12 hours (4.0-fold decrease) and further reduction in the next 4 hours of post stimulation. It declined drastically to the level below the baseline at 24 hours.

Table 1 – EC_{50} values of phyllanthin against HepG2 cells at different incubation periods.

Incubation periods	EC_{50} (µg/ml)
24 hours	39.53 ± 6.47
48 hours	18.04 ± 2.44
72 hours	10.16 ± 0.21
Vincristine sulfate	0.14 ± 0.006

Each value represents the mean ± SD of six replicates (n = 6).

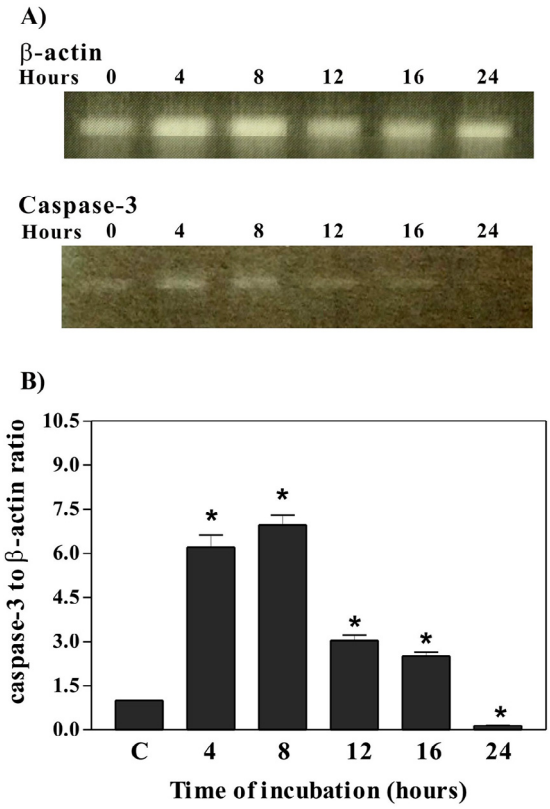

Fig. 3 – (A) mRNA expression of β-actin and caspase-3 in HepG2 cells treated with phyllanthin (10.16 ± 0.21 µg/ml) at different times of incubation. β-actin was used as an internal standard control for each PCR reaction. (B) Semi-quantitative analysis of the caspase-3 mRNA levels in HepG2 treated with phyllanthin using densitometric scanning. Each value represents the mean ± SD (at each point) of the ratio of the RT-PCR product of the respective genes to β-actin, assigning the ratio in unstimulated cells as 1. *P < 0.001 (compared to control).

When compared with control cells, the caspase-3 expression was proven to be significantly different from 4 to 24 hours post-stimulation ($P < 0.05$). Therefore, the results clearly proposed that the mechanism of cell death elicited by the phyllanthin was mediated *via* the activation of caspase-3.

3.3. *Hepatoprotective effects of phyllanthin on CCl_4 intoxicated mice*

As depicted in Table 2, the serum levels of ALT and AST, which are used commonly as biochemical markers for the evaluation of early hepatic injury, were elevated at 210.35 and 332.59 U/l, respectively in the CCl_4-treated mice. Meanwhile, the administration of phyllanthin at doses of 120 and 180 mg/kg effectively attenuated (at least 2-fold reduction) the CCl_4-induced elevation of serums ALT and AST ($P < 0.05$). The decreasing levels of serums were found to be ranging between 74–83 and 62–63%, respectively. These hepatoprotective effects were comparable to the standard drug, silymarin, which shows nearly 66% decrease of the serum levels. This clearly revealed the effectiveness of phyllanthin to limit the leakage of the ALT and AST enzymes.

Histopathological examinations further confirmed the protective properties of phyllanthin and provided the visual evidence for its hepatoprotective efficacy. Histology of the liver sections from the normal group showed typical hepatic architecture with well-preserved cytoplasm, prominent nucleus and radiately arranged hepatocytes around the central vein (Fig. 4A). In contrast, the CCl_4 treated liver sections showed an excessive parenchymal disarrangement, such as ballooning degeneration, infiltration of inflammatory cells and loss of cellular boundaries (Fig. 4B). The hepatic lesions were markedly prevented by pre-treatment with phyllanthin at 120 mg/kg (Fig. 4D). It displayed a more or less normal lobular pattern with a mild degree of necrotic zones, lymphocyte infiltration, hepatocellular degeneration and is almost comparable to the normal control and silymarin treated (Fig. 4C) groups. This clearly indicated that the compound affords a comparable protective effect with its standard reference, silymarin in the CCl_4-mediated hepatic injury.

Table 2 – Effects of the phyllanthin and silymarin on alanine transaminase (ALT) and aspartate transaminase (AST) levels in CCl_4-intoxicated mice.

Group	ALT (1 U/l)	AST (1 U/l)
Group I, normal	16.16 ± 2.59	54.65 ± 1.42
Group II, CCl_4 treated	244.24 ± 59.01[a]	565.21 ± 136.51[a]
Group III, pre-treatment phyllanthin 60 mg/kg + CCl_4	199.69 ± 99.70[a]	612.85 ± 108.41[a]
Group IV, pre-treatment phyllanthin 120 mg/kg + CCl_4	62.26 ± 5.52[b]	92.45 ± 7.85[b]
Group V, pre-treatment phyllanthin 180 mg/kg + CCl_4	90.24 ± 32.16[b]	210.31 ± 40.50[b]
Group VI, silymarin 35 mg/kg + CCl_4	81.49 ± 15.44[b]	188.98 ± 21.96[b]

Each value represents the mean ± SD of six replicates (n = 6).
[a] $P < 0.05$ vs normal control.
[b] $P < 0.05$ vs CCl_4 control.

4. Discussion

Even though phyllanthin is considered to be the main contributor to the hepatoprotective property of *P. niruri*, its abilities to trigger the apoptosis-based growth inhibition on HepG2 carcinoma and *in vivo* hepatoprotective activity of CCl_4-intoxicated mice were unveiled for the first time in this study. Both of these bioactivities are important mechanisms involved in the treatment and prevention of liver disorders. The isolated phyllanthin was subjected to the methylene blue assay for the antiproliferative evaluation, and RT-PCR analysis to determine the mRNA expression level of the apoptotic gene. The hepatic enzymes were measured and histopathological analyses were performed to investigate the hepatoprotective activity.

Phyllanthin was found to possess a concentration- and time-dependent inhibition of HepG2 cells in the preliminary antiproliferative evaluation. This compound displayed a marginal inhibitory reaction at the early stages of treatment (24 hours) and the activity gradually increased when the incubation time was increased to 48 and 72 hours, which reduced nearly 2.0-fold and 4.0-fold, respectively of EC_{50} values than that of 24 hours (Table 1). This clearly indicated that a lower dose of the compound is adequate to abrogate 50% of the HepG2 cell growth when a longer period of exposure is applied. This might be due to the cumulative cytotoxic response of the compound toward HepG2 cells. The antiproliferative potential of this compound is mainly due to the occurrence of the methoxy moieties in the chemical structure, which has been associated with the anticancer activities against various human carcinoma cells (Orfila et al., 2000). Somanabandhu et al. (1993) speculated that phyllanthin acts as a cytotoxic enhancer in vinblastine-mediated multidrug resistant KB oral carcinoma cells through the displacement of vinblastine bound to the cell membrane vesicles, probably by the interaction with P-glycoprotein.

The concentrations used in this study are in fact higher than that of Krithika et al. (2009) against the same cell lines. This means that phyllanthin at lower concentrations can act as an anti-hepatotoxin and at higher concentrations can act as a cytotoxic compound. Other compounds from foods were also found to have these properties in the HepG2 cells. For example, lycopene, at lower concentrations was reported to possess antigenotoxicity and antimutagenicity (Scolastici et al., 2008), and to attenuate arachidonic acid toxicity (Xu, Leo, & Lieber, 2003a) and alcoholic apoptosis (Xu, Leo, & Lieber, 2003b), whereas at higher concentrations, it was found to reduce the cell proliferation (Burgess et al., 2008).

Different mRNA expressions of caspase-3 were observed in HepG2 cells treated with phyllanthin for 24 hours. The peak expression of this gene was significantly observed at 8 hours ($P < 0.05$), indicating that caspase-3 was highly induced at the early phase of the treatment with this compound. This might lead to the amplifying proteolytic cascade of caspases and triggering of the apoptotic morphological alterations and the DNA fragmentation (Salvesen & Riedl, 2008). The sustainability of this gene expression until the late treatment periods could be due to its involvement in the late phase of apoptotic pathway as a downstream stimulator, which is activated after a series of upstream cellular processes during apoptosis (Chowdhury, Tharakan, & Bhat, 2008). In agreement with our findings, the

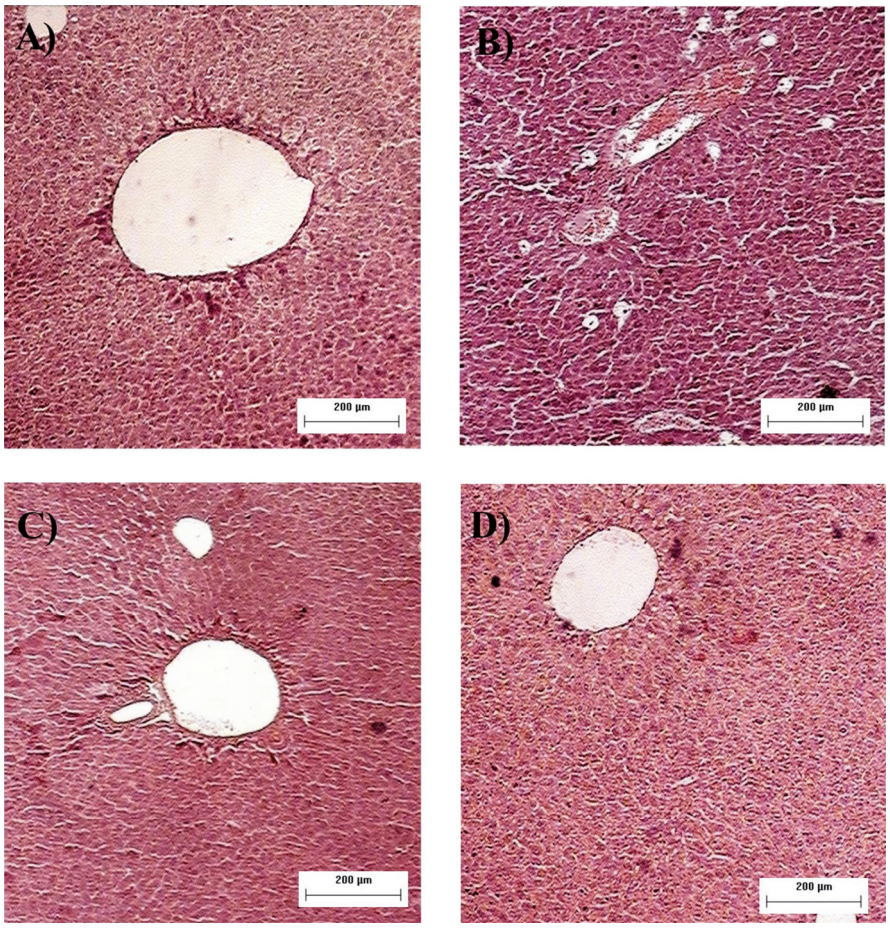

Fig. 4 – Histopathological analyses of CCl_4-induced hepatotoxicity in mice livers. Liver tissue was stained with hematoxylin and eosin: (A) Group I, normal mice liver, (B) Group II, liver after CCl_4 treatment, (C) Group III, liver pre-treated with silymarin 35 mg/kg + CCl_4, (D) Group IV, liver pre-treated with phyllanthin 120 mg/kg + CCl_4.

marked caspase-3 activities in human liver carcinoma cells (HepG2 and Huh-7) were also induced by the spray-dried extract of *P. niruri* (de Araújo Júnior et al., 2012). It is worth noting that the apoptosis triggering potential of phyllanthin might be ascribed to its main dibenzylbutane skeleton, which has been pointed out to evade caspase-3 activity and DNA fragmentation in Huh-7 cells (Mansoor, Ramalho, Rodrigues, & Ferreira, 2012). These findings provide strong evidence to support the role of this compound as the main apoptosis inducer of *P. niruri* extracts in inhibiting the growth of human liver carcinoma cells.

The results obtained from our *in vivo* hepatoprotective study also help to further verify the earlier *in vitro* study of the hepatoprotective potential of phyllanthin (Chirdchupunseree & Pramyothin, 2010). CCl_4 has been extensively highlighted as a hepatotoxicant and its metabolites such as trichloromethyl radicals are reported to be involved in the pathogenesis of liver damage by disrupting the structure and function of lipid and protein macromolecule in the cell membrane (Clawson, 1989). This might lead to hepatic lipid peroxidation and eventually result in the release of ALT and AST enzymes. The abnormally high levels of these intracellular enzymes that were observed in the present study (Table 2) were the consequences of CCl_4 induced liver dysfunction, indicating that these enzymes leaked out from the liver into the circulatory system at the instance of tissue damage.

Meanwhile, treatment with phyllanthin especially at a dose of 120 mg/kg exhibited a significant restoration of the altered biochemical parameters in the CCl_4-intoxicated mice ($P < 0.05$). Lower ALT and AST levels than that of silymarin, a standard hepatoprotective reference, were measured. It is strongly proposed that the compound affords better protection against CCl_4. This hepatic protective effect was also reflected by the histopathological examination. The severe hepatic impairments induced by CCl_4 were effectively attenuated to normal by pretreatment with this compound, which was comparable to that of the silymarin-treated group.

This implies that the compound could increase the stabilization of plasma membrane and effectively alleviate biliary dysfunction thereby preserving the structural integrity of cells as well as the repair of CCl_4-induced liver damage. The results from this study also provide further corroboration of the previous studies that have reported the *in vivo* anti-hepatotoxic activity of phyllanthin and phyllanthin-standardized ethanolic extracts of *P. niruri* (Krithika & Verma, 2014; Krithika et al., 2011). According to Krithika et al. (2009), the antioxidative property of this compound was important in scavenging CCl_4-induced free radicals and protecting the hepatic cell membrane from destruction. It was reported that the methoxy groups of the compound might enhance the hydrogen donating capacities and radical scavenging activities (de Pinedo, Peñalver, & Morales,

2007), suggesting that these moieties indirectly assist in neutralizing and ameliorating the radical injury of intoxicated hepatic cells.

5. Conclusions

Our study revealed the therapeutic potential of phyllanthin for treating hepatic-related diseases by inhibiting HepG2 cell proliferation, inducing apoptosis in HepG2 *via* caspase-3-dependent cell death mechanism and protecting against CCl_4-induced hepatotoxicity. The marked hepatoprotective activity of this compound is also suggested to be associated with the activation of caspase-3 gene, which plays a vital role in modulating and preventing excessive or deregulated apoptosis-caused chronic liver injuries.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

We would like to acknowledge FELDA Foundation, Malaysia (research grant: 304. PBIOLOGI. 650213. Y104) and Universiti Sains Malaysia (research university grant: 1001/PBIOLOGI/813049), for financing the project and providing the laboratory facilities.

REFERENCES

Burgess, L. C., Rice, E., Fischer, T., Seekins, J. R., Burgess, T. P., Skicka, S. J., & Klatt, K. (2008). Lycopene has limited effect on cell proliferation in only two of seven human cell lines (both cancerous and noncancerous) in an *in vitro* system with doses across the physiological range. *Toxicology in Vitro: An International Journal Published in Association With BIBRA*, *22*, 1297–1300.

Chen, S.-Y., Chyau, C.-C., Chu, C.-C., Chen, Y.-H., Chen, T.-H., & Duh, P.-D. (2013). Hepatoprotection using sweet orange peel and its bioactive compound, hesperidin, for CCl_4-induced liver injury *in vivo*. *Journal of Functional Foods*, 5, 1591–1600.

Chirdchupunseree, H., & Pramyothin, P. (2010). Protective activity of phyllanthin in ethanol treated primary culture of rat hepatocytes. *Journal of Ethnopharmacology*, *128*, 172–176.

Chowdhury, I., Tharakan, B., & Bhat, G. K. (2008). Caspases – An update. *Comparative Biochemistry and Physiology. Part B, Biochemistry and Molecular Biology*, *151*, 10–27.

Clawson, G. A. (1989). Mechanisms of carbon tetrachloride hepatotoxicity. *Pathology and Immunopathology Research*, *8*, 104–112.

de Araújo Júnior, R. F., de Souza, T. P., Pires, J. G., Soares, L. A., de Araújo, A. A., Petrovick, P. R., Mâcedo, H. D., de Sá Leitão Oliveira, A. L., & Guerra, G. C. (2012). A dry extract of *Phyllanthus niruri* protects normal cells and induces apoptosis in human liver carcinoma cells. *Experimental Biology and Medicine*, *237*, 1281–1288.

de Pinedo, A. T., Peñalver, P., & Morales, J. C. (2007). Synthesis and evaluation of new phenolic-based antioxidants: Structure–activity relationship. *Food Chemistry*, *103*, 55–61.

Islam, A., Selvan, T., Mazumder, U. K., Gupta, M., & Ghosal, S. (2008). Antitumour effect of phyllanthin and hypophyllanthin from *Phyllanthus amarus* against Ehrlich ascites carcinoma in mice. *Pharmacology Online*, *2*, 796–807.

Kew, M. C. (2010). Epidemiology of chronic hepatitis B virus infection, hepatocellular carcinoma, and hepatitis B virus-induced hepatocellular carcinoma. *Pathologie-Biologie*, *58*, 273–277.

Kim, J. W., Hattori, A., & Phongsamran, P. V. (2010). Drug-induced liver disease. *Critical Care Nursing Clinics of North America*, *22*, 323–334.

Krithika, R., Mohankumar, R., Verma, R. J., Shrivastav, P. S., Mohamad, I. L., Gunasekaran, P., & Narasimhan, S. (2009). Isolation, characterization and antioxidative effect of phyllanthin against CCl_4-induced toxicity in HepG2 cell line. *Chemico-Biological Interactions*, *181*, 351–358.

Krithika, R., & Verma, R. J. (2014). Ameliorative effects of phyllanthin on carbon tetrachloride-induced hepatic oxidative damage in mice. *Asian Pacific Journal of Tropical Disease*, *4*, S64–S70.

Krithika, R., Verma, R. J., Shrivastav, P. S., & Suguna, L. (2011). Phyllanthin of standardised *Phyllanthus amarus* extract attenuates liver oxidative stress in mice and exerts cytoprotective activity on human hepatoma cell line. *Journal of Clinical and Experimental Hepatology*, *1*, 57–67.

Lee, S. H., Jaganath, I. B., Wang, S. M., & Sekaran, S. D. (2011). Antimetastatic effects of *Phyllanthus* on human lung (A549) and breast (MCF-7) cancer cell lines. *PLoS ONE*, *6*, e20994.

MacKenzie, S. H., Schipper, J. L., & Clark, A. C. (2010). The potential for caspases in drug discovery. *Current Opinion in Drug Discovery and Development*, *13*, 568–576.

Mansoor, T. A., Ramalho, R. M., Rodrigues, C. M. P., & Ferreira, M.-J. U. (2012). Dibenzylbutane- and butyrolactone-type lignans as apoptosis inducers in human hepatoma HuH-7 cells. *Phytotherapy Research*, *26*, 692–696.

McClune, A. C., & Tong, M. J. (2010). Chronic hepatitis B and hepatocellular carcinoma. *Clinics in Liver Disease*, *14*, 461–476.

Murugaiyah, V., & Chan, K.-L. (2007). Determination of four lignans in *Phyllanthus niruri* L. by a simple high-performance liquid chromatography method with fluorescence detection. *Journal of Chromatography. A*, *1154*, 198–204.

Olsson, M., & Zhivotovsky, B. (2011). Caspases and cancer. *Cell Death and Differentiation*, *18*, 1441–1449.

Ooi, K. L., Muhammad, T. S. T., Lam, L. Y., & Sulaiman, S. F. (2014). Cytotoxic and apoptotic effects of ethyl acetate extract of *Elephantopus mollis* Kunth. in human liver carcinoma Hep G2 through caspase-3 activation. *Integrative Cancer Therapies*, *13*, NP1–NP9.

Orfila, L., Rodrıguez, M., Colman, T., Hasegawa, M., Merentes, E., & Arvelo, F. (2000). Structural modification of berberine alkaloids in relation to cytotoxic activity *in vitro*. *Journal of Ethnopharmacology*, *71*, 449–456.

Patel, H. J., Patel, J. S., Patel, K. N., Seth, A. K., & Patel, K. D. (2010). Clinical study of hepatoprotective drug *Phyllanthus amarus*. *Research Journal of Pharmaceutical, Biological and Chemical Sciences*, *1*, 335–339.

Patel, J. R., Tripathi, P., Sharma, V., Chauhan, N. S., & Dixit, V. K. (2011). *Phyllanthus amarus*: Ethnomedicinal uses, phytochemistry and pharmacology. *Journal of Ethnopharmacology*, *138*, 286–313.

Pinto, C., Rodrıguez-Galdón, B., Cestero, J. J., & Macıas, P. (2013). Hepatoprotective effects of lycopene against carbon tetrachloride-induced acute liver injury in rats. *Journal of Functional Foods*, 5, 1601–1610.

Qua, C. S., & Goh, K. L. (2011). Liver cirrhosis in Malaysia: Peculiar epidemiology in a multiracial Asian country. *Journal of Gastroenterology and Hepatology*, *26*, 1333–1337.

Rebucci, M., & Michiels, C. (2013). Molecular aspects of cancer cell resistance to chemotherapy. *Biochemical Pharmacology*, *85*, 1219–1226.

Salvesen, G. S., & Riedl, S. J. (2008). Caspase mechanisms. *Advances in Experimental Medicine and Biology*, *615*, 13–23.

Scolastici, C., Lima, R. O. A. D., Barbisan, L. F., Ferreira, A. L. A., Ribeiro, D. A., & Salvadori, D. M. F. (2008). Antigenotoxicity and antimutagenicity of lycopene in HepG2 cell line evaluated by the comet assay and micronucleus test. *Toxicology in Vitro: An International Journal Published in Association With BIBRA*, *22*, 510–514.

Somanabandhu, A., Nitayangkura, S., Mahidol, C., Ruchirawat, S., Likhitwitayawuid, K., Shieh, H. L., Chai, H., Pezzuto, J. M., & Cordell, G. A. (1993). [H.sup.1]-and [C-NMR.sup.14] assignments of phyllanthin and hypophyllanthin: Lignans that enhance cytotoxicity with cultured multidrug-resistant cells. *Journal of Natural Products*, *56*, 233–239.

Song, Z., Deaciuc, I., Song, M., Lee, D. Y., Liu, Y., Ji, X., & McClain, C. (2006). Silymarin protects against acute ethanol-induced hepatotoxicity in mice. *Alcoholism, Clinical and Experimental Research*, *30*, 407–413.

Syamasundar, K. V., Singh, B., Thakor, R. S., Husain, A., Kiso, Y., & Hikino, H. (1985). Antihepatotoxic principle of *Phyllanthus niruri* herbs. *Journal of Ethnopharmacology*, *14*, 41–44.

Tripathi, A. K., Verma, R. K., Gupta, A. K., Gupta, M. M., & Khanuja, S. P. S. (2006). Quantitative determination of phyllanthin and hypophyllanthin in *Phyllanthus* species by high-performance thin layer chromatography. *Phytochemical Analysis*, *17*, 394–397.

Xu, Y., Leo, M. A., & Lieber, C. S. (2003a). Lycopene attenuates arachidonic acid toxicity in HepG2 cells overexpressing CYP2E1. *Biochemical and Biophysical Research Communications*, *303*, 745–750.

Xu, Y., Leo, M. A., & Lieber, C. S. (2003b). Lycopene attenuates alcoholic apoptosis in HepG2 cells expressing CYP2E1. *Biochemical and Biophysical Research Communications*, *308*, 614–618.

Yumnam, S., Park, H. S., Kim, M. K., Nagappan, A., Hong, G. E., Lee, H. J., Lee, W. S., Kim, E. H., Cho, J. H., Shin, S. C., & Kim, G. S. (2014). Hesperidin induces paraptosis like cell death in hepatoblatoma, HepG2 cells: Involvement of ERK1/2 MAPK. *PLoS ONE*, *9*, e101321.