Vietnam Journal of Science and Technology ***60*** *(3) (2022) 410-423*

doi:10.15625/2525-2518/16110

ANTIOXIDANT, LIPID PEROXIDATION INHIBITORY ACTIVITIES, AND HEPATOPROTECTIVE EFFECT OF EXTRACTS OF *PHYLLANTHUS EMBLICA* L. FRUITS

Nguyen Manh Cuong[1,*], Pham Ngoc Khanh[1], Tran Thu Huong[1], Vu Thi Ha[1], Hoang Thi Ngoc Anh[1], Do Thi Thao[2], Nguyen Thi Cuc[2], To Dao Cuong[3,4]

[1]*Institute of Natural Products Chemistry, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Cau Giay District, Ha Noi, Viet Nam*

[2]*Institute of Biotechnology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Cau Giay District, Ha Noi, Viet Nam*

[3]*Phenikaa University Nano Institute (PHENA), Phenikaa University, Yen Nghia, Ha Dong District, Ha Noi, Viet Nam*

[4]*Phenikaa Research and Technology Institute (PRATI), A&A Green Phoenix Group JSC, 167 Hoang Ngan, Cau Giay District, Ha Noi, Viet Nam*

[*]Emails*: nmcuong@inpc.vast.vn*

Received: 3 June 2021; Accepted for publication: 29 July 2021

Abstract. *Phyllanthus emblica* L., commonly known by Vietnamese people as "Me rừng" or "Me mận", is widely distributed in Viet Nam and has been used in folk medicines for treatment of numerous ailments. The preparation of *P. emblica* fruit extracts in different solvent types have significant effects on extraction efficiency, phytochemical profile, and biological activity. This study aims to investigate the effects of six different solvents (methanol, ethyl acetate, water, 50 %, 70 %, and 96 % ethanol) used in the production of the corresponding extracts, denoted PE-M, PE-EA, PE-W, PE-50Et, PE-70Et, and PE-96Et, respectively) on phytochemical yield, *in vitro* antioxidant capacity, *ex vivo* lipid peroxidative inhibitory activity, and hepatoprotective potential of *P. emblica* fruits collected in Viet Nam. Our results showed that the PE-M and PE-96Et extracts of *P. emblica* fruits possessed the highest total phenolic content (321.6 and 287.9 GAE mg/g, respectively). However, because of its low toxicity, PE-96Et turned into the best potential candidate with DPPH antioxidant capacity (SC_{50} of 4.17 µg/mL), lipid peroxidative inhibitory activity via MDA assay (IC_{50} of 3.54 µg/mL), and hepatoprotective activity on CCl_4 intoxicated liver platform (PC_{50} of 56.6 µg/mL). The HPLC-MS profiles of the PE-M and the PE-96Et extracts showed the presence of mucic acid 3-*O*-gallate (**1**), mucic acid 2-*O*-gallate (**2**), mucic acid 1,4-lactone 3-*O*-gallate (**3**), glucogallin (**4**), mucic acid 1,4-lactone 2-*O*-gallate (**5**), gallic acid (**6**), mucic acid dimethyl ester 3-*O*-gallate (**7**), mucic acid 1,4-lactone 1-ethyl ester 2-*O*-gallate (**8**), and quercetin (**9**). The results indicated that the 96 % ethanol extract of *P. emblica* fruits is highly potential for further investigation and development of hepatoprotective products.

Keywords: Phyllanthus emblica, antioxidant, hepatoprotective, lipid peroxidation, phenolic acid.

Classification numbers: 1.1.3, 1.2.1, 1.3.1

1. INTRODUCTION

The genus *Phyllanthus* contains over 600 species distributed throughout the tropical and subtropical regions of the world. The plants of genus *Phyllanthus* have long been used to treat liver diseases [1].

Me rừng (*Phyllanthus emblica* L., syn. *P. taxifolius* D. Don, *Emblica officinalis* Gaertn., and *E. arborea* Raf.), belonging to the *Phyllanthus* of the family Phyllanthaceae (Euphorbiaceae) [2], is commonly known as aonla or Indian gooseberry in English, amla in Hindi, or Me rừng, Me mận, Chùm ruột núi, Mắc kham in Vietnamese [3]. Fruits of *P. emblica* are an important herbal drug used in fork medicine in India, Malaysia, Thailand, China, and Vietnam. The fruits have been used in Ayurveda as a potential medication for treatment of hepatic disorders and in Viet Nam for treatment of diarrhea, sore throat, inflammation, pyretic, urinary retention, and constipation [4]. Recent studies on the *P. emblica* fruits have also revealed that its ethanol crude extract possesses hepatoprotective activity [5 - 6] and is effective in preventing/ameliorating the toxic effects of hepatotoxic agents like ethanol, paracetamol, carbon tetrachloride, heavy metals, ochratoxins, hexachlorocyclohexane, anti-tubercular drugs and hepatotoxicity resulting from iron overload [8]. The polyphenols found in *P. emblica*, especially tannins and flavonoids are key elements for major bioactivities [9]. However, solvent types that have significant effects on extraction efficiency, phytochemical profile and biological activity of fruit extracts have yet to be fully investigated. Therefore, the present study is focused on the investigation of the effects of different solvent extraction types (methanol, ethyl acetate, water, 50 %, 70 %, and 96 % ethanol) used to produce corresponding extracts as PE-M, PE-EA, PE-W, PE-50Et, PE-70Et, and PE-96Et, respectively on phytochemical yield and antioxidant capacity, lipid peroxidation inhibitory activity, and hepatoprotective potential of *P. emblica* fruits collected in Vietnam. The 96 % ethanol extract of *P. emblica* fruits were also studied by HPLC-MS/MS to identify the main compounds responsible for the hepatoprotective effects.

2. MATERIALS AND METHODS

2.1. Chemicals

L-ascorbic acid, Trolox, silymarin and gallic acid were purchased from Sigma Aldrich (USA). Ethyl acetate (EtOAc) and methanol were distilled before use. 50 % and 70 % ethanol were prepared from 96 % ethanol and distilled water.

Methanol (HPLC grade), acetone (HPLC grade), and acetonitrile (HPLC grade) were purchased from Sigma-Aldrich (St. Louis, MO). For the HPLC-DAD-ESI(−)-MS system, acetonitrile (LC-MS CHROMASOL grade) and acetic acid were purchased from Sigma-Aldrich (Steinheim, Germany).

2.2. Materials

The fruits of *Phyllanthus emblica* L. were collected in Son La Province, Northern Vietnam. The plant was identified by botanist Dr. Nguyen Quoc Binh, Vietnam National Museum of Natural History, VAST, Ha Noi, Viet Nam. A voucher specimen (C-629) was deposited in the Herbarium of the Institute of Natural Products Chemistry, VAST, Ha Noi, Viet Nam. The fresh *P. emblica* fruits were washed and dried at 55 °C in an electro-thermostatic blast oven and then ground to fine brown powder with a particle size of 0.1 - 0.3 mm.

2.3. Preparation of extracts

The dried powder of *P. emblica* fruits were extracted with six solvents differing in polarity, including ethylacetate, methanol, 50 %, 70 %, 96 % ethanol, and water using ultrasound-assisted extraction (UAE) method. Briefly, 5 g of dried fruit sample was extracted 3 times with 50 mL of solvent (ratio 10:1, v/w) applying UAE (Elmasonic 100H, Germany) at a power of 150 W and 45 ^oC for 30 min. All extracts were combined and filtered through a Whatman No. 1 filter paper. Further, *P. emblica* extracts were evaporated to dryness by distillation under reduced pressure in a rotary evaporator (Rotavapor R210, Buchi, Germany). An exact amount of collected residue was weighed and dissolved in 96 % ethanol to prepare the extract with the required concentrations for further experiments.

2.4. In Vitro Antioxidant Activity

2.4.1. DPPH radical scavenging

The free radical scavenging ability of the prepared extracts was assessed by DPPH radical scavenging method with slight modification [10]. Briefly, DPPH reagent freshly prepared in absolute ethanol (100 µM, 750 µL) was added rapidly to the test sample (250 µL) and incubated in the dark for 30 minutes. The optical density (OD) value of the solution was taken at 517 nm against a blank solution containing solvents only. Ascorbic acid was used as an antioxidant standard. The analysis was done in triplicate. The scavenging effect was then calculated according to the following equation:

$$\text{DPPH scavenging activity (\%)} = \left(1 - \frac{OD_T}{OD_C}\right) \times 100\% \quad (1)$$

where OD_C = OD value of control group and OD_T = OD value of sample treated group.

The anti-oxidative property of the tested extracts was expressed as IC_{50} value (µg/mL) [10], which is defined as the concentration required for inhibition of DPPH radical by 50 % and determined based on Table Curve 2D v4 Software.

2.4.2. Lipid peroxidation

Albino healthy BALB/c mice at 12 weeks of age (22 - 24 gram, disease-free) were obtained from the Institute of Biotechnology, Vietnam Academy of Science and Technology (Ha Noi, Viet Nam). All mice were housed in plastic cages at a temperature-controlled room on a 12 h light/12 h dark cycle. Mice were provided with food and water *ad libitum*. Experiments were performed in accordance with Vietnamese ethical laws and European Communities Council Directives of November 24, 1986 (86/609/EEC) guidelines for the care and use of laboratory animals.

The ability to inhibit lipid peroxidation of the *P. emblica* fruit extracts was investigated through determination of malonyldialdehyde (MDA) level in the samples. The experiment was conducted according to the method published by Badmus *et al.* with some modification [11]. Briefly, the BALB/c mice were sacrificed by cervical dislocation and their brains were homogenized in phosphate buffer (10 % v/v, pH = 7.4, 0 - 4 ^oC). 1.0 mL of the brain homogenate was added to 0.1 mL of the extract (10 µg/mL) + 0.8 mL phosphate buffer + 0.1 mL Penton ($FeSO_4$ 0.1 mM : H_2O_2 15 mM, 1:1 v/v). Thereafter, the mixture was incubated at 37 ^oC for 15 min. Then, 1.0 mL of 10 % trichloroacetic acid was added, followed by centrifugation at

12000 rpm for 5 min at RT. The organic upper layer was collected and allowed to react with 1 mL of 0.8 % thiobarbituric acid (TBA) in SDS (ratio 2:1). The resulting mixture was mixed and heated up to 100 °C for 15 min. After cooling, the OD value of the mixture was measured at 532 nm. Trolox was used as a positive control. The percentage of inhibition was calculated according to the equation 2:

$$\text{Inhibition of lipid peroxidation (\%)} = \left(1 - \frac{OD_T}{OD_C}\right) \times 100\% \quad (2)$$

where OD_C = OD value of the control group (0.1 % DMSO); OD_T = OD value of the sample treated group.

The IC_{50} value was determined using Table Curve 2D v4 software.

2.4.3. Hepatoprotective activity against CCl_4 induced toxicity in HepG2 cell line

Cell culture

The human liver cancer HepG2 cells were obtained from ATCC, USA and grown on Dulbecco's Modified Eagle's Medium (DMEM, GIBCO, USA) supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (FBS), 2 mM l-glutamine, 10 mM HEPES, and 1.0 mM natri pyruvate. Cells were passed at 80 % confluency by trypsin/EDTA (0.025 %) (Gibco, Thermo Fisher Scientific, MA).

Cell viability assay

3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Celltiter 96-Aqueous One Solution Assay, Promega, Madison, WI, USA) was used to analyze the effect of compounds on cell viability [12]. Briefly, HepG2 cells were cultured overnight in a 96-well plate (~1×10^4 cells/well). Cell viability was assessed after adding the tested extracts at 100 µg/mL for 24 h. The number of viable cells was determined by the OD_{540nm} of the dissolved MTT-formazan product for 30 min to 1 hour, after the addition of 20 µL/well of Dye solution (from the kit), for 4 hours as described by the manufacturer (Promega). Wells without HepG2 cells and with only Dye plus solubilized solutions were blank. Afterwards, the OD value of the mixture was measured at 540 nm. Average values of triplicate experiments were presented.

$$\text{\% Viability} = \left(1 - \frac{OD_T - ODblank}{OD_D - ODblank}\right) \times 100\% \quad (3)$$

where OD_T = OD value of the sample treated group; OD_D = OD value of the control group (only 0.1 % DMSO) and OD_{blank} = OD value of blank group.

CCl_4 treatment and hepatoprotective activity

The HepG2 cells were seeded in 96-well microtitre plates at a concentration of 0.6×10^4 cells/well, treated with different concentration of samples, and incubated for 24 h at 37 °C under 5 % CO_2 to attain confluency. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, and the monolayer was washed once with the medium. The cells were treated with 100 µL of toxicant (1.0 % (v/v) CCl_4) in 0.25 % DMSO prepared in serum-free culture medium and incubated for another 2 h [13] before measuring the OD value using MTT method as described above. The hepatoprotective activity was expressed as percentage of protection, i.e.,

the increased percentage in cell viability compared to the viability of cells treated with CCl_4 alone.

The hepatoprotective activity was calculated according to the equation 4:

$$\% \text{ Protection} = \left(\frac{OD_{T+CCl4} - OD_{CCl4}}{OD_C - OD_{CCl4}}\right) \times 100\% \quad (4)$$

where OD_C = OD value of the control group (only 0.25 % DMSO); OD_{T+CCl4} = OD value of the sample and CCl_4 treated group; OD_{CCl4} = OD value of the CCl_4 treated group. Average values of triplicate experiments were presented.

2.5. Phytochemical Screening

2.5.1. Total phenolic content

The total phenolic content of the *P. emblica* extracts was determined using the Folin–Ciocalteu (FC) colorimetric method as previously described with some modifications [14]. Briefly, 0.5 mL of the extracts was diluted 2 times and mixed with 2.5 mL of 10 % (v/v) Folin–Ciocalteu reagent in distilled water. The mixture was left to settle for 6 min, then 2 mL of 7.5 % (w/v) Na_2CO_3 solution was added and incubated in the dark at room temperature for 1 h. The absorbance of the mixture was measured at 765 nm using a UV–vis spectrophotometer (Jasco V630, Japan). Ethanol and gallic acid were used as control and standard samples. Total phenolic content was calculated from the calibration curve ($y = 0.0011\,x + 0.1185$ ($R^2 = 0.9995$) of gallic acid and expressed as mg gallic acid equivalents (mg GAE)/g dried extract.

2.5.2. Phenolic compounds identification using HPLC analysis

The 96 % ethanol extract of *P. emblica* fruits was filtered through a 0.45 µm filter before being analyzed with HPLC-DAD (using RP-HPLC with Zorbax SB-C_{18} column, 4.6×150 mm, 5 µm particle size) and HPLC-ESI-MS. HPLC system (Agilent 1260 series) was coupled with a DAD detector and an Agilent Single Quadrupole 6120 mass spectrometer (Agilent, Santa Clara, USA). The mobile phase consists of HPLC gradewater with 0.1 % acetic acid (Solvent A) and 100 % methanol (HPLC grade) (Solvent B). Gradient elution program was as follow: Solvents A and B were 0 - 10 % (5 min), 10 - 15 % (5 min), 15 - 20 % (5 min), 20 - 30 % (5 min), and 30 - 40 % (5 min); the flow rate was 1 mL/min and a maximum pump pressure 4000 psi was maintained. Phenolics were identified at a wavelength of 254 nm. Phenolic compounds were tentatively identified by the interpretation of mass spectra as well as by comparison of their retention time with data from the literature. ^{1}H-and ^{13}C-NMR spectra were obtained from Bruke 500 Avance (Bruker, Germany).

2.6. Statistical analysis

The result of the DPPH assay was expressed as mean ± SEM. The IC_{50} values of the *P. emblica* extracts were calculated by regression analysis. The HepG2 *in vitro* data were statistically analyzed using SPSS software, version 10. The results were expressed as mean ± SEM. The hypothesis testing method included one-way Analysis of Variance (ANOVA) followed by least significant difference (LSD) multiple comparison tests. The level of significance was accepted with $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Extraction yield

Extraction yields referring to the percentage of crude extract obtained from dried fruit samples with six different solvent types are presented in Fig. 1.

Figure 1 shows that under the same extract conditions, (UAE, at a power of 150 W, 45 ^{o}C and for 60 min), among six solvents used, PE-M had the highest extraction yield (11.2 g of dried extract/100 g of dried sample, equal to 11.2 %). PE-70Et, PE-96Et, and PE-W extracts followed with extraction yields of 9.1, 8.6, and 6.4 %, respectively. These findings are similar to the results published by Nguyen *et al.*, who demonstrated that the phytochemical content and antioxidant potential of *Paramignya trimera* were affected by extraction solvent polarities and solvent types, such as water, acetonitrile, methanol, ethyl acetate, and hexane [14]. However, our results are slightly different from those published by Alagar *et al*, who demonstrated that the water extraction yield of *P. emblica* fruits was higher than alcoholic solvents [15]. Figure 1 indicated that the extraction yield was greatly affected by the type of solvents, among which methanol, 70 % ethanol, and 96 % ethanol are the best solvents for extracting natural compounds from *P. emblica* fruits.

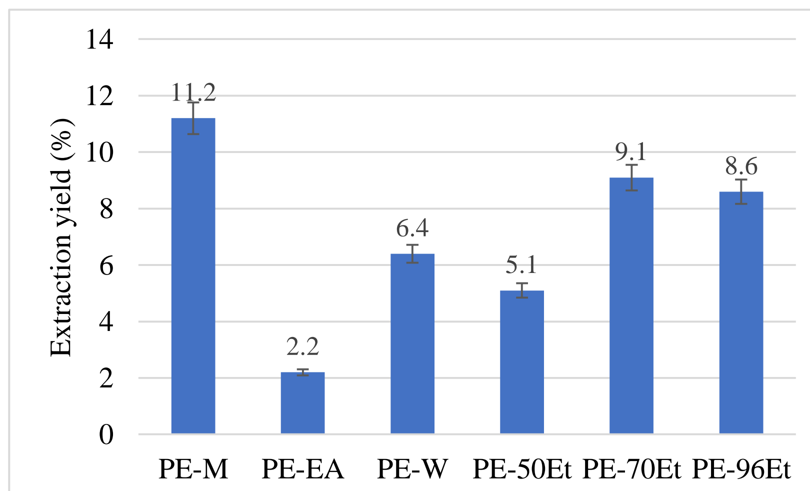

Figure1. Extraction yields (w/w % of dry weight) of *P. emblica* fruits with different solvents.

3.2. Effects of extracted solvents on antioxidant capacity and hepatoprotective activity

3.2.1. DPPH radical scavenging capacity

1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical was used to determine hydrogen donating ability of the plant extracts. DPPH reacts with hydrogen donors (free radical scavengers) to yield the stable product 1,1-Diphenyl-2-picrylhydrazine resulting in a color change from purple to yellow [10].

The DPPH radical scavenging activity of the *P. emblica* fruit extracts was shown in Fig. 2. The antioxidant activity of the fruit extracts was concentration-dependent in DPPH free radical scavenging assays, with SC_{50} values in the range of 4.05 to 6.01 µg/mL. The highest antioxidant activity was observed by the PE-M, followed by PE-96Et, and PE-EA extracts. The results also

indicated that the hydrogen donating ability of PE-M, PE-96Et, and PE-EA extracts of *P. emblica* fruits is higher than that of L-ascorbic acid. The results are also similar to those in a research by Kumari *et al.*, where the antioxidant activities of different fruits of *P. emblica* varieties decreased in the order of PE-M > PE-95Et > PE-EA extract [16].

Notably, in our results, the DPPH scavenging activity (SC_{50}) of the PE-96Et was similar to that of the PE-M extract (4.17 µg/mL vs 4.05 µg/mL). In comparison to the PE-M extract, the PE-96Et extract is worthy of further investigation and development of hepaptoprotective herbal products due to its low-level toxicity.

3.2.2. Effects of extracted solvents on lipid peroxidation

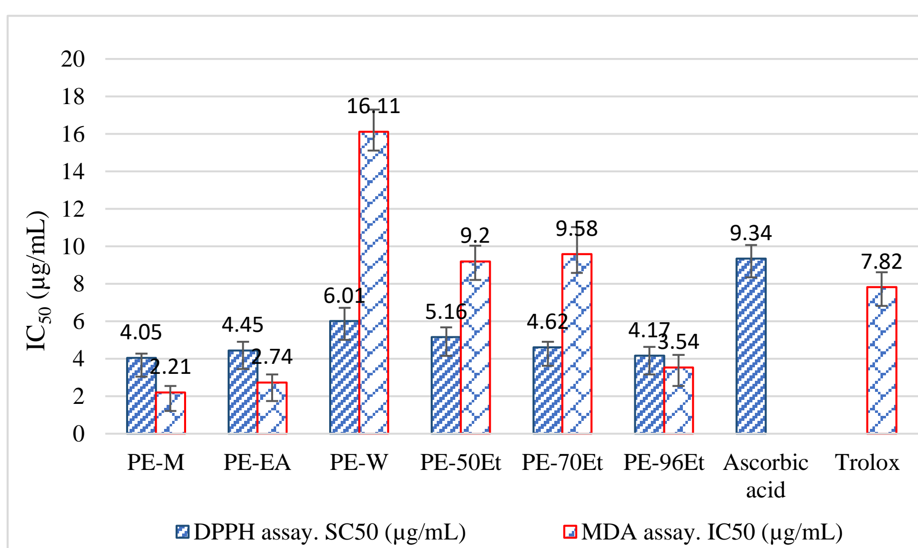

Figure 2. DPPH and MDA assays of *P. emblica* fruit extracts with different solvents.

Malondialdehyde (MDA) is one of the final products of polyunsaturated fatty acids peroxidation in the cells. The overproduction of MDA caused by increased free radicals indicates the oxidative stress and antioxidant status in cancerous liver cells. In this paper, the extent of lipid peroxidation represented by MDA level was measured by thiobarbituric acid reactive substance (TBARS) assay (Fig. 2).

It is observed that these *P. emblica* extracts inhibited lipid peroxidation of cell membrane in a concentration-dependent manner. The PE-M extract inhibited MDA formation higher than that of the PE-EA, PE-96Et, and Trolox (positive control), PE-50Et, PE-70Et, and PE-W extracts with IC_{50} values of 2.21, 2.74, 3.54, 7.82, 9.20, 9.58, and 16.11 (µg/mL), respectively. The PE-EA and PE-96Et extracts have lipid peroxidation inhibitory activity as high as that of the PE-M extract (2.21 (µg/mL) with IC_{50} values of 2.74 and 3.54 (µg/mL), respectively. It seems that natural compounds from those organic extracts of *P. emblica* fruits possess higher inhibitory activity of lipid peroxidation and antioxidant capacity than water (PE-W) and water-containing ethanol extracts (PE-50Et and PE-70Et).

3.2.3. Hepatoprotective activity against CCl_4 induced toxicity in HepG2 cells

CCl_4 is a common reagent used in the laboratory to mediate liver injury *in vitro* and *in vivo* as it is capable of generating free radicals and subsequent lipid peroxidation processes in an

organism [11]. To ascertain the hepatoprotective activity against CCl_4 induced toxicity in HepG2 cell line, we performed two experiments: (i) cytotoxic assay of *P. emblica* extracts at different concentrations by MTT assay to see if these extracts are toxic to the HepG2 cells or in other words, the cells can survive in the presence of *P.emblica* fruit extracts; and (ii) hepatoprotective assay against CCl_4-induced toxicity in HepG2 cells to determine if these extracts can protect the cells from CCl_4-induced injury. Figure 3 shows that the tested samples, even at the highest concentration (100 µg/mL), are not toxic to HepG2 cells. The cell viability is in the range of 89.67 - 110.64 %. Therefore, these concentrations can be further used to determine the hepatoprotective properties against CCl_4-induced oxidative damage in HepG2 cell model.

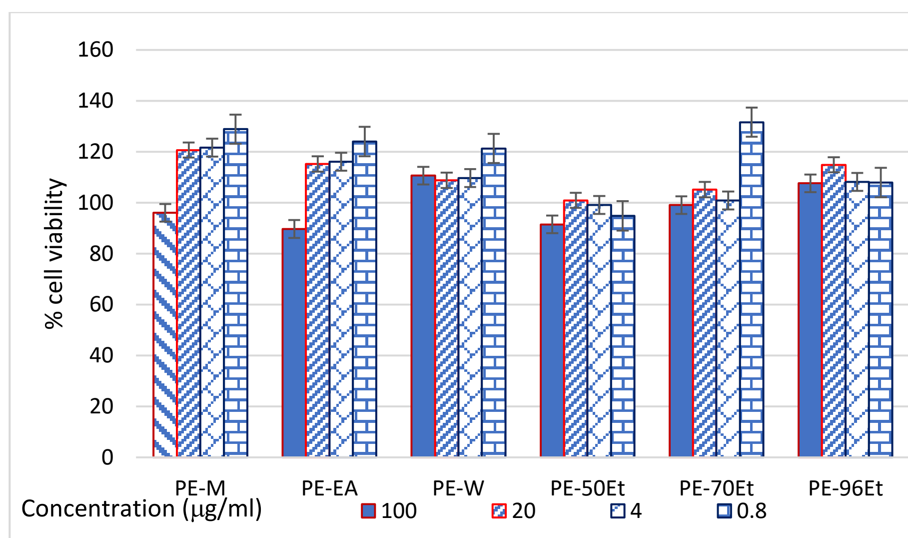

Figure 3. The HepG2 cell viability under the treatment of the *P. emblica* fruit extracts at different concentrations.

As can be seen from Figure 3, all fruit extracts of *P. emblica* were submitted to further assess their hepatoprotective effects at different concentrations of 0.8, 4, 20, and 100 µg/mL. The hepatoprotection were calculated and expressed as the percentage of cells alive in comparison to the control group.

In the hepatoprotective experiment, for the group with CCl_4-induced damage, only 27.89 % of HepG2 cells were survived and its percent protection (%) was set to zero. The cell protective concentrations represented using PC_{50} values (PC-Protective concentrations at 50 %) are also displayed in Figure 4. In the presence of the *P. emblica* fruit extracts, the percentage of viable HepG2 cells increased. Three extracts (PE-M, PE-70Et, and PE-96Et) had the highest hepatoprotective effects against CCl_4-induced toxicity in HepG2 cells with PC_{50} values of 47.68, 60.14, and 56.56 µg/mL, respectively. The PE-M extract had the highest hepatoprotective effect with PC_{50} value of 47.68 µg/mL. Silymarin, the positive reference control, had hepatoprotective effect against CCl_4 damage with $PC_{50} > 100$ µg/mL. As far as our knowledge, this is the first report of hepatoprotective effect against CCl_4 damage in *in vitro* HepG2 cell model of *P. emblica* fruit extracts. In 2011, Sharma S. K. *et al*. reported the hepatoprotective activity of five *Phyllanthus* species (*P. amarus*, *P. fraternus*, *P. rotundifolius*, *P. urinaria,* and *P. maderaspatensis*) on *tert*-butyl hydroperoxide (*t*-BH)-induced cytotoxicity in HepG2 cells [17]. The methanol extract of *P. urinaria* exhibited the highest hepatoprotective activity with IC_{50} =

72 μg/mL. However, all the extracts showed less potency when compared with the reference standard, silymarin (IC_{50} = 49.0 μg/mL) [17].

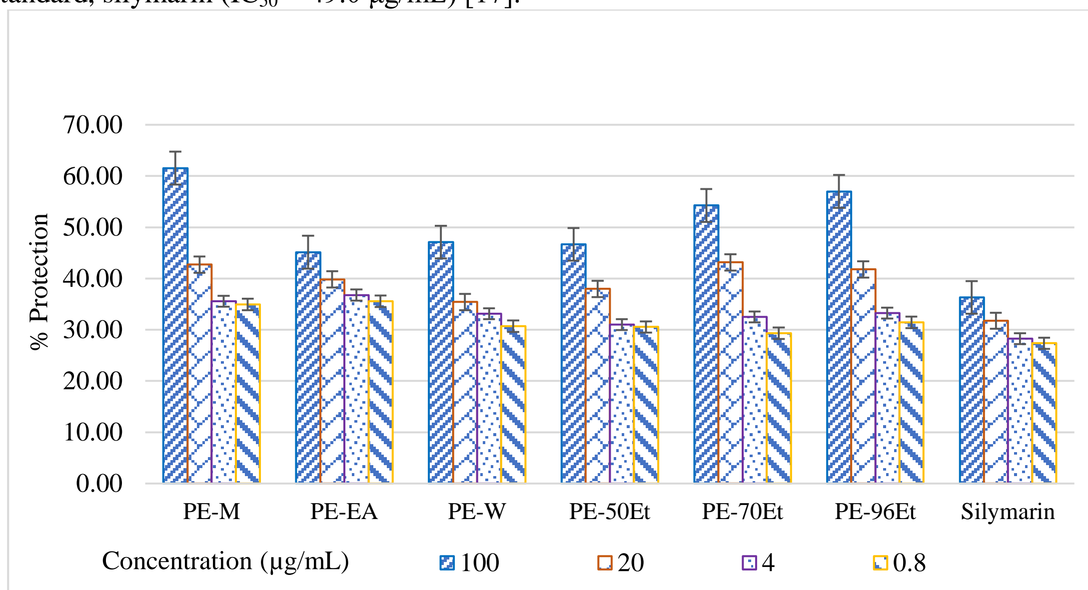

Figure4. Hepatoprotective activity of fruit extracts of *P. emblica* with different solvents against CCl_4-induced toxicity in HepG2 cells.

3.3. Phytochemical Screening

3.3.1. Total phenolic content (TPC)

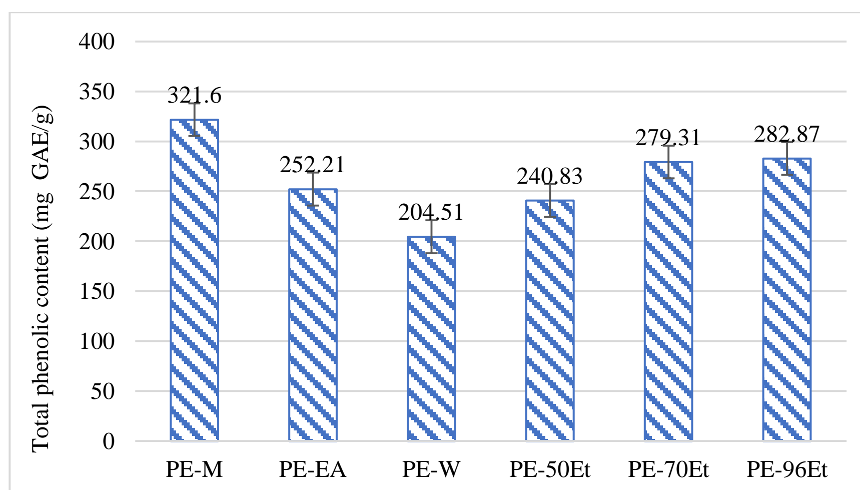

Figure 5. Total phenolic contents of fruits extract of *Phyllanthus emblica*.

Phenolic compounds in plants have strong links to their antioxidant and anticancer activities [18]. As can be seen in Figure 5, among the six solvents used, the PE-M extract had the highest TPC from *P. emblica* fruits (321.60 mg GAE/g dried extract). TPCs level extracted by PE-W was the lowest (204.51 mg GAE/g dried extract). TPCs were obtained by PE-96Et > PE-70Et >

PE-EA > PE-50Et (282.87, 279.31, 252.21, and 240.83 mg GAE/g dried extract, respectively) (Fig.5). In 2014, Ha *et al*. reported that the 96 % ethanol extract of *P. emblica* fruits collected in Cao Bang province possessed a higher TPC than the water extract (439.81 vs 352.08 mg GAE/g) [19]. Correspondingly, this result means that the greater the total phenol content, the higher antioxidant and hepatoprotective activities. It is also indicated by the smaller value PC_{50} of hepatoprotective effect. Comparison of TPC content in *P. emblica* fruits collected in other countries showed that, the TPC of *P. emblica* fruits was very different from place to place, and from solvent to solvent used for the extraction. For example, the highest TPC of *P. emblica* fruits collected in China was 133.58 mg GAE/g 66 % ethanol dried extract [20], in Indonesia only 128.18 mg GAE/g ethyl acetate fruit extract [21], while in Thailand was 342.2 mg GAE/g water extract [22].

3.3.2. Phenolic compounds identification using HPLC analysis

A typical HPLC profile of the phenolic and flavonoids constituents of ethanol extract of *P. emblica* is presented in Fig 6.

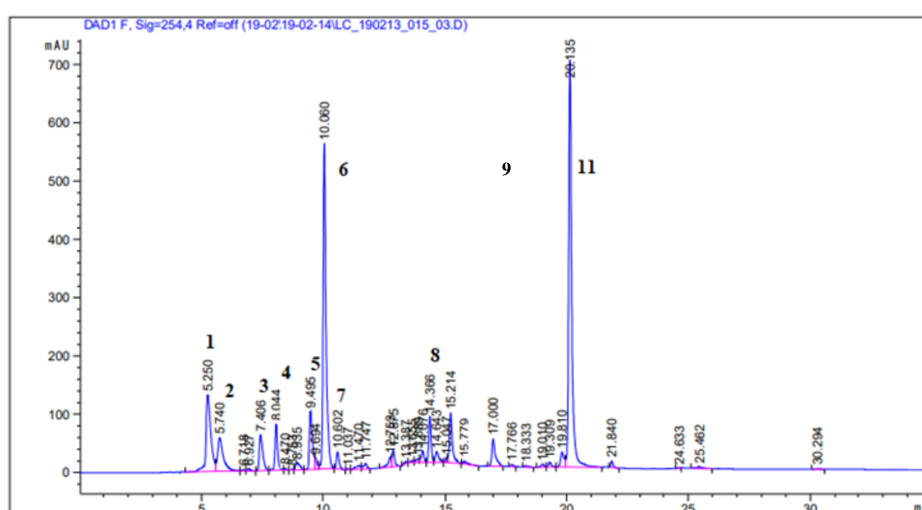

Figure 6. HPLC profile of 96 % ethanol extract of *P. emblica* fruits at 254 nm.

Gallic acid (**6**) was isolated and identified based on its MS and NMR spectra by comparison to a reference compound. Gallic acid had an ion at *m/z* 169 $[M-H]^-$ and 2 aromatic protons at δ_H = 7.086 ppm in its 1H-NMR spectrum. HPLC-ESI-MS analysis of the ethanol extract of *P. emblica* fruits showed the presence of gallic and mucic acid derivatives including mucic acid 3-*O*-gallate (**1**), mucic acid 2-*O*-gallate (**2**), mucic acid 1,4-lactone 3-*O*-gallate (**3**), glucogallin (**4**), mucic acid 1,4-lactone 2-*O*-gallate (**5**), mucic acid dimethyl ester 3-*O*-gallate (**7**), and mucicacid1,4-lactone 1-ethyl ester 2-*O*-gallate (**8**), tentatively based on their MS spectrum with $[M-H]^-$ pseudo-molecular ions at *m/z* of 361.2, 361.2, 343.2, 331.2, 343.2, 389.2, and 371.2, respectively, as well as in comparison of their retention time with data from the literature [23 - 25]. Quercetin (**9**) was detected based on its MS pseudo molecular peak $[M-H]^-$ at *m/z* of 301.2 [23] (Table 1 and Figure 7). Phenolic compounds are the most abundant phyto-compounds present in the ethanol extract of *P. emblica* as compared with flavonoids. These findings are similar to the results published by Yang *et al*. [23], Olenniko *et al*. [24] and

Zhang *et al.* [25], who demonstrated the phytochemical content and antioxidant potential of mucic acid derivatives isolated from the fruits of *P. emblica*.

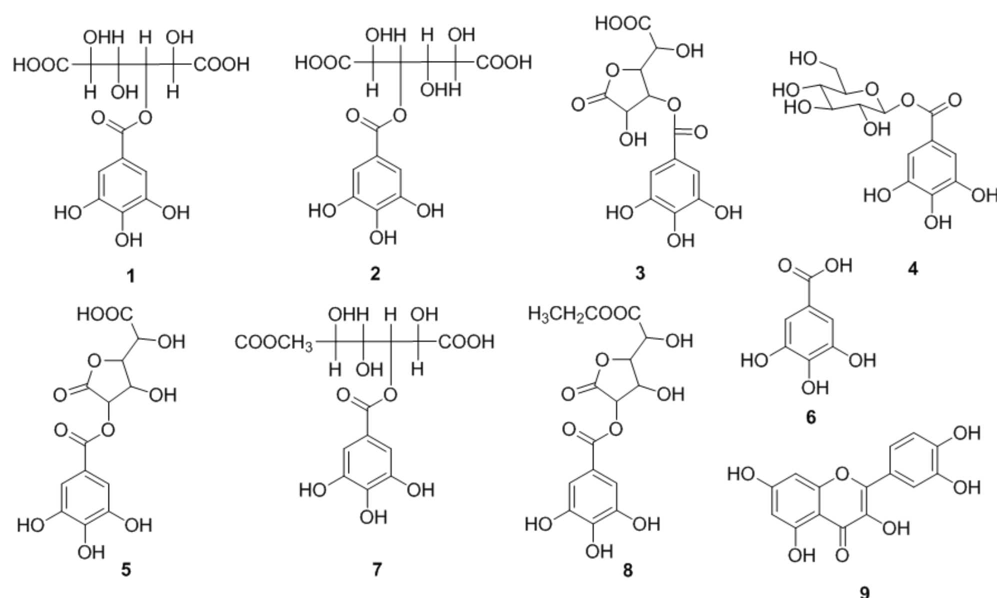

Figure 7. Structure compounds identified in ethanol extract of *P. emblica.*

Table1. Composition of phenolic compounds tentatively identified in ethanol extract of *P. emblica.*

Peak No.	Retention time R_f (min)	Compound	Molecular formula Molecular weight (MW)	Pseudo-molecular ion $[M–H]^−$
1	5.250	Mucic acid 3-*O*-gallate (**1**)	$C_{13}H_{14}O_{12}$ (MW = 362)	$[M–H]^−$ = 361.2
2	5.740	Mucic acid 2-*O*-gallate (**2**)	$C_{13}H_{14}O_{12}$ (MW = 362)	$[M–H]^−$ = 361.2
3	7.406	Mucic acid 1,4-lactone 3-*O*-gallate (**3**)	$C_{13}H_{12}O_{11}$ (MW = 344)	$[M–H]^−$ = 343.2
4	8.044	Glucogallin (**4**)	$C_{13}H_{16}O_{10}$ (MW = 332)	$[M–H]^−$ = 331.2
5	9.495	Mucic acid 1,4-lactone 2-*O*-gallate (**5**)	$C_{13}H_{12}O_{11}$ (MW = 344)	$[M–H]^−$ = 343.2
6	10.060	Gallic acid (**6**)	$C_7H_6O_5$ (MW = 170)	$[M–H]^−$ = 169.2
7	10.602	Mucic acid dimethyl ester 3-*O*-gallate (**7**)	$C_{15}H_{18}O_{12}$ (MW = 390)	$[M–H]^−$= 389.2
8	14.366	Mucic acid 1,4-lactone 1-ethyl ester 2-*O*-gallate (**8**)	$C_{15}H_{16}O_{11}$ (MW = 372)	$[M–H]^−$ = 371.2
9	20.135	Quercetin (**9**)	$C_{15}H_{10}O_7$ (MW = 302)	$[M–H]^−$ = 301.2

4. CONCLUSIONS

In this study, the fruits of *P. emblica* were extracted with different solvents including methanol, ethyl acetate, water, 50 %, 70 %, and 96 % ethanol. Antioxidant capacity was

assessed using DPPH radical scavenging and lipid peroxidation inhibition (MDA) methods. The PE-M extract possessed both the highest antioxidant, followed by the PE-96Et, and lipid peroxidation inhibitory activities, while PE-EA had the second highest inhibitory value in MDA assay. The hepatorprotective activity of the extracts was evaluated against CCl_4-induced toxicity in HepG2 cells. The results showed that the treatment with the methanol and 96 % ethanol extracts appears to enhance the recovery from hepatic injury induced by CCl_4 with PC_{50} of 47.68 ± 2.24 and 56.56 ± 5.01 (µg/mL), respectively. The total phenolic content in the PE-M and PE-96Et dried extracts of *P. emblica* fruits were 321.60 and 282.87 mgGAE/g, respectively. Although methanol had higher extraction yield and the methanol extract possessed higher antioxidant activity, the use of ethanol is more suitable because of its low toxicity to human. The HPLC-MS analysis of the ethanol extract of *P. emblica* fruits tentatively identified the presence of polyphenolic compounds including mucic acid 3-*O*-gallate (**1**), mucic acid 2-*O*-gallate (**2**), mucic acid 1,4-lactone 3-*O*-gallate (**3**), glucogallin (**4**), mucic acid 1,4-lactone 2-*O*-gallate (**5**), gallic acid (**6**), mucic acid dimethyl ester 3-*O*-gallate (**7**), mucic acid 1,4-lactone 1-ethyl ester 2-*O*-gallate (**8**), and quercetin (**9**). The results also indicate that the 96 % ethanol extract of *P. emblica* fruits is highly potential for further investigation and development of hepatoprotective herbal products.

Acknowledgements. The research funding from Vietnam Academy of Science and Technology (VAST) for financial support (Grant number: VAST.UDPT.06/18-19) was acknowledged.

CRediT authorship contribution statement. Author 1: Methodology, Investigation, Funding acquisition, Writing paper. Author 2: Writing paper, Formal analysis. Author 3: Investigation, Formal analysis, Writing paper, Supervision. Authors 4-5: Investigation. Authors 6-7: Investigation, Formal analysis, Supervision. Author 6: Formal analysis. Author 8: Formal analysis, Writing paper.

Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

REFERENCES

1. Calixto J., Santos A., Cechinel F. V., and Yunes R. - A review of the plants of the genus *Phyllanthus*: their chemistry, pharmacology, and therapeutic potential, Med. Res. Rev. **18** (4) (1998) 225-258. https://doi.org/10.1002/(sici)1098-1128(199807)18:4<225::aidmed2 > 3.0.co;2-x.
2. The_Plant_List. http://www.theplantlist.org/tpl1.1/record/kew-153790 (accessed on 31 May 2021).
3. Wikipedia. https://vi.wikipedia.org/wiki/Phyllanthus_emblica (access on 1st Jun 2021).
4. Gaire B. P. and Subedi L. - Phytochemistry, Pharmacology and Medicinal Properties of *Phyllanthus emblica* Linn., Chin. J. Integr. Med. (2015) 1-8.
   https://doi.org/10.1007/s11655-014-1984-2.
5. Chaphalkar R., Apte K. G., Talekar Y., Ojha, S. K., and Nandave M. - Antioxidants of *Phyllanthus emblica* L. Bark Extract Provide Hepatoprotection against Ethanol-Induced Hepatic Damage: A Comparison with Silymarin, Oxid. Med. Cell Longev. (2017) 1-10. https://doi.org/10.1155/2017/3876040.
6. Duong N. T., Uyen N. T., Vinh P. D., Son N. T., Ha L. V., Ha D. T., and Thuy P. T. - Hepatoprotective Effect of Standardized Dry Extract from *Phyllanthus emblica* L. Fruits

on Hepatotoxicity Model Induced by Carbon-Tetrachloride in Mice, J. Med. Materials **24** (2) (2019) 108-112 (in Vietnamese).

7. Jose J. and Kuttan R. - Hepatoprotective activity of *Emblica officinalis* and Chyavanaprash, J. Ethnopharmacol **72** (1-2) (2000) 135-40. https://doi.org/10.1016/s0378-8741(00)00219-1.
8. Thilakchand K., Mathai R., Simon P., Ravi R., Baliga-Rao M., and Baliga M. - Hepatoprotective properties of the Indian gooseberry (*Emblica officinalis* Gaertn): a review, Food Funct **4** (10) (2013) 1431-41. https://doi.org/10.1039/c3fo60237k.
9. Yadav S., Singh M., Singh P., and Kumar V. - Traditional knowledge to clinical trials: A review on therapeutic actions of *Emblica officinalis*, Biomed. Pharmacother. **93** (2017) 1292-1302. https://doi.org/10.1016/j.biopha.2017.07.065.
10. Molyneux P. - The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity, Songklanakarin J. Sci. Technol. **26** (2) (2004) 211-219.
11. Badmus J. A., Adedosu T. O., Fatoki J. O., Adegbite V. A., Adarmoye O. A., and Odunola A. O. - Lipid peroxidation inhibition and antiradical activities of some leaf fractions of *Mangifera indica*, Acta Poloniae Pharmaceutica Drug Research **68** (1) (2011) 23-29. PMID: 21485698.
12. Kim J. A., Son J. H., Song S. B., Yang S. Y., and Kim Y. H. - Sterols isolated from seeds of *Panax ginseng* and their antiinflammatory activities, Pharmacogn. Mag. **9** (34) (2013) 182-185. https://doi.org/10.4103/0973-1296.111288.
13. Krithika, R., Mohankumar, R., Verma, R.J., Shrivastav, P.S., Mohamad, I.L., Gunasekaran, P., and Narasimhan, S. - Isolation, characterization and antioxidative effect of phyllanthin against CCl_4-induced toxicity in HepG2 cell line, Chemico-Biological Interactions **181** (2009) 351-358. https://doi.org/10.1016/j.cbi.2009.06.014.
14. Nguyen, V. T., Bowyer, M. C., Vuong, Q. V., Altena, I. A. V., and Scarlett, C. J. - Phytochemicals and antioxidant capacity of Xao tam phan (P*aramignya trimera*) root as affected by various solvents and extraction methods, Industrial Crops and Products. **67** (2015) 192-200. https://doi.org/10.1016/j.indcrop.2015.01.051.
15. Alagar, R. M., Shailaja, V., Banji, D., Rao, K. N. V., and Selvakumar, D. - Evaluation of standardisation parameters, pharmacognostic study, preliminary phytochemical screening and in vitro antidiabetic activity of *Emblica officinalis* fruits as per WHO guidelines, J. Pharmacog. Phytochem. **3** (4) (2014) 21-28.
16. Kumari, P. and Khatkar, B. S. - Assessment of total polyphenols, antioxidants and antimicrobial properties of aonla varieties, J. Food Sci. Technol. **53** (7) (2016) 3093-3103. https://doi.org/10.1007/s13197-016-2282-0.
17. Sharma S. K., Arogya S. M., Bhaskarmurthy D. H., Agarwal A., Velusami C. C. - Hepatoprotective activity of the *Phyllanthus* species on *tert*-butyl hydroperoxide (*t*-BH)-induced cytotoxicity in HepG2 cells, Pharmacognosy Magazine, **7** (27) (2011) 229- 233. https://doi.org/10.4103/0973-1296.84237.
18. Fitriansyah, S. N., Aulifa, D. L., Febriani, Y., and Sapitri, E. - Correlation of Total Phenolic, Flavonoid and Carotenoid Content of *Phyllanthus emblica* Extract from Bandung with DPPH Scavenging Activities, Pharmacogn. J. **10** (3) (2018) 447-452. https://doi.org/10.5530/pj.2018.3.73.

19. Ha D. T., Oanh T. T., and Khoi N. M. - Determination of total phenol content of fruits of *Phyllanthus emblica* collected in Cao Bang using UV- spectroscopy, J. Med. Materials **19** (3) (2014) 182-188 (in Vietnamese).
20. Yongyu L., Bingchun G., Wenting W., Liang L., Lili C., Chao Y., Jingyuan L., Qin L., Jianjun C., Shaohua W., and Liaoyuan Z. - Characterization of phenolic compounds from *Phyllanthus emblica* fruits using HPLC-ESI-TOF-MS as affected by an optimized microwave-assisted extraction,Int. J. Food Prop. **22** (1) (2019) 330-342.
 https://doi.org/10.1080/10942912.2019.1583249.
21. Fitriansyah S. N., Aulifa D. L., Febriani Y., and Sapitri E. - Correlation of Total Phenolic, Flavonoid and Carotenoid Content of *Phyllanthus emblica* Extract from Bandung with DPPH Scavenging Activities. Pharmacogn. J. **10** (3) (2018) 447-452.
 https://doi.org/10.5530/pj.2018.3.73.
22. Juree C., Chatri N., Noppamas S., Kanjana J., and Seewaboon S. - Antioxidant activities of the standardized water extract from fruit of *Phyllanthus emblica* Linn., Songklanakarin J. Sci. Technol. **32** (6) (2010) 599-604.
23. Yang B., Kortesniemi M., Liu P., Karonen M., and Salminen J. P. - Analysis of Hydrolyzable Tannins and Other Phenolic Compounds in Emblic Leafflower (*Phyllanthus emblica* L.) Fruits by High Performance Liquid Chromatography−Electrospray Ionization Mass Spectrometry, J. Agric. Food Chem. **60** (2012) 8672-8683.
 https://doi.org/10.1021/jf302925v.
24. Olennikov D. N., Kashchenko N. I., Schwabl H., Vennos C., and Loepfe C. - New mucic acid gallates from *Phyllanthus emblica*, Chem. Nat. Compd. **51** (4) (2015) 666-670. https://doi.org/10.1007/s10600-015-1380-y.
25. Zhang Y. J., Tanaka T., Yang C. R., and Kouno I. - New Phenolic Constituents from the Fruit Juice of *Phyllanthus emblica*. Chem. Pharm. Bull. **49** (5) (2001) 537-540. https://doi.org/10.1248/cpb.49.537.