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Chromatographic Isolation and Characterization of Certain Bioactive Chemical Ingredients of *Phyllanthus emblica* Extracts and Assessment of Their Potentials as Antiviral and Anticancer Agents

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Abstract

The current study aims to evaluate the antioxidant, anticancer and antiviral activities of *Phyllanthus emblica* leaves, as well as characterization of bioactive chemical constituents. The total phenolic and flavonoid contents of plant extracts were determined using Folin-Ciocalteu's and aluminium trichloride assays, respectively. The antioxidant activities were evaluated by different methods, the anticancer activity was evaluated via MTT assay, while the antiviral activity was evaluated using cytopathic effect (CPE) inhibition and anti-HCV assays. Moreover, the chemical profiling of extracts was performed using reversed phase high-performance liquid-chromatography method. Our finding revealed that *P. emblica* is rich in glycosides, flavonoids, phenolics, terpenoids and tannins. The *n*-butanol fraction exhibited the highest total phenolic and flavonoid contents with values 654.78 mg GAE /g ext and 110.14 mg RE / g ext, respectively. Also, it possessed the most effective antioxidant activities (DPPH; IC_{50}=19.59 µg/mL, RPAA; 0.948 mg AAE /g ext., and TAC; 543.62mg AAE /g ext.) and anticancer activity against HepG-2 and MCF-7 cell lines with an IC_{50} of 25.63 and 22.80µg/mL, respectively. Moreover, *P. emblica* extracts exhibited anti-HCV activity. The HPLC finger print results of *P. emblica* extracts showed 14 peaks superimposed to the standards. Structure elucidation of pure isolates was achieved via IR, UV, ^{1}H & ^{13}C-NMR

Keywords: *Phyllanthus emblica*, HPLC, Secondary metabolites, Antioxidants, Antiviral, Anticancer.

1. Introduction

Oxidative stress is the imbalance between the production of free radicals and their stabilizing antioxidant in the biological systems. Reactive oxygen species (ROS) can be produced by cellular metabolism and react with molecules like lipid, protein and DNA leading to cellular damage and cause various diseases, such as diabetes, atherosclerosis, cardiovascular diseases, liver diseases, acute respiratory distress syndrome, neurological diseases Alzheimer's disease, viral disease, ageing and cancer [1-3]. The antioxidants lower the oxidants by donating electron to free radical and make it not reactive compound so as to reduce the harmful effects generated by these radicals in the cell. Several natural antioxidant compounds, originated from medicinal plant can prevent oxidation of molecules thus help in repairing the oxidative damage caused by the oxygen radicals to the cells and also play a very important role in health maintenance and protection of chronic diseases [4]. Recently, interest has become greater than before in finding naturally occurring antioxidants to replace synthetic antioxidants due to their availability, limited side effects and low cost [5].

The liver is an essential organ in the body which plays a vital role in the metabolism, bile secretion and drugs detoxification. [6-7]. Many toxic chemicals, viral infections and also autoimmune disorders can cause liver diseases **[8]**. Hepatitis C virus (HCV) is a universal health concern which is responsible for most of the liver diseases and can finally progress to chronic infection and can cause hepatocellular carcinoma (HCC), cirrhosis and death [9]. Now, there is no vaccine available for HCV due to the high degree of strain change. For that reason, there is a need to develop new antiviral agents that interfere with different stages of the HCV life cycle. Several

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plant species and their constituents have been tested against HCV, and some have been shown to have great potential use in HCV therapy as Silymarin, which is isolated from Silybum marianum has been tested against HCV and is found to be effective in inhibiting the viral activity of HCV [10].

Phyllanthus emblica (Euphorbiaceae family), is commonly known as Indian gooseberry or Amla, is native of tropical and subtropical countries. Amla is a magic herb and one of the valuable gifts of nature to human kind. *P. emblica* has a variety of Phytochemical such as tannins, flavonoids, terpenoids and alkaloids which reported to indicate several pharmacological properties such as antioxidant, anticancer, antitumor, antigenotoxic, and anti carcinogenic effects and is commonly used in treating various ailments as asthma, cough, diabetes, jaundice, diarrhea, vomiting, and kidney and urinary bladder disturbances [11-12]. The anticancer potential of Phyllanthus species has been reported in a many papers, for example *P. amarus* protects from hepato carcinogenesis [13-14]. Also *P. niruri* used in colorectal cancer patients **[**15]. Thus, it is believed that *P. emblica* might possess anticancer properties as well. On the other hand, some Phyllanthus species have proven to be strong sources of antiviral agents where it has a diversity of phytochemical constituents aiding in viruses inhibition [16-17]. Previous phytochemical reports revealed that several bioactive secondary metabolites have been isolated and identified from different parts of the plant including tannins**,** alkaloid, carbohydrates, saponins, phenolics and flavonoids [18-20].

Therefore, the aim of our study was to evaluate the in vitro antioxidant activity of some extracts of *P. emblica* growing in Egypt using different antioxidant assays such as total antioxidant activity, DPPH free radical scavenging potential and reducing power activity. Also, the total phenolic content and flavonoids contents were determined from plant extracts and their correlations with the antioxidant activities were confirmed. Furthermore, the anticancer and antiviral of these extracts were examined. Moreover, we concerned with the isolation of some plant components which supposed to be physiologically active. Our investigations on *n*-butanol extract of the plant have resulted in the separation of compound (1) and compound (2). The structures of the compounds were elucidated by the spectroscopic data including the ^{1}H-NMR and ^{13}C-NMR experiments.

2. Experimental

2.1. Chemicals and reagents

Gallic acid, rutin, DPPH (1,1-diphenyl-2-picrylhydrazyl) were purchased from Sigma-Aldrich Co. (St. Louis, Missouri, USA), Folin-Ciocalteu's reagent, ammonium molybdate, ascorbic acid and sodium carbonate were purchased from Merck Chemical Co. (Darmstadt, Germany). All other chemicals and reagents were the highest analytical grade and purchased from common sources.

2.2. Plant materials

The leaves of *P. emblica* were collected from the Giza Zoo garden during March 2018, Giza, Egypt. The plant was kindly identified by Eng. Teresa Labib, General Manager and head of plant Taxonomy in El-Orman Botanical Garden, Giza, Egypt. A voucher specimen was kept at the Medicinal Chemistry Department, Theodor Bilharz Research Institute under accession number (P18/3/2018). The collected leaves were washed, air dried, grounded into coarse powder and stored for further processing.

2.3. Extraction and fractionation

The finely powdered leaves (1.5 kg) were extracted using 90% methanol then filtered using Whatman Filter paper No.1 and dried under reduced pressure using rota-vapour. The crude methanolic extract (230g) was defatted with diethyl ether and then fractionated by using different organic solvents; petroleum ether (60-80°C), methylene chloride, ethyl acetate and *n*-butanol. Each fraction was filtered and then concentrated to afford petroleum ether (4.6 g), methylene chloride (18.5 g), ethyl acetate (25.2 g) and *n*-butanol (65.4 g).

2.4. Preliminary phytochemical analysis

The prepared extracts were qualitatively investigated to show the presence of phytochemical constituents such as alkaloid, tannin, flavonoid, saponin, carbohydrate, glycoside, steroid and phenols. These were recognized by characteristic changes using standard reported methods [21-25].

2.5. Determination of total phenolic content (TPC)

Total phenolic content in each extract was determined quantitatively by the modified Folin-Ciocalteu method [26]. A100 µl of the extract (100 µg/ml) was mixed with a 500 µl of Folin-Ciocalteu reagent and 1.5 ml of sodium carbonate (20%). The mixture was vortexed for 15 sec and made up to 10 ml using distilled water then allowed to stand for 120 min at 25°C for color development. Absorbance was then measured at 765 nm using a spectrophotometer (UV-VS spectrophotometer, Milton Roy 601, Co, USA). Gallic acid was used as standard and samples were evaluated at a final concentration of 100 µg/ml. Total phenolic contents were expressed as mg Gallic acid equivalent, mg of GAE/g extract [27].

2.6. Determination of total flavonoid content (TFC)

Total flavonoid contents were determined using the method described by Ordon ez *et al* [28]. By

mixing 5 ml extract (0.01 mg/ml) with 5 ml $AlCl_3$(2%), the mixture was incubated at room temperature for 40 minutes ,then the absorbance was measured at 415 nm using spectrophotometer (UV-VS spectrophotometer, Milton Roy 601) against a blank sample consisting of a 5 ml extract solution with 5 ml methanol without AlCl3. Also a rutin solution (0.5 mg/ml) used as standard [29]. The amount of flavonoids in plant extracts were expressed as rutin equivalents (RE).

2.7. Determination of phenolics and flavonoids in plant materials by HPLC finger print with diode-array detection

Phenolics and flavonoids were determined by HPLC according to the method of Goupy *et al*. [30]. as follow: 0.5 g of sample were mixed with methanol and centrifuged at 10000 rpm for 10 min and the supernatant was filtered through a 0.2 µm Millipore membrane filter then 1-3 ml was collected in a vial for injection into HPLC Agilent (series 1260) equipped with autosamplling injector, solvent degasser and quaternary HP pump (series 100). The separation was carried out using Eclipse Plus C18 column (4.6 mm x 250 mm i.d., 5 µm). The mobile phase consisted of water (A) and 0.02% tri-floro-acetic acid in acetonitrile (B) at a flow rate 1 ml/min. The mobile phase was programmed consecutively in a linear gradient as follows: 0 min (80% A); 0–5 min (80% A); 5-8 min (40% A); 8-12 min (50% A); 12-14 min (80% A) and 14-16 min (80% A). The multi-wavelength detector was monitored at 280 nm. The injection volume was 10 µl for each of the sample solutions [31-32]. Standards of phenolic acids and flavonoids were obtained from Sigma Co. and were dissolved in a mobile phase and injected into HPLC. Retention time and peak area were used to calculate the concentration of the compounds by the data analysis of Agilent software.

2.8. Antioxidant activity

2.8.1. DPPH free radical scavenging activity

The different concentrations of each of the extracts were prepared in methanol and were added to 3ml of 0.1mM methanolic solution of DPPH. The tubes were shaken vigorously and allowed to stand for 30 min at room temperature in the dark. Changes in absorbance of samples were measured at 517 nm. A control reading was obtained using methanol instead of the extract. Ascorbic acid served as the standard Free radical scavenging activity was expressed as inhibition percentage and was calculated using the following formula,

$$\% \text{ inhibition} = ((A0 - At) / A0 * 100$$

Where, A0 is the absorbance of the control, At is the absorbance of test samples. All the tests were performed in triplicates and the results are reported as IC_{50}, which is the amount of antioxidant necessary to decrease the initial DPPH• concentration by 50% [33].

2.8.2. Total antioxidant capacity (TAC)

The total antioxidant capacity (TAC) of each extract was determined according to the Phosphomolybdenum Assay. Briefly 0.3ml diluted concentration of leaf extracts was mixed with 3ml of reagent solution (0.6M Sulfuric acid, 28mM Sodium phosphate and 4mM Ammonium molybdate) in labeled tubes. The tubes were capped and incubated in a boiling water bath at 95°C for 90 min. The tubes were cooled and the absorbance of the solution was measured at 695nm using a spectrophotometer. In case of blank 0.3ml of methanol was used in place of extracts. Ascorbic acid was used as a reference standard and the antioxidant capacity of extracts was expressed as µg ascorbic acid equivalents (AAE)/mg of extract [34-35].

2.8.3. Reducing power antioxidant assay (RPAA)

For this 2.5 ml of each extract was mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide (10 mg/ml). The mixture was incubated at 50 ºC for 20 min, then rapidly cooled, mixed with 2.5 ml of 10% trichloroacetic acid and centrifuged at 6500 rpm for 10 min. An aliquot (2.5 ml) of the supernatant was diluted with distilled water (2.5 ml) and then ferric chloride (0.5 ml, 0.1%) was added and allowed to stand for 10 min [36]. The absorbance was read spectrophotometrically at 700 nm. Ascorbic acid used as standard. Three replicates were made for each test sample. The percentage of reducing power was calculated by using the formula**:**

$$\text{Reducing power (\%)} = A_{control} - A_{sample} / A_{control}$$

Where, $A_{control}$ was the absorbance of solution without extract and A_{sample} was the absorbance with different dilutions of extract, Ascorbic acid was used as a standard.

2.9. Anticancer activity via Microculture Tetrazolium (MTT) assay

The anticancer activity was done according to Mauceri *et al*. [37] using two human tumor cell lines, namely, hepatocellular carcinoma (HepG-2) and mammary gland breast cancer (MCF-7). The cell lines were obtained from American Type Culture Collection (ATCC) via Holding company for biological products and vaccines (VACSERA), Cairo, Egypt. 5-Fluorouracil was used as a standard anticancer drug for comparison. Briefly, the different cell lines mentioned above were used to determine the inhibitory effects of extracts and compounds on cell growth using the MTT assay. This colorimetric assay is based on the conversion of the yellow tetrazolium bromide (5MTT) to a purple formazan

derivative by mitochondrial succinate dehydrogenase in viable cells. The cells were cultured in RPMI-1640 medium with 10% fetal bovine serum. Antibiotics added were 100 units/ml penicillin and 100 µg/ml streptomycin at 37 °C in a 5% CO_2 incubator. The cells were seeds in a 96-well plate at a density of 1.0 × 10^4 cells/well at 37 °C for 48 h under 5% CO_2. After incubation, the cells were treated with different concentrations of compounds and incubated for 24 h. After 24 h of drug treatment, 20 µL of MTT solution at 5 mg/mL was added and incubated for 4 h. Dimethyl sulfoxide (DMSO) in volume of 100 µL was added into each well to dissolve the purple formazan formed. The colorimetric assay was measured and recorded at absorbance of 570 nm using a plate reader (EXL 800, USA). The relative cell viability in percentage was calculated as:

(Absorbance of treated samples/ Absorbance of control sample) X 100

The extracts cytotoxicity was calculated according to the following equations: Cell cytotoxicity (%) = 100-cell viability(%) [36, 38-39].

2.10. Detection of cytotoxic activity by haemolysis assay

The erythrocytes of mammals provide a good model to test the cytotoxicity of molecules by measuring cellular damage. Certainly, it is important to investigate the cytotoxicity of any molecule before studying its mechanism of action. The rate of haemolysis is among the numerous cytotoxicity assays that determine the potential toxicity of red blood cells. Red blood cells are the main cells in circulation, and they are responsible for transporting oxygen; in fact, any alterations of this process could be lethal. One method of toxicity assessment is based on the measurement of the release of haemoglobin from suspended red blood cells which is called haemolysis. Therefore the loss of haemoglobin is the signal stability of the cell membrane of the erythrocytes [40]. The blood was collected from a healthy human volunteer in a Heparin tube. The tube was centrifuged at 1000 rpm, 4ºC for 5 min and washed three times with an equal volume of PBS (pH7.4). The volume of blood was measured and reconstituted as a 10% v/v suspension with PBS (pH7.4). Samples at different concentrations were added to Erythrocytes suspension at ratio 4:1. After incubation for 30 min at 37ºC, centrifugation was carried out at 1000 rpm; 4ºC for 3 min. Spectrophotometric measurement detected the haemoglobin release at 540 nm. The performance of the haemolysis assay was tested by two controls that were prepared without the extracts. The negative control received PBS (pH7.4) [41].

The Haemolysis percentage was calculated using the following equation:

aemolysis= (Abs of sample/Abs of (-) control) x 100

All experiments were performed in triplicate and mean values were used for the calculation. The degree of in vitro cytotoxicity to hemolytic activity is evaluated using the mortality rate observed: 0% to 9%= non-toxic; 10% to 49% = slightly toxic; 50% to 89% = toxic; and 90% to 100% = highly toxic. The LC0%-9% is referred to as a non-toxic concentration [42].

2.11. Detection of antiviral activity

2.11.1. Cytopathic effect (CPE) inhibition assay

The *in vitro* antiviral activity of different extracts and purified compounds were evaluated through the assessment of the inhibition caused by the compound on the cytopathic effect produced by hepatitis A virus; HAV (Faculty of medicine for girls, Egypt) in Vero cells (ATCC CCL-81™, Vacsera), Three folds serial dilutions of each sample in DMEM media (60 µl/well). The started concentration for each sample was safe to normal cells (Vero cells) as this concentration was lower than the identified IC_{50}. Vero cells (1x10^3 cells/ 100µl media/ well) were plated in a 96 well plate and incubated (37ºC, 5% CO_2) overnight to allow the cells to attach to the wells. An equal volume (1:1 v/v) of non-lethal dilution of the tested sample and the virus suspension were incubated for one hour. Then this mix was incubated with confluent cells (37ºC, 5% CO_2) for 24 h to allow the virus to take effect. To measure cellular metabolic activity as an indicator of cell viability, proliferation, and cytotoxicity; MTT dye was used at a concentration (5 mg/ml). Twenty µl MTT was added to each well. The plate was then placed on a shaking table at 150rpm for 5 min to thoroughly mix the MTT into the media. The plate was incubated (37ºC, 5% CO_2) for 1-5 h to allow the MTT to be metabolized. The media was discarded and 200 µl of DMSO (Dimethyl sulfoxide), the plate was placed on a shaking table at 150rpm for 5 min. Finally, the plate was read at 560 nm [43]. The percentage of cells that survive after virus addition is relative to the antiviral effect of the compound present in the assay sample [44].

2.11.2. Anti-HCV assay

HepG2 cell line was infected with a serum from a patient with chronic hepatitis C according to a previous study [45]. Cells were grown for 24 h to semi-confluence in 1640 RPMI medium w/o PYR (Thermo Fisher Scientific). The media were supplemented with 10% fetal bovine serum (FBS) (LONZA), 1% antibiotic, fungizone (LONZA), and 1% HEPES buffer (Biowest). The cells were washed twice with Dulbecco's phosphate-buffered saline (DPBS) (Gibco), then inoculated with 500 µl of serum patients, 500 µl FBS free-RPMI media, and

4% PEG /3 × 10^6 cells. The viral load in the used serum was quantitated by real-time PCR and the average copy number was 1.7 × 10^6 copies/ml. After 90 min, RPMI containing FBS was added to a final volume of 8 ml. Cells were maintained overnight at 37°C in 5% CO_2. On the next day, adherent cells were washed three times with a culture medium to get rid of the remaining infection serum and incubation was continued in a complete medium containing 10% FBS with regular medium changes for 3 days. In 24 well tissue culture plates, the infected HepG2 cells (7x10^5 cells/ml of RPMI, supplemented with 10% FBS, 1% antibiotic fungizone, and 1% HEPES buffer) were grown for 48h to a confluent sheet [46]. After that, media were aspirated from monolayer cultures and each sample was solubilized in RPMI media containing 2% FBS to detect its anti-HCV activity using an effective concentration that was detected from CPE assay. Incubation was done 72 h. After incubation, the media was collected. The viral infection in HepG2 cells throughout the culture duration was assessed qualitatively by detection of core antigen in media using an immunological technique (HCV Core Antigen ELISA, Cell Biolabs INC, Cat no. VPK-151) [47]. According to the ELISA kit protocol, each sample was tested in triplicate. The negative control had PBS buffer saline instead of the sample. The positive control was the media of infected HepG2 which was not treated with any sample.

2.12. Statistical analysis

The test of significance was performed using the GraphPad Prism 8 (San Diego, California, USA). The experimental results were expressed as the mean ± Standard Deviation (SD). Group comparisons were performed using One Way ANOVA and multiple T-test. A *p*-value< 0.05 was considered statistically significant* and A *p*-value< 0.005 was considered statistically significant **.

2.13. Ethics approval and consent to participate

Ethical approval had been granted approval by the Ethics Committee of Theodor Bilharz Research Institute (TBRI) number [FWA00010609].

3. Results and Discussion

3.1. Phytochemical screening

Identification of the major phytoconstituents of methanol extract and its derived fractions was carried out using the conventional reported methods. The results were evaluated by change in color or precipitation, showed the presence of certain primary and secondary metabolites as glycosides, flavonoids, phenolics, Terpenoids and tannins. These constituents played a vital role in the treatment of different diseases.

3.2. Determination of total phenolic content (TPC) and total flavonoid content (TFC)

The systematic literature indicates that the plant phenolics are one of the main groups used as primary antioxidants. Therefore, it is worthwhile to evaluate their total content in the plants of the study. [48]. Therefore, the content of phenolics of *P. emblica* is determined in the extracts (Table 1). It can be observed that the phenolic contents, being highest in butanol extract (654.78mg GAE /g ext) followed by 90% methanol extract (439.75), ethyl acetate extract (432.56) and others. The concentration of phenols and flavonoids also depends on the polarity of the solvents used for extraction and fractionation [49]. In addition from analysis, it was found that all extracts of *P. emblica* are rich in flavonoids content (Table 1). Flavonoids are probably the most important natural phenolics. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging properties [50].

Table (1): Total phenolic content (TPC) and total flavonoids content (TFC) of *P. emblica* extracts

Sample	TFC (mg RE / g ext.)[a]	TPC (mg GAE / g ext.)[b]
90% MeOH	102.7 ± 0.68	439.75 ± 0.69
Petroleum ether	39.17 ± 1.25	211.06 ± 1.24
Methylene chloride	51.41± 2.08	214.82 ± 1.38
Ethyl acetate	97.74 ±1.74	432.56 ± 1.22
n-butanol	110.14 ± 0.94	654.78 ± 1.97

Results are expressed as mean values ± standard deviation (n = 3).
[a] TPC (Total phenolic content) values are expressed as mg gallic acid equivalent/g extract (mg GAE/g ext.).
[b] TFC (Total flavonoid content) values are expressed as mg rutin/g extract (mg RE/ g ext.).

3.3. Determination of phenolics and flavonoids by reverse phase HPLC finger prints with diode-array detection

High performance liquid chromatography (HPLC) has been the most sensitive, accurate and reliable tool for the separation of complex plant extracts. HPLC finger prints allow us to recognize unknown compounds by comparison of their HPLC retention times with known standards. HPLC is the technique of choice for the analysis of phenolics and flavonoids. It is safe for flavonoids as it can be operated even at room temperature thus avoiding decomposition of flavonoids with high temperature [51-52].

Due to the high phenolic and flavonoid contents of the ethyl acetate and butanol extracts of *P. emblica* so, these extracts were subjected to further analysis via reverse phase HPLC with diode-array detection to identify their chemical constituents. Results of HPLC finger print analysis at 280 nm (Figures 1-3; Tables 2

and 3), shows presence of various constituents at various retention times (3.150, 3.543, 3.804, 4.904, 5.314, 5.783, 6.883, 7.706, 8.534, 9.141, 9.450, 10.328, 10.697, and 11.253).

By comparing the resulted peaks with the retention time of standards, 14 peaks superimposed to the standards as gallic acid, chlorogenic acid, catechin, caffeic acid, syringic acid, rutin, ellagic acid, coumaric acid, vanillin, ferulic acid, naringenin, propyl gallate and cinnamic acid as illustrated in tables (2 & 3) and Figure (1), which agree with previous reports [53-55]. It is reported that, *P. emblica* is rich in polyphenols and hydrolysable tannin derived compounds that act as antioxidants. Also, it seems reasonable that several of the anticancer activity of this plant is resulting from the tannins as Gallic acid and ellagic acid and from the flavonoid quercetin [56-58]. The study proved the presence of biologically and pharmacologically significant constituents making *P. emblica* useful for preparation of phytopharmacon. Further, the technique is reliable and reproducible for the determination of flavonoids and phenolics in plant extracts.

3.4. Chromatographic isolation of compounds 1 and 2

N-butanol fraction (50 g) was chromatographed on polyamide column chromatography (100 X 6 cm, 250 gm). Elution was started with 5% methanol and the polarity was gradually increased, similar fractions were pooled according to their pattern upon paper chromatogram. Elution with 20% methanol gave fraction I (Fr. I). Fr. I (800 mg) was further purified on the Sephadex LH-20 column with 40% methanol led to isolation of compound (1).

While Fr. II eluted with 50% methanol from the polyamide column and purified over Sephadex LH-20 by 30% methanol to afford compound 2 (35 mg), physical and chemical analyses of the compound confirm its steroidal nature (Salkowski test and Lieberman-Burchard test) [59].

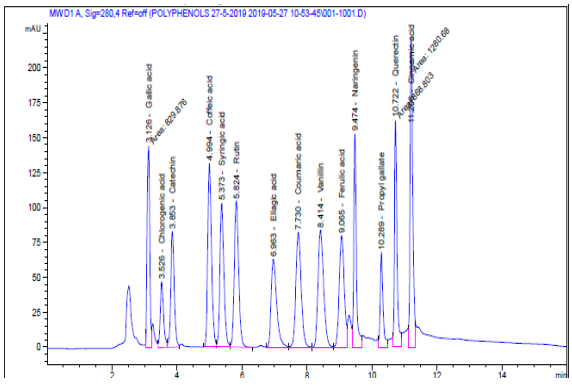

Fig. 1. HPLC-fingerprint chromatogram of 14 standard phenolic compounds

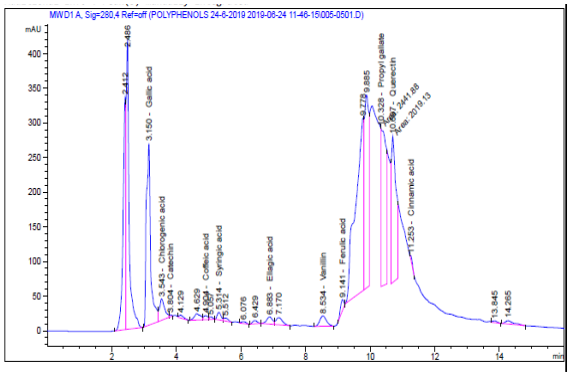

Fig. 2. HPLC-fingerprint chromatogram of EtOAc extract of *P. emblica* leaves

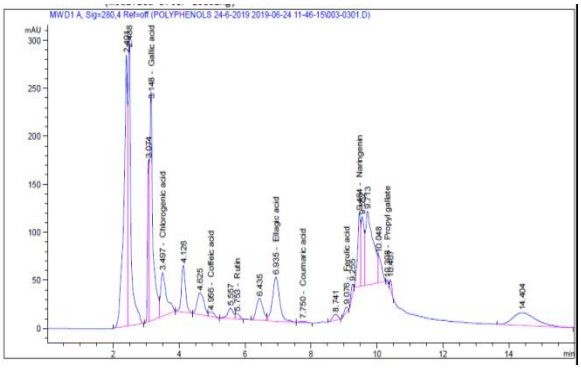

Fig. 3. HPLC-fingerprint chromatogram of *n*-butanol extract of *P. emblica* leaves

Table (2): The constituents of the *n*-butanol extract of *P. emblica* by reversed phase HPLC finger print with diode-array detection

Name of the component	Retention time (min)	Area%	Concentration (mg/g extract)
Gallic acid	3.148	14.2063	7.28
Chlorogenic acid	3.497	4.7173	7.01
Catechin	3.833	0.00	0.00
Caffeic acid	4.956	0.4688	0.14
Syringic acid	5.346	0.00	0.00
Rutin	5.753	0.4218	0.55
Ellagic acid	6.935	5.7601	5.66
Coumaric acid	7.750	0.2090	0.05
Vanillin	8.395	0.00	0.00
Ferulic acid	9.076	0.3176	0.08
Naringenin	9.464	3.8856	0.99
Propyl gallate	10.308	0.0859	0.02
Quercetin	10.703	0.00	0.00
Cinnamic acid	11.187	0.00	0.00

Table (3): The constituents of the ethyl acetate extract of *P. emblica* by reverse phase HPLC with diode-array detection

Name of the component	Retention time (min)	Area%	Concentration (mg/g extract)
Gallic acid	3.150	11.9419	11.51
Chlorogenic acid	3.543	1.7232	4.82
Catechin	3.804	0.0770	0.37
Caffeic acid	4.904	0.2620	0.15
Syringic acid	5.314	0.4579	0.31
Rutin	5.783	0.00	0.00
Ellagic acid	6.883	0.6411	1.19
Coumaric acid	7.706	0.00	0.00
Vanillin	8.534	0.00	0.64
Ferulic acid	9.141	0.5606	0.26
Naringenin	9.450	0.00	0.00
Propyl gallate	10.328	12.2119	4.97
Quercetin	10.697	10.0978	13.94
Cinnamic acid	11.253	0.1473	0.02

3.4.1. Structural elucidation of identified compounds 1 and 2

Compound (1) was isolated as yellow powder with melting point (225-229 ˚C). ^{1}H-NMR (DMSO-d_6) spectra of compound 1 showed peaks characteristic for a flavonol moiety. (ring A) a tetra substituted phenyl ring system was presented by two signals at δ_H 6.3 (1H, s, H-6) and 6.9 (1H, s, H-8). Ring-B a symmetrically disubstituted phenyl ring was observed at δ_H 7.9 (2H, d, H-3', H-5'), 8.0 (2H, d, H-2', H-6'), while the anomeric proton was resonated at δ_H 5.26 (d, *J*= 7.0 Hz, H-1") (S1**)**. The ^{13}C-NMR (DMSO-d_6) spectra showed 15 carbon signals, including 7 quaternary aromatic carbons at δ_C 10425-164.58 ppm and a carbonyl group at δ_C 178.56 (C-4), two methine aromatic carbons at δ_C 98.55 (C-6) and δ_C 93.46 (C-8) of ring A, four methine carbons of ring-B at δ_C 130.6 (d, C-2', C-6'), and 115.15 (d, C-3', C-5') and δ_C 104.0 (C-1") (S2). The compound (1) is a phenolic compound containing a flavonoid moiety which is *O*-glycosidically linked at the C-3 position; it was elucidated as flavonol glycoside based on its spectral data and comparison with literature help. It was identified as kaempferol-3-*O*-β-D-glucoside (Figure 4), with trivial name astragalin [60-61].

Compound (2) was isolated as off white crystalline compound, m.p. 87-89 °C; it shows white in visible light, blue in UV. IR (KBr) absorptions bands appeared at 2926 cm^{-1} (aliphatic CH_2 stretching), 2857 cm^{-1} (aliphatic CH stretching), 1677 cm^{-1} (C=O), 1627 cm^{-1} (C=C), 1063.34 cm^{-1} (C-O) and 801 cm^{-1}signifies cycloalkane. In the ^{1}HNMR ($CDCl_3$, 500 MHz) of this compound six methyl group appeared at a range 1.06 -1.26 ppm, an olefinic proton at δ_H 5.37 and anomeric proton at δ_H 3.71 ppm (The low field signal may be due to the attachment of β-OH group at C-3 carbon). ^{1} H NMR ($CDCl_3$): δ_H 0.68 (3H, s, H-18), 0.78 (3H, d, *J* = 6.8 Hz, H-27), 0.80 (3H, d, *J* = 6.8 Hz, H-26), 0.81 (3H, t, *J* = 6.8 Hz, H-29), 0.89 (3H, d, *J* = 6.5 Hz, H-21), 1.15 (3H, s, H-19), 2.34 (2H, m, H-2), and 5.70 (1H, s, H-4) **(**S3). ^{13}C NMR spectrum showed 29 signals at δ_C 11.1, 12.5, 17.4, 18.6, 18.9, 19.8, 21.0,24.6, 24.7, 25, 27.2, 29.4,29.6 , 31.92, 32.7,32.8, 34, 36.0, 36.1, 37.29 37.4, 37.64, 39.3, 42.0, and 76.7 ppm in the aliphatic range (S4).

This data indicated that the compound (2) is sterol with a double bond at C-4 and C-5 and carbonyl carbon at C-3. This was confirmed by presence of two carbon signals at δ_C 127.8 and 180.6 ppm for C-4, C-5 and carbonyl carbon at δ_C 198.6 ppm. These spectral data are in closed agreement to those observed for Stigmast-4-en-3-one (Figure 4) [62-63].

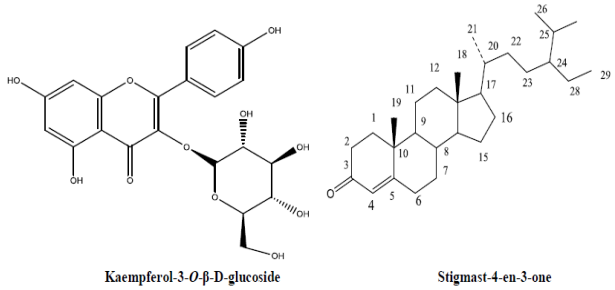

Fig. 4. Chemical structures of the isolated compounds

3.5. Antioxidant activity

It is well known that reactive oxygen species (ROS) produced *in vivo*, such as hydroxyl radical, superoxide anion and hydrogen peroxide, are highly reactive. Tissue damage resulting from an imbalance between ROS-generating and scavenging systems has been concerned in a variety of disease as cancer, diabetes mellitus, atherosclerosis, hypertension and aging [64-66]. Antioxidant is a compound that can postpone or prevent the oxidation of lipids and other molecules by inhibiting the oxidative chain reactions and which can thus stabilize and prevent cell damage done by oxygen. The consumption of natural antioxidant was reported to protect the human body against damage by ROS [67]. In recent years, there has been a great interest in finding natural antioxidants from plant resources. The antioxidants from plants, mainly polyphenols and flavonoids [68], have been reported to protect the human body from disease [69]. Additionally, the synthetic antioxidants have been questioned because of their toxicity. Therefore, there have been many researches to search for potential natural and possibly economic and effective antioxidants to replace the synthetic ones [70]. In our study, we aimed to investigate the

antioxidant and free radical scavenging properties of the extracts of *P. emblica* using DPPH, TAC, and RPAA methods

3.5.1. DPPH radical scavenging activity

The method is based on the scavenging of DPPH by antioxidants; DPPH is a stable free radical which forms a violet solution in methanol. It is reduced in the presence of antioxidant compounds, and decolorizes the DPPH methanol solution. The DPPH assay is an easy and rapid way to assess antioxidants. Results were reported as IC_{50}; the effective concentration value at which the DPPH radical is scavenged by 50% [71]. All the extracts were capable of scavenging DPPH free radicals. *n*-butanol extract had the lowest IC_{50} value 19.59 µg/ml (Table 4). This result could be due to the presence of a large amount of flavonoid and phenolic compounds in *n*-butanol extract as mentioned in table 1. From HPLC finger print results, *P. emblica* displayed to contain gallic acid, chlorogenic acid, catechin, caffeic acid, syringic acid, rutin, quercetin, ellagic acid, coumaric acid, vanillin, ferulic acid, naringenin, propyl gallate and cinnamic acid as illustrated in table 2. Quercetin and propyl gallate are potent antioxidant compounds in both *in vitro* and *in vivo* [72-73]. In our study the correlation of the total antioxidant potential and the total phenolic content is observed. These results are in agreement with others, who have shown that there is a linear correlation between phenolic content of plants and antioxidant effect. Phenolic acids, flavonoids and polyphenols characterized by antioxidant activities due to their redox properties which make them act as hydrogen donors, reducing agents and singlet oxygen quenchers [74]. The antioxidant potential of phenolic constituents varies, depending on their structure. Also, there may be some interference rising from other chemical ingredients present in the extract [75].

3.5.2. Reducing power antioxidant assay (RPAA)

This assay used to measure the transferring capacity of Fe^{3+} to Fe^{2+}, which then reacts with $FeCl_3$ to form blue colored $(Fe^{3+})_4[Fe^{2+}(CN^-)_6]^3$ complex that has an absorption maximum at 700 nm. The reducing power is correlated with electron transfer ability of the sample. Increased absorbance of the reaction mixture indicated increased reducing power of the plant extract, reducing power was reported as ascorbic acid equivalent per gm of dry sample [76]. The absorbance values of the reducing power antioxidant assay (RPAA) of *P. emblica* were 0.876, 0.432, 0.618, 0.871 and 0.948 respectively for 90% methanol, 85% methanol, 70% methanol, petroleum ether, methylene chloride, EtOAc and *n*-BuOH extracts compared to 0.970 of the positive standard ascorbic acid at concentration 200 µg/ml (Table 4). Based on the results the *P. emblica* extracts able to transfer the Fe^{3+} into Fe^{2+}, and have an antioxidant potential on a concentration dependent manner with respect to the ascorbic acid as a standard [77]. The reducing power was found to be varied in different solvent extracts. The difference in the antioxidant potential of these extracts may be attributed to the differences in the amount and types of antioxidant compounds present in the extractives.

3.5.3. Total antioxidant capacity (TAC)

The total antioxidant capacity of the extracts was measured by phosphomolybdenum method which is based on the reduction of Mo $^{+6}$ to Mo $^{+5}$ and then formation of green phosphate/Mo (V) compound with a maximum absorption at 695 nm. This method is a quantitative one, since a high absorbance value of the sample indicates its strong antioxidant capacity [77-78]. Total antioxidant capacity of *P. emblica* extracts and its fractions were ranked as follows: 90% MeOH (615.41 ± 0. 79) > *n*- butanol (543.62 ± 1.12) > ethyl acetate (500.41 ± 1.27)> methylene chloride (281.65 ± 2.14) > petroleum ether (216.92 ± 1.06) (Table 4).

Table (4): The antioxidant potential of the different extracts *P. emblica* leaves

Sample	Free radical scavenging potential DPPH IC_{50} [µg/ml]	Reducing Power antioxidant assay (RPAA)	Total antioxidant capacity (mg AAE /g ext.)
90% MeOH	28.19. ± 1.09	0.876	615.41 ± 0. 79
Petroleum ether	87.11 ± 0.99	0.432	216.92 ± 1.06
Methylene chloride	76.84 ± 1.92	0.618	281.65 ± 2.14
Ethyl acetate	33.23 ± 0.09	0.871	500.41 ± 1.27
n-butanol	19.59 ± 0.98	0.948	543.62 ± 1.12
Ascorbic acid		0.970	

Results are means ± SD, All experimental carried in triplicates (n=3).
A higher DPPH radical-scavenging activity is associated with a lower IC_{50} value.

3.6. Anticancer activity

Plant extracts are a complex mix of compounds and it is not trivial to interpret which molecules mediate their biological effects. It may be that combinations of molecules from these extracts work in synergy with one another to attain diverse biological activity. In our study the anticancer activity of different extracts of *P. emblica* was evaluated against two human tumor cell lines, namely, (HepG-2) hepatocellular carcinoma and (MCF-7) mammary gland breast cancer. The

American National Cancer Institute guidelines reported that Samples with IC_{50} less than 30µg/mL were considered active [80]. The results showed that *P. emblica* *n*-butanol extract was active against HepG-2 and MCF-7 cell lines with an IC_{50} of 25.63 and 22.80µg/mL, respectively, compared to Doxorubicin (DOX) (Table 5, Figure 5). The cytotoxic activity of the *n*-butanol extract on all cell lines was much higher than that of the other extracts, likely due to the *n*-butanol extract possessing much higher contents of phenolics and flavonoids when compared to the other extracts. Many previous reports revealed that some of the known molecules held within the *P. emblica* extract, identified by (HPLC) high pressure liquid chromatography, have proven anticancer properties as hydrolysable tannin (ellagic acid, corilagin, pyrogallol, chebulagic acid, gallic acid etc), the flavonoid quercetin, vitamin C [56, 81-84].

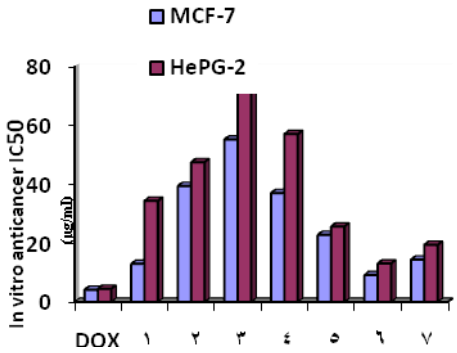

Fig. 5. The anticancer activity of *P. emblica* extracts and isolated compounds against human cancer cells (MCF-7 and HepG2) compared to DOX as standard

3.6.1. *Haemolysis assay*

The Red blood cells (RBCs) are the most abundant cells in the circulation that act as oxygen transporters. RBCs are an excellent model to investigate the cytotoxicity of any substances. One method of toxicity assessment is based on measuring the released haemoglobin from suspended RBCs which is called Haemolysis. Definitely, the rate of haemolysis is one of the cytotoxicity assays that evaluate the potential toxicity of RBCs [40]. Comparing samples and positive control that gave 100% haemolysis using RM One way ANOVA showed that samples were significant at different concentrations (p= 0.004). Results in Table (6) and Figure 6 showed that all samples were safe at a concentration of 125 µg/ml (LC0-9). This concentration was considered a safe dose to be used to study bioactivity.

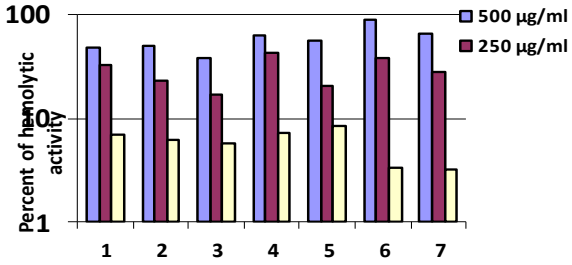

Fig. 6. The percent of haemolysis of samples at different concentrations (500, 250, and 125 µg/ml)

Table (5): The anticancer activity of the different extracts *of P. emblica* leaves and the isolated compounds against human cancer cells (MCF-7 and HepG2) compared to DOX as standard

No.	Sample	*In vitro* anticancer (IC_{50} ± SE) (µg/ml)	
		MCF-7	HepG2
1	90% MeOH	12.69±1.0	34.40±2.5
2	Petroleum ether	39.43±2.9	47.49±3.2
3	Methylene chloride	55.23±3.6	75.70±3.9
4	Ethyl acetate	37.05±2.7	57.09±3.4
5	*n*-butanol	22.80±1.9	25.63±2.1
6	Compound (1)	9.12±1.3	13.07±0.84
7	Compound (2)	14.39±1.1	19.47±0.58
	DOX	4.17±0.2	4.50±0.2

3.6.2. *Cytopathic effect (CPE) inhibition assay*

Cytopathic effect (CPE) means structural changes in host cells that are caused by viral attack. The cytopathic effect (CPE) inhibition assay is one of the most common bioassays for antiviral potential. This bioassay depends on the ability of the compound to provide animal cells resistant to viral attack. It involves the incubation of compounds with animal cells that sensitive to damage by a specific virus. The percentage of cells that survive after virus addition is relative to the antiviral effect of the compound present in the assay sample [44]. The *in vitro* antiviral activity of different extracts and purified compounds were evaluated through the assessment of the inhibition caused by the compound on the cytopathic effect produced by hepatitis A virus in Vero cells. The results in Table (7) and Figure (7) showed that most of the samples had an observed antiviral activity at the first concentration (50 µg/ml). 90% MeOH and petroleum ether had remarkable antiviral activity (Anti HAV). Methylene chloride and compound (1)

had moderate antiviral activity. The n-butanol extract and compound (2) had low antiviral activity. Ethyl acetate extract has no antiviral activity. Statistical analysis using One-way ANOVA showed that samples were highly significant at different concentrations (p= 0.002).

Table (6): Percent of hemolytic activity of different extract of *P. emblica* and the pure isolated compounds

No.	Sample	Percent of hemolytic activity		
		500 µg/ml	250 µg/ml	125 µg/ml
1	90% MeOH	47±3	32±3.5	7±1
2	Petroleum ether	49.7±2	23±2.6	6.3±0.8
3	Methylene chloride	38±4	17±1	5.8±4.6
4	Ethyl acetate	63±3	41.7±2.5	7.3±1
5	*n*-butanol	55.7±2	20.7±2.1	8.5±1.3
6	Compound (1)	86.7±2.1	38.3±1.5	3.3±1
7	Compound (2)	65±3.6	28±2	3.2±0.7

Table (7): The percent of antiviral activity of samples against HAV

No.	Sample	Percent of antiviral activity against HAV		
		12.5µg/ml	25 µg/ml	50µg/ml
1	90% MeOH	11.2±0.54	43.2±1	88.3±0.4
2	Petroleum ether	13.7±0.4	48.3±0.75	78.2±1.7
3	Methylene Chloride	9.6±0.23	23.2±1.3	55.3±0.15
4	Ethyl acetate	0	0	0
5	*n*-butanol	0.12±0.11	0.7±0.15	9±0.15
6	Compound (1)	1.4±0.2	12.65±0.27	51.3±2.6
7	Compound (2)	0.42±0.3	3.8±0.36	10.3±0.86

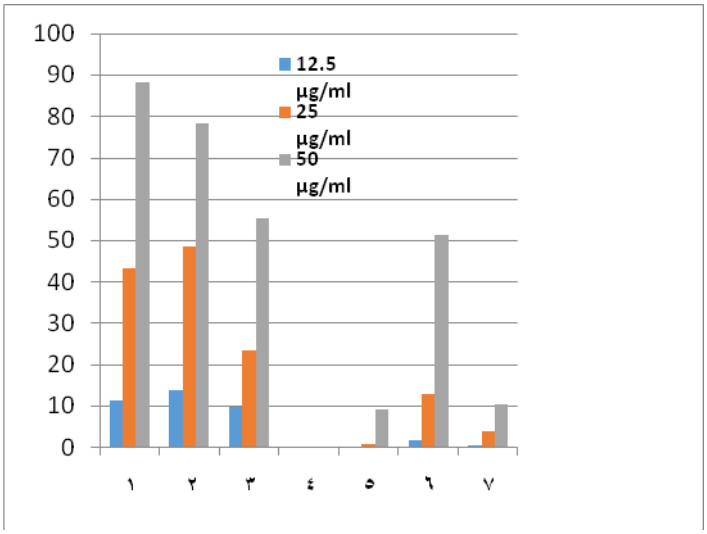

Fig. 7. Antiviral activity of the tested samples determined by the CPE method.

3.7. Anti-HCV assay

There is no hepatitis C virus vaccine available till now so the finding of potent antiviral natural plants and their bioactive components will help to evolve preventive cures against HCV infection. In the current study, *P. emblica* extracts and the Isolated compounds (1&2) were evaluated for their antiviral activities against hepatitis C virus (HCV) Tables (8 & 9) using a concentration (50, 25, 12.5µg/ml) according to IC_{50} detected on HepG2. The extracts showed anti HCV activities compared to the positive control (OD = 0.37± 1.1). The results in Figures (8 & 9) are compatible with the traditional antiviral assay; CPE. Our results revealed that the extracts showed antiviral activity against HCV infection, where the methanolic extract showed a higher antiviral effect than other extracts, this result may be attributed to presence of several constituents such as flavonoids, phenolics, alkaloids, terpenes, steroids and glycosides in the methanol extract. These constituents react with each other in methanol extract leading to a synergistic effect and so higher antiviral activity than the purified isolated compounds.

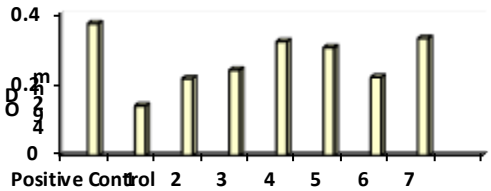

Fig. 8. This graph represents the results obtained in ELISA to detect the anti-HCV activity. The Bars represent the mean ± SD of OD for each sample in triplicate.

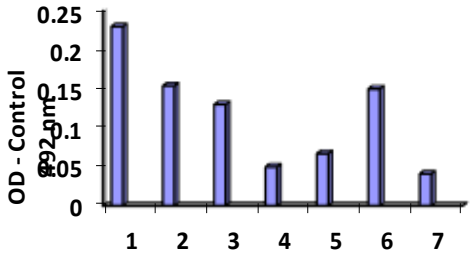

Fig. 9. This graph represents the results obtained in ELISA to detect the anti-HCV activity after subtracting the positive control value. So, in this graph high value represents high anti-HCV activity.

Table (8): The Anti -HCV activity of the samples obtained in ELISA

No.	Sample	Anti -HCV activity (OD)
	Positive Control	0.37± 1.1
1	90% MeOH	0.14± 0.13
2	Petroleum ether	0.216± 0.84
3	Methylene chloride	0.24±0.97
4	Ethyl acetate	0.320± 0.58
5	*n*-butanol	0.303±1.4
6	Compound (1)	0.220± 0.35
7	Compound (2)	0.329± 0.71

Table (9): The Anti -HCV activity of the samples obtained in ELISA after subtracting the positive control value

No.	Sample	Anti -HCV activity after subtracting the positive control value
1	90% MeOH	0.23
2	Petroleum ether	0.154
3	Methylene chloride	0.13
4	Ethyl acetate	0.05
5	n-butanol	0.067
6	Compound (1)	0.15
7	Compound (2)	0.041

4. Conclusion

In our study, *P. emblica L.* extracts and the isolated compound (1&2) from *n*-butanol extract were assayed for its potential as anti-hepatitis A virus (HAV) and anti-hepatitis C virus (HCV) agents via observing cytopathic effects. The cytotoxicity on (HePG-2) hepatocellular carcinoma as well as (MCF-7) mammary gland breast cancer cells was also evaluated by MTT assay. Results revalued that there are obvious variation between the tested extracts in their bio-activities which could be due to different chemical classes in each extract. Furthermore, the antioxidant and cytotoxic bio-assays revealed that *n*-butanol extract was proven to be more effective than the other extracts. Thus further exploration can be carried out on *P. emblica* extracts to enhance their use as a valuable drug.

5. Acknowledgments

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6. Conflicts of interest

There are no conflicts of interest

7. Abbreviations

°C: Degree in Celsius; ^{13}C-NMR: Carbon 13 Nuclear Magnetic Resonance; ^{1}H-NMR: Proton Nuclear Magnetic Resonance; AAE: Ascorbic Acid Equivalent; Ac: Absorbance of Control; ACI: American Cancer Institute; As: Absorbance of Sample; ATCC: American Type Culture Collection ; cm: Centimeter; cm^{-1}: $Centimeter^{-1}$; Conc.: Concentration; CPE: Cytopathic Effect; d: Doublet; dd: Doublet of Doublet; DMSO- d6: Dimethyl Sulfoxide-deutrated6; DNA: Deoxyribonucleic acid; DOX: Doxorubicin; DPPH: 1,1'- Diphenyl-2-PicrylHydrazyl; FBS: fetal bovine serum; g/ml: Gram/Milliliter; g: Gram; GAE /g ext: Gallic acid equivalents per gram extract; HCC: hepatocellular carcinoma; HCV: Hepatitis C virus; HepG-2: hepatocellular carcinoma; HPLC: High Performance Liquid; Chromatography; hr: hour; IC_{50}: Median Inhibitory concentration; IR: Infra-Red; *J*: Coupling Constant; l: Liter; M: Molar; MCF-7: mammary gland breast cancer; mg: milligram; MHz: Mega Hertz; min: minute; ml/min: milliliter per minute; ml: Milli Liter ; mM: Milli Molar; mm: Milli Mole; MTT: *Microculture Tetrazolium*.; PBS: Phosphate Buffer Saline; PCR: Polymerase Chain Reaction; ppm: Part Per Million; RBCs: Red blood cells; RE: Rutin Equivalent; ROS: Reactive Oxygen Species; RPAA: Reducing Power Antioxidant Assay; Rpm: Round per minute; S.D.: Standard Deviation; s: singlet; S1: Supplementary number 1; SC50: Median Scavenging Concentration; TAC: Total Antioxidant Capacity; TFC: Total Flavonoid Content; TMS: Tetramethyl Silane; TPC: Total Phenolic Content; UV: Ultraviolet; UV-Vis: Ultraviolet-Visible; v/v: Volume/Volume; δ: Chemical Shift; δ_C: Carbon Chemical Shift; δ_H: Proton Chemical Shift; λmax: Wave Length Maximum; µg/ml: Microgram / Milliliter; µg: Microgram; µl: Microlitre; µM: Micro Molar; µm: Micro Mole; ν_{max}: Frequency Maximum.

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