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Chemical and biological evaluation of *phyllanthus amarus* as antiviral agent

Doha H Abou Baker[1], Waleed M El Senousy[2] and Souad El Gengaihi[1]*

[1]Medicinal and Aromatic Plants Dept., Pharmaceutical and Drug Industries Division, National Research Centre, Cairo, **Egypt.**
[2]Virology lab., Water Pollution Research Dept., Environmental Research Division, National Research Centre, Cairo, **Egypt.**

***Correspondence:** souadgengaihi@hotmail.co.uk **Accepted: 23** Dec.2019 **Published online:** 26 Feb. 2019

Phyllanthus amarus (Schum and Thonn) family Euphorbaceae, is a medicinal herb used extensively in many countries for variant medical purposes. The objective of the present study was to investigate the antiviral activity of the extracts as well as isolation and identification of the bioactive compounds. Promising HCV reduction was recorded with nontoxic dose of ethyl acetate extract (5.8 µg/ml). Two lignans were isolated and identified by spectral tools; phyllanthin and hypophyllanthin, in addition to lignan lactone patavin.

Keywords: *Phyllanthus amarus* ; antiviral; lignans; isolation; identification.

INTRODUCTION

The genus Phyllanthus consists of about 650 species of the family Euphorbaceae in 300 genus. Two hundred genera are American, One hundred African, 70 from Madagascar while the remaining are from Asia and Australia **(**Webster et al., 1994)The name Phyllanthus means leaf and flower because the two organs become or combined together (Cabieses, 1993).

The most famous and important species of this genera is *P. amarus, P. niruri* and *P. urinaria*.

This investigation is focused on *P. amarus* shum and Thoon, in which 76% of the market samples contained or included this species worldwide.

Alkaloids flavonoids, tanins (hydrolysable) polyphenols, sterols and triterpenes in addition to volatile oils, all are the secondary products found in most of the species (Chevallier, 2000, (Srivastava et al., 2008, Patel et al., 2011 and (Khatoon et al., 2006)

The analytical techniques used are concentrated on HPLC analyses which are developed and validated as an easily methods for standardized extract of *P. amarus*. The important lignan found in Phyllanthus species are: hypophyllanthin, phyllanthin, are confirmed by their abundant molecular adduct ions, and mass spectra as compared with reference compounds (Shanker et al., 2011)

P. amarus has been reported to exhibit pharmacological effects as: antimicrobial, anti-viral against hepatitis viruses, antimutagenic and possess hypoglycaemic activities. The leaves exhibit immunomodulatory activity. Ellagitanins were found to be the most potent madiators of the antiviral HIV activity. The two major lignans found in the leaves phyllanthin and hypophyllanthin possessed antitumor activities against EAC in swiss albino mice (Adeneye and Benebo 2008, (Krithika and Verma 2009 and Lee et al., 2011)

The present investigation aims to introduce this plant to the Egyptian condition in order to use it as a hepatoprotective plant with antiviral

activities against different haptic viruses specially virus C. However the condition of adaptation failed to produce successful yield of herb as a material for the investigation. So, a quantity of leaves was obtained from prolabo company U.S.A. which we acknowledge this gift in a trial to isolate its components then evaluate its biological activity.

MATERIALS AND METHODS

Virology Studies

Cytotoxicity test

It was done according to Simões [11] and Walum [12]. Briefly, all samples (100 mg) were dissolved in 500-1000ul of water or solvents. Decontamination of samples was done by adding 12-24µl of 100 x of antibiotic-antimycotic mixture to 500-1000µl of reach sample. Then, bi-fold dilutions were done to 100µl of original dissolved samples and 100µl of each dilutions were inoculated in Hep-2 and Hep G2 cell lines (obtained from the Holding Company for Biological Products & Vaccines VACSERA, Egypt) previously cultured in 96 multi well plates (Greiner-Bio one, Germany) to estimate the non toxic dose of the tested samples. Cytotoxicity assay was done using cell morphology evaluation by inverted light microscope and cell viability test applying trypan blue dye exclusion method.

Cell morphology evaluation by inverted light microscopy

Hep-2 and Hep G2 cell cultures (2 x 10-5 cells/mL) were prepared in 96-well tissue culture plates (Greiner-Bio one, Germany). After 24h incubation at 37°C in a humidified 5% (v/v) CO2 atmosphere cell monolayers were confluent, the medium was removed from each well and replenished with 100µl of bi-fold dilutions of different samples tested prepared in DMEM (GIBCO BRL). For cell controls 100µl of DMEM without samples was added. All cultures were incubated at 37°C in a humidified 5% (v/v) CO2 atmosphere for 72h. Cell morphology was observed daily for microscopically detectable morphological alterations, such as loss of confluence, cell rounding and shrinking, cytoplasm granulation and vacuolization. Morphological changes were scored as adopted by Simões (Simões et al., 1999)

Cell viability assay

It was done by trypan blue dye exclusion method (Wallum et al., 1990)

. Hep G2 cell cultures (2 x 10-5 cells/mL) were grown in 12-well tissue culture plates (Greiner-Bio one, Germany). After 24h incubation, the same assay described above for tested samples cytotoxicity was followed by applying 100µl of tested samples dilutions (bifold dilutions) per well. After 72h the medium was removed, cells were trypsinized and an equal volume of 0.4% (w/v), trypan blue dye aqueous solution was added to cell suspension. Viable cells were counted under the phase contrast microscope.

Antiviral effect of tested samples on adenovirus type 40

Seventy five microliters of nontoxic dilutions were mixed with 75µl of 1 x 10-5 infections viral particles/mL of adenovirus type 40 provided by American Type Culture Collection (ATCC). Then the mixture was incubated overnight at 4°C. Inoculation of 100µl of 10 fold dilutions of treated and untreated adenovirus was done into Hep-2 cell line in 12 multi well-plates. After 1h incubation for adsorption at 37°C, 1 mL medium (DMEM) was added to each well. The cell line was observed daily for one week then, three times freezing and thawing for tested plates were done. Nested PCR was done for confirmation of adenovirus (presence/absence) in each well [13].

Cell culture-RT-PCR (CC-RT-PCR) test to quantify the number of infections particles of HCV before and after treatment of natural products

Serial dilutions (10 fold dilutions) of HCV untreated or treated with natural products was done and then inoculated on Hep G2 cell line or 7721 cell line, RT-PCR for all the dilutions was done at intervals after inoculation (3,6,9,12,15 days). The number of infectious units was calculated as the reciprocal of the end dilution positive for HCV.

Extraction and isolation

Dried herb of *P. amarus* obtained from Prolabo Comp. USA, was extracted with 50%. MeOH then evaporated, and the residue obtained was mixed with silica gel for column (small amount) put on the top of the column filled with silica gel. The column was eluted with hexane 100% then with EtOAc with the following ratios: Hexan: EtoAc (9:1), (8:2), (7:3) and then (6:4) the eluted portions (50ml each) were monitored on HPLC plates with solvent system hexane: acetone: EtOAc (74:12:8) (Krithika et al., 2009), fractions proved to contain lignan (give blue color

with vanillin) were combined together, evaporated and then purified by small column of sephadex LH20 eluted with 50,80 and 100% then with MeOH.

On another portion of the dried powder, successive extracts were performed using gradient solvents with increasing polarities i.e. pet-ether, $CHCl_3$, EtOAc and finally methanol each solvent was evaporated under reduced pressure and the residues obtained were used in virology investigations.

HPLC fractionation of phenolic compounds

The MeOH extract of *P. amarus* leaves was HPLC evaluated for its phenolic acids. The HPLC system was HP 1100 chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with an auto-sampler, quaternary pump and a diode array detector. The measurements were integrated by Chemstation chromatographic software interfaced to a personal computer.

The analytical column was ZORBAX Eclipse XDB C18 column (15 cm x 4.6μm I.D., 5μm, USA). Operative conditions were: mobile phase A, MeOH; mobile phase B, 2% acetic acid; flow rate, 1mL min; fixed wavelengths, 280, 320 and 360nm; injected quantity, 20 uL; elution program, A (%)/B (%): 0 min 5/95; 10 min 25/75; 20 min 50/50; 30 min 100/0; 40 min 5/95. Identification of phenolic compounds was performed by comparison with the retention times of standard substances **(Leonardis et al.,2005)**

RESULTS AND DISCUSSION

Antiviral Activity

The extracts showed potential antiviral activity with low toxicity were studied as follow:

Serial dilutions $1x10^{-6}$ of the tested antiviral was prepared and its toxicity tests were checked using Huh 7.5 cell line.

Inoculation of 100µl of the antiviral dilution (from undiluted to 10^{-6}) or PBS plus 20µl of HCVCC per well was performed using Huh 7.5 cell line.

The medium was changed after one day incubation.

Analysis 72 hrs later using immune fluorescence and flow cytometry was done.

Extracts of pet ether, $CHCl_3$ and EtOAc of phyllanthus showed an antiviral activity against adenovirus type 40, resulted in 70, 50, and 70% reduction of initial viral dose respectively, Table (1).

Anti HCV of tested extracts

Pet-ether, $CHCl_3$ and EtOAc extracts of *P. amarus* were checked for the infectivity of HCV on Hep G2 cell line. About 10 to 30% reduction of the initial HCV infectious units were recorded, Table (2).

In the present investigation, the antiviral activity of plant extracts were checked by two model of viruses, enteric adenovirus type 40 which considered as one of the most important causes of gastroenteritis within entric viruses, and hepatic C virus which is a blood transmitted virus responsible for a lot of liver infections in Egypt. This virus causes cirrhosis, and liver cancer in advanced cases.

When comparing the effect of phyllanthus herb on both viruses *in vitro,* considerable percentage of reductions were observed. The effect of these extracts were more potent against adenovirus 40 than HCV virus (Table 2).

The extracts showing higher antivirus activity were tested for their cytotoxic activity which was compiled in Table 3.

Table 1. Antiviral activity of tested extracts against infectious adenovirus type 40.

Extract	Initial infectious adeno virus 40 titre	Final infectious adeno virus 40 titre	% Reduction
$CHCl_3$	1×10^{-5}	5×10^{-4}	50%
Pet. Ether	1×10^{-5}	3×10^{-4}	70%
EtOAC	1×10^{-5}	3×10^{-4}	70%

Table 2. Antiviral activity against infectious HCV.

Extract	Initial infectious	Final infectious HCV	% Reduction
Pet.ether	1×10^{-3}	8×10^{-2}	20%
$CHCl_3$	1×10^{-3}	9×10^{-2}	10%
EtOAc	1×10^{-3}	9×10^{-2}	30%

Table 3. Cytotoxicity and HCV reduction of different doses of tested extracts on Huh 7.5 cell line:

Tested material	Tested concentration	% of cytotoxicity	% of HCVcc reduction
Pet. Ether extract	34.4 mg/ml	10%	100%
	3.44 mg/ml	10%	100%
	344 µg/ml	0%	60%
	34.4 µg/ml	0%	50%
	3.44 µg/ml	0%	30%
	0.34 µg/ml	0%	40%
	0.03 µg/ml	0%	10%
EtOAc extract	58 mg/ml	30%	100%
	5.8 mg/ml	0%	100%
	580 µg/ml	0%	90%
	58 µg/ml	0%	70%
	5.8 µg/ml	0%	20%
	0.58 µg/ml	0%	10%
	0.05 µg/ml	0%	100%
MeOH extract	10 mg/ml	100%	100%
	1 mg/ml	100%	100%
	100 µg/ml	100%	100%
	10 µg/ml	70%	85%
	1 µg/ml	15%	30%
	0.1 µg/ml	10%	20%
	0.01 µg/ml	0%	5%

On Huh 7.5 cell line, the non- toxic doses of pet.ether and EtOAc extracts were 344 µg/ml and 5.8 mg/ml respectively. The two extracts have promising anti HCV activities ranged from 60 to 100% for the non-toxic concentrations respectively.

Non considerable reduction of HCV (5%) with non-toxic dose of MeOH extract was detected. The high toxicity of the previous extract in relation to other tested extracts may return to the toxicity of Methanol which is suitable for dissolving the residues obtained.

Chemical Investigation

To detect the compounds found in the extracts proved to have antiviral activity, these extracts were subjected to HPLC analyses to reveal its active constituents by two methods:

Fractionation and determination by HPLC.

Isolation using silica gel column then purification of the isolated compounds by sephadex LH_{20} column.

HPLC analyses using three waves length to detect all the compounds found in the 50% alcohol extract were performed. Table (4) complied the data recorded from the charts of HPLC obtained. The HPLC reveal the occurrence of different types of phenolic acids, caffeic acid was the major, followed by cinnamic.

Lignans were presented by phyllanthin and hypophyllanthin in amount of 21 and 29 µg/mg. These two lignans were considered as a marker for *P. amarus* spp. Flavonoids were also detected in the alcoholic extracts i.e. quercetin, rutin and kampferol with amounts ranged from 0.13 – 0.18 µg/mg.

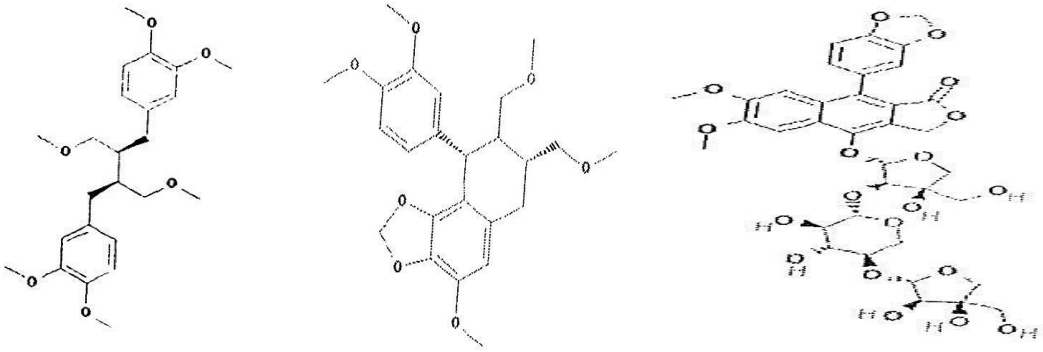
Isolation and Identification

Dried herb of *P. amarus* were obtained as a gift from Prolabo Comp. USA, was defatted with hexane then extracted with 50% MeOH at room temperature MeOH was evaporated under reduced pressure by a rotary evaporator at 40°C.

The residue obtained was mixed with silica gel to from an admixture and put on the top of silica gel column 500mm length x 20mm diameter. Gradient elution was done in the following sequence, hexane, hexane. EtOAc 98:2, 95:5 then with ratio of 90:10, 80:20 till 100 EtOAC in fractions of 50 ml.

Table 4. HPLC analysis of 50% MeOH extract

nm	Compounds	RT	µg/mg
280	Gallic acid	5.5	0.27
	Protochatchuic acid	9.5	0.11
	4-hydroxybenzoic acid	14.6	0.78
	Vanillic acid	25.1	1.72
	Phyllanthin	28.2	21.0
	Hypophyllanthin	29.7	29.0
320	Genitistic acid	16.5	5.1
	Caffeic acid	21.2	99.9
	Ferulic acid	32.1	10.9
	Sinapic acid	33.3	0.02
	Catchin	19.2	1.28
360	Rutin	36.0	0.19
	Rosmarinic acid	40.0	0.15
	Kampferol	46	0.13
	Syringic acid	22.4	0.03
	p-cumaric	37.2	2.09
	Quercetin	44.0	0.16
	Cinnamic acid	42.5	40.0



Phyllanthin (1) Hypophyllanthin (2) Patavine (3)

Figure 1. Isolated compounds from *P. amarus*

The fractions obtained were evaporated and rechromatographed on TLC using hexane-acetone-EtOAC (v/v 74:12:8) as mobile phase, described by krithika et al., (Krithika and Verma 2009)

. The compound revealed to be pure were again purified with small sephadex column LH_{20} eluted with 50-100% MeOH.

Compound No. 1, eluted from hexane-EtOAc 7:3 afford white needles. Electron impact mass with probable fragments at m/z 418 (molecular ion peak) 386, 354, 303, 177, 151 (the base peak) corresponding to dimethoxybenzyl fragment. The probable fragmentation pathway reveals ion of 386 and 354 due to successive elimination of MeOH. H NMR: δ 6.53 (2H, d), 6.60 (6H, dd), 2.52 (7H, dd), 2.03 (8H, m), 3.76 (oMe-3, s), 3.81(oMe-4, s), 3.25 (oMe-9, s). These fragmentation signals and HNMR coincide with phyllanthin, $C_{24}H_{34}O_6$.

The second compound eluted by hexane-EtOAc 6: 4 reveal the following electron impact mass with probable fragments of 430nm (molecular ion peak) 400, 354, 323, 208, 177, 166, 151, 135 (base peak), 91, 89, 77 which holds true with hypophyllanthin having molecular formula of $C_{24}H_{30}O_7$.

Patavine:

The third compound eluted by EtOAc as a

yellowish amorphous powder, give a bluish fluorescence under uv light similar to lignans. M/Z 799, 645, 381. The H-NMR spectrum showed three anomeric proton signals at d 5.67 (1H, d, J51.8 Hz), 5.06 (1H, d, J 52.0 Hz), and 4.57 (1H, d, J57.5 Hz) which go parallel with patavine. The isolated compounds were identified by their ESI-MS and NMR data, and compared with those previously reported by Somanabandhu (Innocenti et al., 2002) and (Somanabandhu et al., 1993) Fig 1.

CONCLUSION

In view of the results obtained from this investigation it can be deduced that *P. amarus* can be considered as an important remedy for liver diseases due to the lignans found in the plant.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

SEG, Corresponding author PI of the project, the role of SEG is designed the study, brought the plant material, arranged the results and discussion and wrote the manuscript.
DHA, performed the extraction, isolation and identification of the isolated compounds, help in formatting process of the journal.
WHE, performed the viral experiments (cytotoxicty and antiviral effect).

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