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(Research Article)

In vitro cytotoxic activity of *Phyllanthus amarus* Schum. & Thonn.

S. S. Sravanthi Pammi [1,*] and Archana Giri [2*]

[1] Department of Botany, SVRK (M) GDC, Nidadavole, W.G.Dt., Andhra Pradesh, India.
[2] Centre for Biotechnology, Institute of Science and Technology, Jawaharlal Nehru Technological University Hyderabad, Kukatpally, Hyderabad, India.

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Abstract

Some bioactive compounds from plants are excellent sources of anticancer drugs. These natural phytochemicals are used in active research for cancer prevention and treatment. In our present study *invitro* anticancer activity was evaluated using dimethylformamide leaf extract of *Phyllanthus amarus* as its GC-MS analysis revealed many active principles which exhibited good antimicrobial and antioxidant properties. There were reports that anti-proliferative activity is always coupled with antioxidant activity. Anti-cancer activity of the *P. amarus* leaf extract was tested against HCT 15 and T47D cell lines and inhibitory effect on HCT 15 cell line was found to be greater than T47D cell line. With the increasing concentration of extract, the percentage of viability of cell lines was found to be decreased for both the cell lines. The anticancer activity of leaf extract of *P. amarus* is comparable to positive control drug doxorubicin. N-Hexadecanoic acid, lignans and polyphenol compounds in leaf extract may be responsible for the anticancer activity. These phytochemicals block cancer cell propagation by controlling cancer stem cells and can influence all the stages of cancer development effectively.

Keywords: Anticancer; *Phyllanthus amarus*; Medicinal plants; GC-MS; Cell lines; MTT assay

1. Introduction

Cancer is a serious disease caused by invasive growth of cells which tend to proliferate rapidly causing malignancies in body. Cancer cells are formed as a result of imbalance in body metabolism and destroy healthy cells of our body [1]. Cancer cells overlook the signals that normal cells take; thereby disturbing the process of programmed cell death (apoptosis). After cardiovascular disease, cancer is the leading cause of death [2] as it is related with complex mechanisms both at cellular and molecular level. Natural sources such as plants, micro-organisms and marine-organisms serve about 60% of the total anti-cancer agents [3]. Medicinal herbs exhibit anti-cancer activity because of their excellent anti-oxidant and immunomodulatory properties. Phytochemical rich diet lessens cancer risk by 20%. These phytochemicals are generally natural plant derived secondary metabolites. Many challenges were faced during cancer treatment as patients undergo various types of therapies such as radiation, chemotherapy etc. In low and middle income countries (LMIC), over 20 million new cancer cases are expected annually as early as 2025[4]. Anti-cancer agents avoid or repress the growth of cancer. Plant derived compounds are gaining insight for exploiting novel pathways in cancer therapy. Plant based drugs have fewer side effects and research in plants is in continuous progress for isolating the active principles for curing various types of cancers in an effective way. Medicinal plants such as *Podophyllum peltatum, Taxus brevifolia, Camptotheca acuminate, Cephalotaxus harringtonia, Catharanthus roseus etc.* have been reviewed and compounds such asbetulinic acid, combrestatin and silvesterol were found to be responsible for

*Corresponding author: Archana Gir; E-mail: archanagiriin@jntuh.ac.in
Centre for Biotechnology, Institute of Science and Technology, Jawaharlal Nehru Technological University Hyderabad, Kukatpally, Hyderabad, India.

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anticancer activity [5]. Lignan compounds present in plants proved to play an important role in reducing risk of many cancers such as breast cancer, uterus cancer, ovarian cancers and other estrogen-related cancers [6].

P. amarus is a well-known medicinal herb belonging to family Euphorbiaceae. It is commonly known as Bhui amla and contains many active constituents which cure wide range of diseases. Alkaloids, terpenoids and other secondary metabolite compounds of plant are found to exhibit anti-cancer properties [7]. Lignan compounds such as phyllanthin, hypophyllanthin, nirtetralin, phyltetralin have been identified from extracts of *P. amarus* [8]. This plant contains an array of flavonoids such as quercetin, rutin, kaempferol, astragalin and quercitrin [9]. Tannin precursors such as Gallic acid, ellagic acid, simple tannins such as 1,6-digalloylglupyranose, 4-O-galloylquinic acid and complex tannins such as geraniin are also present in this plant [8]. Plant parts of *Phyllanthus amarus* were studied using different solvents for their metabolic fingerprinting studies [9] [10], antimicrobial activity [11] [12] and anti-oxidant activity [13] [14]. Gallic acid and quercetin inhibited cell cycle in G1 phase by inactivating cdc25A phosphorylation thereby inducing apoptosis in tumor cells. This is done by activating caspase activity and reducing cyclin D production [15] [16]. Phytochemical investigation revealed the presence of lignan and polyphenol compounds [17] [12]. The presence of gallic acid, gereniin, quercetin and rutin in *P. amarus* which exhibit anticancer properties were reported [18]. Plant derived bioactive compounds exert anticancer effects by different mode of action such as interferons induced cell death, cell death by DNA damage, autophagy induction by activation of tyrosine kinase and proteosome inhibitors[19]. Induction of programmed cell death is by use of glucocorticoid hormones and disconnection of cellular metabolism by limiting tumor cell growth. Other mechanisms include triggering apoptosis by inducing mutations in cancer cells, interfering with DNA replication by use of alkylating agents, cross linking in DNA strands by use of heavy metals, blocking of nucleic acid synthesis in cell cycle by use of antimetabolite compounds, DNA fragmentation by using mixture of glycopeptides, preventing reunion of DNA double helix during replication by stabilizing the DNA tomoisomerase II complex, preventing DNA replication by Topoisomerase inhibitors and inhibiting mitotic spindle formation by blocking tubulin synthesis [20]. Phyllanthus species were also reported to arrest cell cycle at different phases. The mechanism of action is due to interference of protein synthesis and DNA synthesis machinery. The property of uncontrolled proliferation is lost when cells cycle is arrested [21]. *P. amarus* was found to treat breast cancer by diminishing the potential of mitochondrial membrane, increasing reactive oxygen species intracellularly, upregulation of caspase -3 expression and down-regulation of Bcl-2 expression [18]. Four species of Phyllanthus *viz. P. amarus, P.niruri, P.urinaria* and *P.watsonii* were found to exhibit anti-angiogenic effect by inhibiting capillary tube formation and anti-metastatic effect by decreasing the ability of cancer cell invasion and migration [22]. Hepatocarcinoma was treated using *Phyllanthus urinaria* by inducing the production of TNF by inhibiting the expression of antiapoptotic genes and secondary tumour development [23]. GC-MS analysis of dimethylformamide leaf extract revealed the presence of a compound N-Hexadecanoic acid that is responsible for anticancer activity. Our study reports the effect of dimethylformamide extract of *P. amarus* leaf on cancer cell lines (HCT 15 and T47D).

2. Material and methods

2.1. Anti-cancer activity of Dimethyl formamide leaf extracts of *P. amarus*

The cytotoxic effect of Dimethyl formamide leaf extract was tested against Human colorectal adeno carcinoma cancer cell line (HCT 15) and Human breast cancer cell line (T47D). Dulbecco's Minimal Essential Medium (DMEM) was used for cell culture studies.

2.2. Cancer cell line development and maintenance

The Human colorectal adeno carcinoma (HCT 15) and Human breast cancer (T47D) cell lines were obtained from the National Center for Cell Sciences (NCCS), Pune (Table 1). HCT 15 and T47D cancer cell lines were maintained in Dulbecco's Modified Essential Medium (DMEM) supplemented with 4.5 g/l glucose, 2mM L-glutamine and 5% fetal bovine serum (FBS) at 37^0C in 5% CO_2 incubator (Thermo scientific, USA). Cells from exponentially growing culture were used for experimental purpose. The cancer cell lines were maintained successfully in required laboratory conditions and used for further studies i.e., Trypan blue staining and MTT assay.

Table 1. Nature of cancer cell lines used for evaluating anti-cancerous activity of *Phyllanthus amarus*

Cell line	Morphology	Origin	Species	Growth mode	Supplier
HCT15	Epithelial	Colon	Human	Adherent	NCCS, Pune
T47D	Epithelial	Breast	Human	Adherent	NCCS, Pune

2.3. Cell viability assay using Trypan blue dye

Trypan Blue is an essential blue coloured acid dye, consisting of two azochromophore group widely used for studying the number of viable cells present in a population. This dye does not penetrate into the cell wall of live cells which are grown in culture.

Procedure: The cells were suspended in a known quantity of PBS and the cell count was adjusted to $1x10^6$ cells/ml. The *P. amarus* leaf extract at various concentrations (0-0.2 mg/ml) were prepared from the stock solution (10 mg/ ml) in phosphate buffer saline solution (PBS). The PBS solution was added to all the tubes containing plant extract and the final volume was made to 800 µl with PBS. 100µl of HCT 15 and T47D cell lines in phosphate buffered saline was added to the tubes. A control having solvent alone was also prepared. They are then incubated at 37^0C for 3 hours and 100 µl of trypan blue was added to all test tubes. Cell counts were done using trypan blue dye exclusion method on haemocytometer by counting stained (non-viable) and unstained (viable) cells. Cell viability assay results were expressed as percentage of cell viability [24].

Calculation:

$$\text{Viability percentage } = \frac{\text{live cell count}}{\text{total cell count}} \times 100$$

2.4. Cytotoxic assay of cell lines by MTT assay- (4, 5-diMethyl Thiazol-2-yl) - 2, 5- diphenylTetrazolium bromide

MTT assay is based on the capacity of Mitochondrial succinate dehydrogenase enzyme in living cells to reduce the yellow water soluble substrate 3- (4, 5-dimethyl thiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT) into an insoluble, purple blue colored formazan product which can be measured. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells [25]. The trypsinized cells from T-25 flask were seeded in each well of 96-well flat bottom tissue culture plate at a density of $5x10^3$ cells/well in growth medium and cultured at 37^0C in 5% CO_2 adhere. After 48hrs incubation, the supernatant was discarded and the cells were pre-treated with growth medium and were subsequently mixed with different concentrations of leaf extract (0-0.2mg/ml) using dimethylformamide solvent and then incubated for 48 hrs at 37^0C in Co_2 incubator. The supernatant growth medium was removed by aspiration. Each well is then added with 5 µl of fresh MTT (0.5 mg/ml in PBS) followed by incubation for 2hr at 37^0C in dark. Formazan crystals formed after incubation were solubilised with 100µl of DMSO and incubated for 30min. The absorbance (OD) of the colored product in culture plate was read at a wavelength of 570nm on an ELISA reader (Thermo scientific multiscan, USA). Optical density is directly correlated with cell quantity. Culture medium along with DMSO without plant extract was used as control. The absorbance values which are lower than the control cells indicate a reduction in the rate of cell proliferation. Anticancer drug doxorubicin was used as a reference compound for determination of anticancer activity of cell lines. IC_{50} values were calculated from the graph of percentage inhibition against sample concentration.

Calculation:

% Cell survival= $\{(A_t\text{-}A_b)/ (A_c\text{-}A_b)\}$ x100

Where,
A_t= Absorbance value of test compound.
A_b= Absorbance value of blank.
A_c=Absorbance value of control.
% cell inhibition= 100 - cell survival.

2.4.1. Statistical analysis

The triplicate data were analyzed as mean ± standard deviation. Data was statistically analyzed by Graph pad prism (ver. 7.0.1).

3. Results and discussion

3.1. Anti-cancer activity of dimethylformamide leaf extract of *Phyllanthus amarus* against HCT 15 and T47D cell lines

The cytotoxic effect of plant extract was tested by the MTT assay, which showed the effect of its secondary metabolites on the cell viability in HCT 15 and T47D cancer cell lines. The percentage viability of the HCT 15 and T47D cancer cells were calculated before treating with various concentrations of plant extract. 97.87% of viability was shown by HCT 15 cell lines and 98.44% of viability was shown by T47D cell lines which are most suitable to perform MTT cytotoxicity studies (Table 2).

Table 2 Percentage of cell viability of cancer cell lines.

Cell line	% viability	Live cell count	Total cell count	pH
T47D	98.44%	2.473×10^3	2.512×10^3	7.5
HCT 15	97.87%	2.528×10^3	2.583×10^3	6.9

3.2. Trypan blue staining

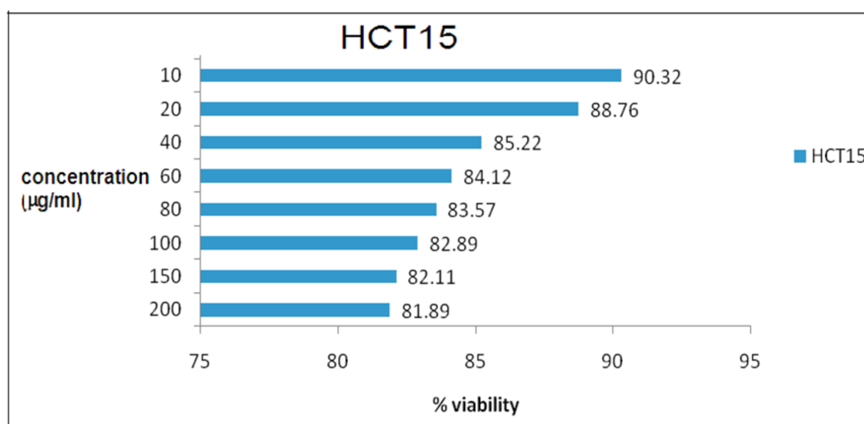

Figure 1 Effect of different concentrations of leaf extract of *P. amarus on* viability percentage of HCT15 cell lines

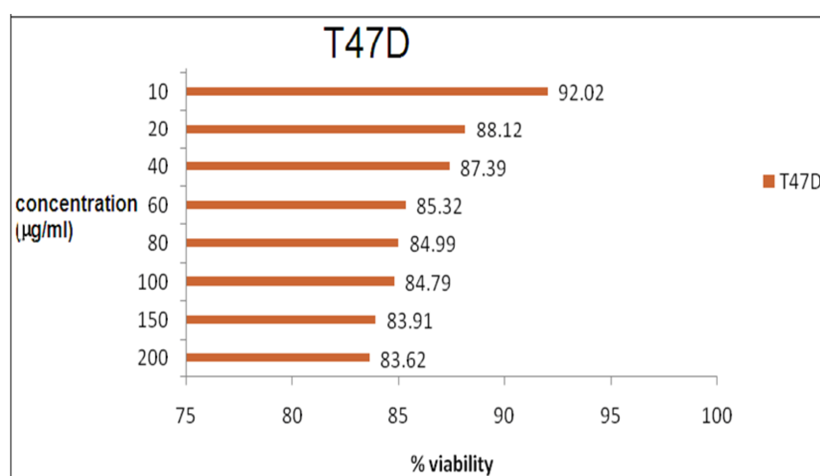

Figure 2 Effect of different concentrations of leaf extract of *P. amarus* on viability percentage of T47D cell lines

Percentage of cell viability of cell lines were carried out by Trypan blue staining using haemocytometer. After treating the cells with different concentrations of leaf extract (0-0.2mg/ml), it was found that with the increasing concentration of leaf extract, the percentage viability of cancer cell lines was decreased (Fig.1,2).

3.3. MTT Assay results of *Phyllanthus amarus* dimethyl formamide leaf extract

A plotted graph of anticancer activity of dimethylformamide leaf extract of *P. amarus* on HCT 15 and T47D cell lines using MTT assay was shown in Fig.5 and Fig.6 respectively. With the increasing concentration of dimethylformamide leaf extract from 10 to 200 µg/ml, the HCT 15 cancer cell line growth inhibition increased from 8.86% to 87.22 % (Table 3, Fig.3) and the T47D cancer cell line growth inhibition increased from 8.39 % to 86.01% (Table 4, Fig.4). This shows that the leaf extract inhibit the growth of the cancer cells and the inhibitory effect on HCT 15 cell line is comparatively greater than T47D cell line. The IC_{50} value was found to be 106.7 µg/ml for HCT 15 cancer cell line and 90.3 µg/ml for T47D cancer cell line, which shows that the anticancer activity of leaf of *P. amarus* is comparable to positive control drug doxorubicin and can be used as good anticancer agent.

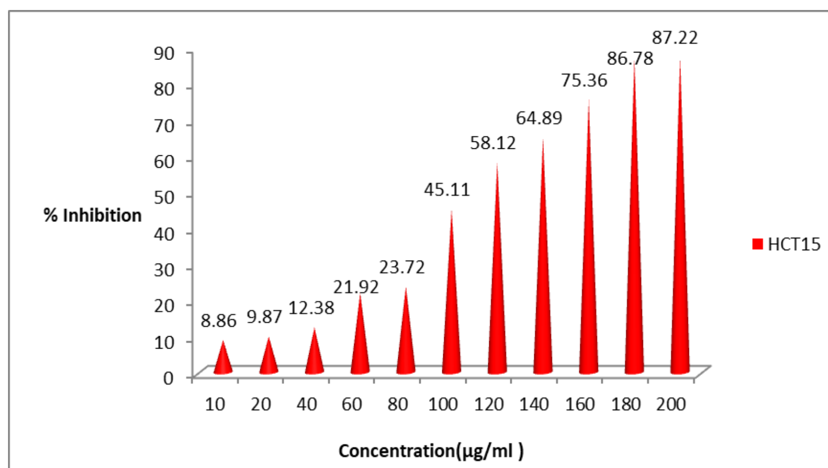

Figure 3 Cytotoxic activity of various concentrations of leaf extract of *P. amarus* against HCT15 cell line by MTT assay

Table 3 Inhibition percentage of HCT15 cell line at various concentrations of leaf extract by MTT assay.

Concentration µg/ml	% of HCT 15 cell inhibition	IC_{50}	R^2 Value
200	87.22±0.75	106.7±0.62	0.96
180	86.78±0.64		
160	75.36±0.49		
140	64.89±0.77		
120	58.12±0.58		
100	45.11±0.83		
80	23.72±0.66		
60	21.92±0.52		
40	12.38±0.69		
20	9.87±0.65		
10	8.86±0.52		
	22.88±0.53		

Doxorubicin			

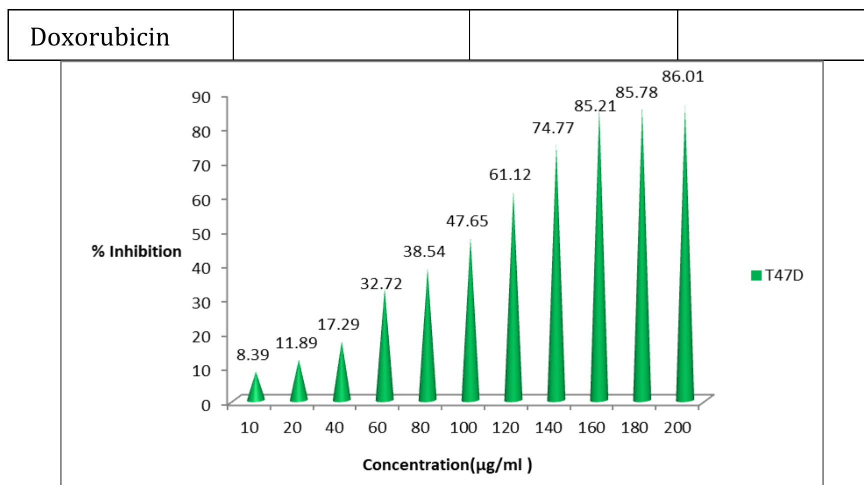

Figure 4 Cytotoxic activity of various concentrations of leaf extract of *P. amarus* against T47D cell line by MTT assay

Table 4 Inhibition percentage of T47D cell line at various concentrations of leaf extract by MTT assay

Concentration µg/ml	**% of T47D inhibition**	**IC_{50}**	**R^2 Value**
200	86.01±0.53	90.3±0.57	0.963
180	85.78±0.62		
160	85.21±0.49		
140	74.77±0.66		
120	61.12±0.71		
100	47.65±0.43		
80	38.54±0.58		
60	32.72±0.68		
40	17.29±0.70		
20	11.89±0.56		
10	8.39±0.60		
Doxorubicin	46.87±0.53		

MTT Assay of DMF leaf extract of *P. amarus* against HCT 15 Cell line

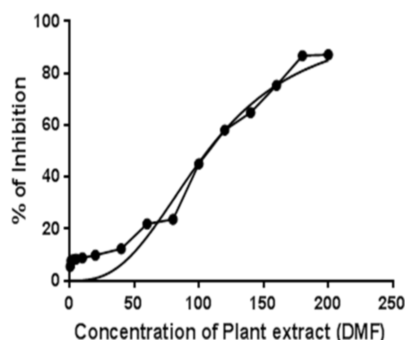

Figure 5 Anticancer activity of dimethylformamide leaf extract of *P. amarus* on HCT 15 cell line using MTT assay

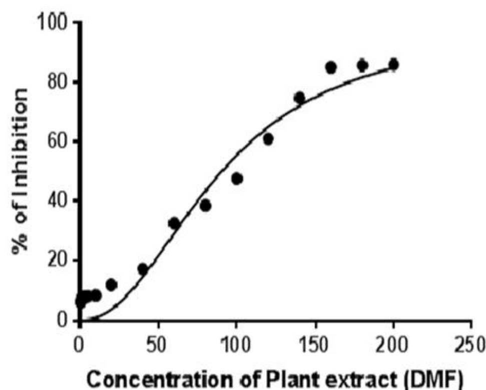

Figure 6 Anticancer activity of dimethylformamide leaf extract of *P. amarus* on T47D cell line using MTT assay

In a previous report, the compounds n-Hexadecanoic acid, N-Methoxy-N-methyl acetamide, Ursa-9 (11), 12-dien-3-ol, gammasitosterol were found to be responsible for biological activity [26]. There is no report in literature regarding testing the cytotoxic potential of dimethylformamide leaf extract of *P. amarus* against Human colorectal adeno carcinoma (HCT 15) and Human breast cancer (T47D) cell lines by MTT assay. n-hexane and chloroform extracts of heart wood of *Albizia adianthifolia*[27] and ethanolic extract of whole plant of *Aristolochia krysagathra*[28] were reported to have trans-13-octadecanoicacid and 9,12-Octadecadienoic acid compounds respectively, which belong to methyl ester group and display anti-cancer properties. The presence of a diterpene compound called 2-Cyclopenten-1-one, 2-hydroxy in ethanolic leaf extract of *Bruguiera cylindrica* showing antimicrobial and anticancer properties have been reported [29]. They also reported phenolic compounds possessing antimicrobial, antioxidant and anticancer properties. Whole plant extracts of *Calanthe triplicate* in ethyl acetate extracts revealed the presence of 4H-Pyran-4-one, 2,3-dihydro-3,5- dihydroxy-6-methyl which is a flavonoid compound exhibiting antimicrobial, anticancer and anti-inflammatory properties [30]. Phytol, which is a diterpene compound, is reported to have antioxidant, anti-inflammatory and anticancer properties in ethanolic leaf extract of *Cyperus rotundus* [31]. Antioxidant, anti-inflammatory and anticancer properties in methanolic leaf extract of *Eupatorium triplinerve* have been reported [32]. An Isoprenoid compound named Squalene showing antioxidant and anticancer properties and a ketone compound named 3, 7, 11, 15-Tetramethyl-2-hexadecen-1-ol exhibiting anti-inflammatory and anti-cancer properties were reported in methanolic leaf extract of *Eupatorium triplinerve* [32]. These extracts also have 2, 6, 10-trimethyl, 14-ethylene-14-pentadecane, which exhibit anti-fungal, antibacterial and anti-cancer properties and 5-Hydroxymethyl furfural which exhibits anti-oxidant and anti-cancer properties. Tetradecanoic acid, which is a Myristic acid, is found to have antioxidant and anticancer properties in ethanol extract of bark of *Hugonia mystax* [33]. Tetradecanoic acid, which is a Myristic acid, is found to have antioxidant and anticancer properties in ethanolic leaf extracts of *Hyptis lanceolata* Poir. [34]. Steroid compounds 7-dehydrodiosgenin and lupeol were also found to have anticancer properties in *n*-hexane and chloroform extracts of stem bark of *Pterocarpus angolensis* [27]. An ester, 10 Dotriacontylpentafluoropropionate is found to have cytotoxic properties in wild and mutant strains of *Schizophyllum commune* [35]. All the above reports show that various esters, acids, phenols, flavonoids, isoprenoids, ketones, hydrocarbons, steroids compounds present in plants are responsible for various types of activities. All the above reports confirm that esters, acids, and flavonoid compounds which occur in *P. amarus* were the major cause for its anti-cancer activity and other medicinal properties.

4. Conclusion

These findings report *Phyllanthus amarus* as a potential plant exhibiting anticancer properties. Scientific study of the plant for its chemical constituents helps in understanding of their functional properties for development and designing of effective anticancer drugs. Safe and effective use of this plant in prevention of cancer helps to recommend it as a

dietary supplement. The mechanism of action against cancer cell proliferation and the regulation of apoptotic pathway are yet to be investigated using preliminary *in vitro* data for removing the barriers towards *in vivo* applications.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare that they have no conflict of interest.

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