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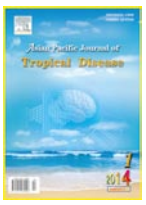

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Investigation of anticancer potential of hypophyllanthin and phyllanthin against breast cancer by *in vitro* and *in vivo* methods

Madhukiran Parvathaneni[1*], Ganga Rao Battu[1], Alexander I Gray[2], Pitchaiah Gummalla[1]

[1]*NAIP/ICAR Project, University College of Pharmaceutical Sciences, Andhra University, Visakhapatnam 530 003, India*

[2]*Department of Pharmaceutical Sciences, Strathclyde Institute for Biomedical Sciences, University of Strathclyde, Glasgow G4 0NR, Scotland, UK*

PEER REVIEW

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Dr. Murty Devarakonda, Professor, Faculty of Pharmacy, Asia Metropolitan University, Cheras, Kuala Lumpur, Malaysia.
Tel: 006-01116241939
E-mail: devarakonda@masterskill.edu.my

Comments

The manuscript is well documented and the results are discussed in a good way with suitable references. This research will be helpful for exploring highly potent new anticancer molecules for better therapy.
Details on Page S75

ABSTRACT

Objective: To investigate the *in vitro* and *in vivo* anticancer activities of hypophyllanthin and phyllanthin isolated from *Phyllanthus amarus* Schum & Thonn against breast cancer.
Methods: *In vitro* anticancer activity was evaluated against two cell lines (MCF-7 and MDA-MB-231) using MTT assay. *In vivo* anticancer activity was tested using Sprague-Dawley rats with N-methyl-N-nitrosourea induced mammary cancer.
Results: *In vitro* studies demonstrated a dose-dependent inhibitory effect on cell growth with IC_{50} values of (35.18±1.48) μg/mL (hypophyllanthin) and (32.51±0.95) μg/mL (phyllanthin) for MCF-7; (38.74 ±1.24) (hypophyllanthin) and (32.2±1.17) (phyllanthin) for MDA-MB-231 breast cancer cell lines. Tumor weights per group at doses of 5 and 10 mg/kg/day for hypophyllanthin (12.82 and 12.06 g) and phyllanthin (11.95 and 8.87 g) treated groups were significantly (P<0.001) lower than untreated N-methyl-N-nitrosourea group (35.85).
Conclusions: Results of the present research work indicated that the isolated lignan compounds, hypophyllanthin and phyllanthin showed significant anticancer activities against breast cancer, *in vitro* and *in vivo*.

KEYWORDS

Phyllanthus amarus, Hypophyllanthin, Phyllanthin, Breast cancer

1. Introduction

Phyllanthus amarus Schum & Thonn (Euphorbiaceae) (*P. amarus*) is locally well known as bhumi-amla and is officially listed in Indian herbal medicine system of Ayurveda. It is widely distributed in tropical areas of the world such as China, Java, Southern Florida, Bahmas, West Indies and Tropical America[1]. The main active constituents of the herb are sterols[1], lignans[2-5], triterpenes, tanins, flavonoids[6-8], alkaloids and volatile oils[9,10]. *P. amarus* is highly valued in the treatment of liver ailments[11], kidney stones and cancer[12-14]. It also acts as anti-viral, HIV replication and reverse transcriptase inhibitor agent[15,16].

However, most of the pharmacological activities were conducted on the complex extracts, and how each of these components contributed to the effects was not clearly understood, probably because of the commercially unavailable compounds. The lignans hypophyllanthin and phyllanthin of *P. amarus* were found to have greater importance due to their potent bioactive nature against leukemia[17], hepatic diseases[18,19], free radical scavenging activity and inflammation[19-21].

Based on the structural chemistry of lignans (phytoestrogens) and their antioxidant properties (Figures 1 and 2), we hypothesized that isolated lignans hypophyllanthin and phyllanthin may have anticancer activities against breast cancer cell lines in *in vitro* and *in vivo* animal models. However, there are no such existing reports. To confirm this hypothesis, the inhibitory effect of hypophyllanthin and phyllanthin on the growth of breast cancer cells *in vitro* and on the growth of N-methyl N-nitrosourea (MNU) induced mammary cancer in Sprague Dawley (SD) rats *in vivo* were

*Corresponding author: Madhukiran Parvathaneni, M. Pharm, PhD, Senior Research Fellow, NAIP/ICAR Project, University College of Pharmaceutical Sciences, Andhra University, Visakhapatnam, A.P, India-530003.
Tel: +91 9966647666
E-mail: madhukiran.parvathaneni@gmail.com

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investigated in this experimental study.

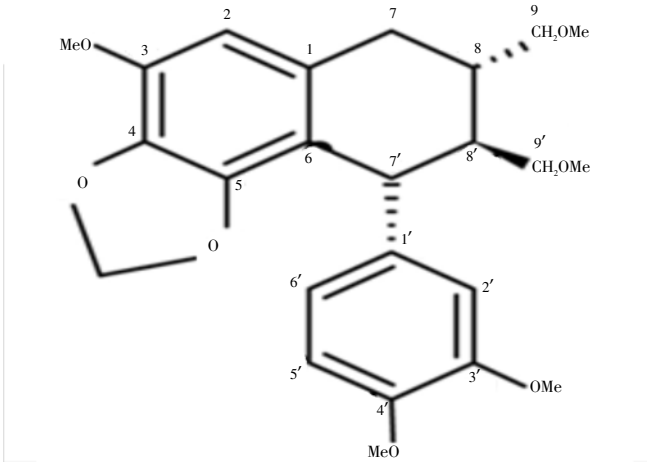

Figure 1. Hypophyllanthin.

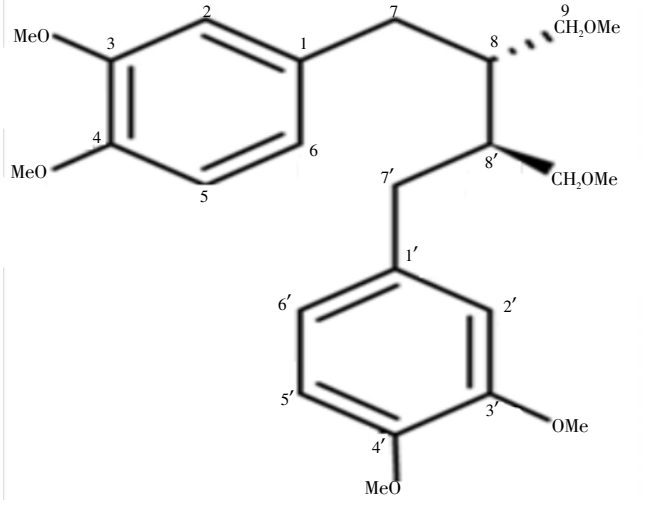

Figure 2. Phyllanthin.

2. Materials and methods

2.1. Chemicals

All the chemicals and reagents used were of analytical grade. Column chromatography was performed on silica gel (60-120 mesh; Merck, Germany). Thin-layer chromatography was carried out on precoated silica gel 60 GF_{254} (Merck, USA) plates by using hexane:ethylacetate (1:2, v/v) solvent system. MTT reagent and MNU were purchased from Sigma, USA.

2.2. Plant collection and extraction

The aerial parts of *P. amarus* were collected in November 2011 from Paderu region, Visakhapatnam District, Andhra Pradesh, India and authenticated by Dr. M. Venkayya, Taxonomist. Voucher specimen (BG/PMK/PA-11-11) was deposited in the herbarium, University College of Pharmaceutical Sciences, Andhra University. The dried and pulverized material (5 kg) was subjected to Soxhlet extraction with hexane. The solvent thus obtained was concentrated under vacuum at 40 °C (Buchi rotavapor, Switzerland) to give crude extract.

2.3. Separation of lignan fraction by column chromatography

Hexane extract (25 g) was fractionated over a silica gel column (100.0×3.5 cm; Borosil, India). Gradient elution was done in the following sequence, hexane (100, v/v), hexane:ethylacetate (95:5, v/v) and then hexane:ethylacetate (90:10, v/v). The fractions (Fr150-189) collected for hexane:ethylacetate (90:10, v/v) showed the presence of lignans on thin-layer chromatography with R_f values of 0.36 (bluish green) and 0.45 (violet)[22]. These fractions were pooled together, concentrated and subjected to preparative HPLC separation for obtaining pure (>95%) phyllanthin and hypophyllanthin.

2.4. In vitro cytotoxicity assay

The *in vitro* cytotoxicity assay of 1 and 2 was carried out on human estrogen receptor positive breast cancer cell lines (MCF-7) and human estrogen receptor negative breast cancer cell lines (MDA-MB-231) using MTT assay[23,24]. Cells (15×10^3) were plated in 100 μL of medium/well in 96-well microtiter plate. After 24 h incubation at 37 °C, hypophyllanthin and phyllanthin were added at different concentrations (10, 20, 40 and 80 μg/mL). Five wells were included in each compound concentration.

After 48 h of treatment, 10 μL of 5 mg/mL MTT reagent (Sigma, USA) was added to each well and cultivated at 4 °C for 1 h. The supernatant was then removed, 100 μL of dimethylsulfoxide was added and shaken at 960 r/min for 3 min. Absorbance was measured at 570 nm on a Tecan microtiter plate reader, (Switzerland). All experiments were performed in triplicate. The cytotoxicity effect of hypophyllanthin and phyllanthin on the growth of cancer cells was expressed as % cell growth inhibitions [(absorbance of control cells-absorbance of treated cells)/absorbance of control cells]×100.

2.5. In vivo anticancer activity

Female virgin SD rats were obtained from Mahaveer Enterprises (Hyderabad) at 35 d of age. Rats were housed at 4 per cage and maintained at (25±2) °C under 12 h dark/light cycle with access to standard diet (Nutrimix Std-1020, Nutravet Pvt. Ltd, Pune) and water *ad libitum*. Animals were experimented with prior approval from the Institutional Ethics Committee (Regd. No. 516/PO/c/01/CPCSEA), University College of Pharmaceutical Sciences, Andhra University. MNU (Sigma, USA) was dissolved in 0.9% NaCl containing 0.05% acetic acid (pH 5). Rats were given intraperitoneally 50 mg/kg of MNU on the 50th day[25,26]. Animals were divided into different groups with eight animals in each group. Group I (intact control) received 0.9% NaCl *i.p.*, adjusted to pH 5.0 with acetic acid. Groups II-VII were induced with MNU and after 85 d (all rats developed at least one tumor), animals were treated with tamoxifen at 2 mg/kg/day (Fresenius Oncology Ltd., India) and isolated compounds, hypophyllanthin and phyllanthin (5 mg/kg and 10 mg/kg in 1% sodium carboxymethyl cellulose orally by gavage once per day) for 4 weeks (from 86th to 113th day). Animals in intact control group and untreated MNU group were given vehicle (sodium carboxymethyl cellulose suspension) according to experimental protocol.

After completion of treatment, blood was collected from retro orbital puncture and the hematological parameters like red blood cells (RBC), white blood cells (WBC), hemoglobin (Hb), and platelets (PLTs) were estimated by fully-auto-

hematology analyzer (Erba Scientific, India)[27]. At the end of the experiment all animals were sacrificed, tumors were excised for the measurement of different tumor parameters like tumor incidence (number of animals with tumors), tumor multiplicity (number of tumors per rat observed in different treated groups) and tumor weight (tumor weight per rat in each group)[27,28]. Tumors were fixed in 10% buffered formalin. Paraffin sections (5 μm thickness) of the excised tissue were made using microtome and were stained with haematoxylin and eosin for histopathological evaluation. The breast cancer disease pathology and histological types were studied.

2.6. Statistical analysis

The results of the *in vitro* experiments were expressed as mean ±SEM and were statistically analyzed using Dunnett's multiple comparison test. The IC_{50} values were obtained from nonlinear regression analysis by using Graphpad prism-5 software. In the *in vivo* experiments the difference in % tumor incidence, tumor multiplicity per rat and tumor weight per group were statistically evaluated by One-way analysis of variance (ANOVA), followed by Dunnett's test. Values of $P<0.05$ were considered to be significant.

3. Results

3.1. In vitro cytotoxicity assay

Figures 3 and 4 reveal the dose-dependent inhibitory effect exhibited by hypophyllanthin and phyllanthin on *in vitro* growth of MCF-7 and MDA-MB-231 breast cancer cells. Furthermore the study demonstrated that the anticancer activity of phyllanthin is more potent than hypophyllanthin as detailed in Table 1.

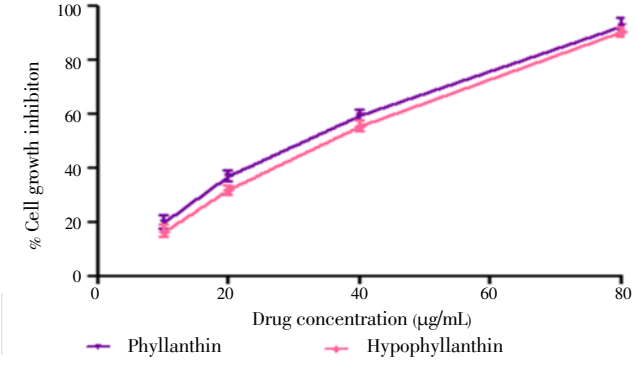

Figure 3. Percentage cell growth inhibition of MCF-7 cells treated with hypophyllanthin and phyllanthin (mean±SEM, n=3).

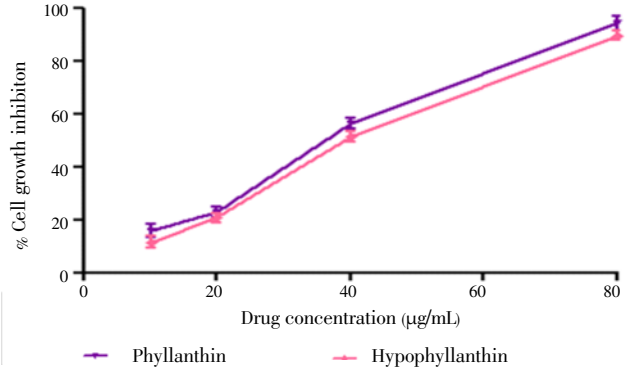

Figure 4. Percentage cell growth inhibition of MDA-MB-231 treated with hypophyllanthin and phyllanthin (mean±SEM, n=3).

Table 1
Percentage cell growth inhibition (%CGI) and IC_{50} values of hypophyllanthin and phyllanthin.

Compounds	MCF-7		MDA-MB-231	
	%CGI	IC_{50} (μg/mL)	%CGI	IC_{50} (μg/mL)
Hypophyllanthin	90.17±1.62	35.18±1.48	89.56±2.85	38.74±1.24
Phyllanthin	92.27±2.84	31.51±0.95	94.14±3.14	32.22±1.17

MCF-7: Human estrogen receptor positive breast cancer cell lines; MDA-MB-231: Human estrogen receptor negative breast cancer cell lines. Results are given as mean±SEM, n=3.

3.2. In vivo anticancer activity

Oral administration to SD rats bearing MNU induced mammary cancer with hypophyllanthin and phyllanthin at doses of 5 mg/kg and 10 mg/kg significantly decreased the mammary cancer. The incidence rate in untreated MNU group was found to be 85.7%. Mammary tumors first appeared after 12 weeks of the MNU injection, six rats developed at least one tumor out of remaining seven rats in the untreated MNU group (one rat out of eight died after four days of MNU injection). Table 2 reflects the tumor incidence, tumor multiplicity and tumor weights of different treatment groups. Phyllanthin at a dose of 10 mg/kg showed highest reduction of mammary tumor incidence of 31.5% next to tamoxifen treated group (25%). There was nearly 54.2% reduction in the incidence rates of phyllanthin (10 mg/kg) treated group when compared to the untreated MNU group and this better explains the anticancer potential of phyllanthin.

Table 2
Effect of hypophyllanthin and phyllanthin treatment against MNU induced mammary carcinogenesis.

Treatment/groups	No. of rats with tumor	Incidence (%)	Tumor multiplicity per rat[d]	Tumor weight per group (g)
Control (DV oral)	–	–	–	–
MNU	6/7	85.7	2.14±0.58	35.85
MNU+tamoxifen (2 mg/kg)	2/8	25.0	0.50±0.26[c]	4.67[b]
MNU+(1) (5 mg/kg)	5/8	62.5	1.38±0.32	12.82[a]
MNU+(1) (10 mg/kg)	4/8	50.0	1.25±0.41	12.06[a]
MNU+(2) (5 mg/kg)	4/8	50.0	1.12±0.47	11.95[a]
MNU+(2) (10 mg/kg)	3/8	31.5	0.87±0.27	8.87[a]

[a]: $P<0.001$; [b]: $P<0.01$; [c]: $P<0.05$ compared with control, [d]: Results are given as mean±SEM, (1): hypophyllanthin; (2): phyllanthin, MNU: N-methyl N-nitrosourea, DV: Drug vehicle.

The weight of grossly detectable mammary tumors in untreated MNU group was 35.85 g ranged from 0.06 g to 4.9 g per tumor. The mean tumor weight per rat in untreated MNU group was (5.12±0.26) g. The weight of total mammary tumors per group (total tumor mass) ranged from 4.67 g to 35.85 g. The majority of rats (65%) had a total tumor mass lower than 4.50 g per rat. Tamoxifen treated group showed significant ($P<0.01$) decrease of total tumor weight (4.67 g). Furthermore the groups treated with hypophyllanthin and phyllanthin showed significant ($P<0.001$) decrease in tumor weight. The total tumor weight of phyllanthin treated groups was decreased from 11.95 g to 8.87 g with increase in dose from 5 mg/kg to 10 mg/kg whereas no such remarkable change was observed with increase in hypophyllanthin dose.

Table 3 reflects the hematological analysis data of tumor bearing rats treated with hypophyllanthin and phyllanthin. Untreated MNU group showed significant decrease ($P<0.05$) in number of RBC, PLTs and Hb indicating a tendency of anemia, whereas concomitant increase in WBC was observed indicating the diseased state. Tamoxifen (standard drug for breast cancer treatment) treated group showed significant ($P<0.05$) reversal of

all hematological parameters to normal levels. Upon treatment with hypophyllanthin and phyllanthin all hematological parameters which were altered by induction of mammary cancer were considerably restored.

Table 3

Hematological analysis of tumor bearing rats treated with hypophyllanthin and phyllanthin.

Treatment/group	WBCs (10^3/μL)	RBCs (10^6/μL)	Hb (g/L)	PLTs (10^3 /μL)
Control (DV oral)	6.52±1.27	7.17±1.22	13.81±2.08	660.07±31.61
MNU	12.62±2.65	6.18±2.21	11.22±2.35	448.24±28.11
MNU+tamoxifen (2mg/kg)	7.51±1.31[a]	6.97±1.17[c]	13.25±3.12	571.70±21.25[b]
MNU+(1) (5 mg/kg)	9.25±0.98[a]	6.73±1.74	11.95±2.29	522.41±32.24
MNU+(1) (10 mg/kg)	8.77±1.35 [a]	6.79±1.67	12.48±2.51	536.08±31.97
MNU+(2) (5 mg/kg)	8.95±1.87 [a]	6.75±1.64	12.25±2.46	517.58±17.50
MNU+(2) (10 mg/kg)	8.63±1.24 [a]	6.82±1.71	12.72±2.77	547.58±25.84[c]

[a]: $P<0.001$; [b]: $P<0.01$; [c]: $P<0.05$ compared with control, Results are given as mean±SEM, (1): hypophyllanthin; (2): phyllanthin, MNU: N-methyl N-nitrosourea, DV: Drug vehicle.

Figure 5 clearly depicts the MNU induced mammary tumor in untreated rat. Histology of normal mammary gland (Figure 6A) shows the presence of lobules (L) with numerous acini and clear basement membrane (BM). Photomicrographs (Figures 6B-F) of mammary tissue of untreated MNU group clearly shows marked proliferation of stroma (SP) resulted due to stromal reaction with carcinogen. Figure 6B depicts the distribution of congested blood vessels (BV) in stroma. Figure 6C reveals complete tumor tissue necrosis (NC) owing to increased tumor size followed by congestion of blood vessels and insufficient blood supply to the tumor tissue. Figure 6D demonstrates a well circumscribed tumor composed of closely packed tubules (T), giving rise to 'back to back fashion'. The tubules are lined by inner epithelial cells and basal myoepithelial cells. In between the tubules, stroma (S) was very scanty with mixed mononuclear cell infiltrate and prominent mast (M) cells.

Figure 6E shows the presence of intraluminal secretions (SE) of tubules (T). The epithelial component of tumors was organized into acinar structures but the growth pattern was variable from one region to another in the same tumor, with areas of typical well differentiated adenocarcinoma showing papillary and cribriform patterns. Figure 6F clearly demonstrates the formation of well-defined capsule (CP) by fibrous collagenous tissue in between adipose (AD) and cancerous tissues (CT), which is a characteristic feature of breast cancer. Upon comparison with untreated MNU group, the animals treated with hypophyllanthin and phyllanthin showed small sized tumors. Histology of phyllanthin treated rat tumors showed reduced necrosis (NC) (Figure 6H) when compared with hypophyllanthin treated rat tumors which showed moderate number of necrotic cells (NC) (Figure 6G). This reduced necrosis in Figure 6H was due to potent inhibition of mammary tumor growth with significant decrease in tumor multiplicity and tumor weight associated with phyllanthin treatment.

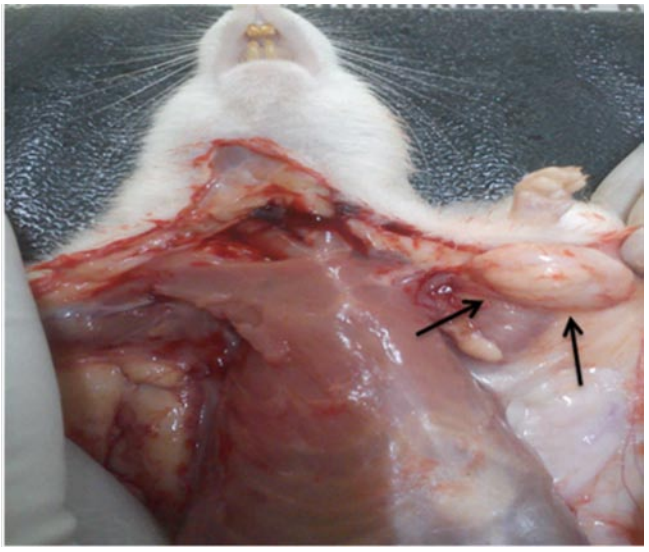

Figure 5. MNU induced mammary tumor in SD rat.

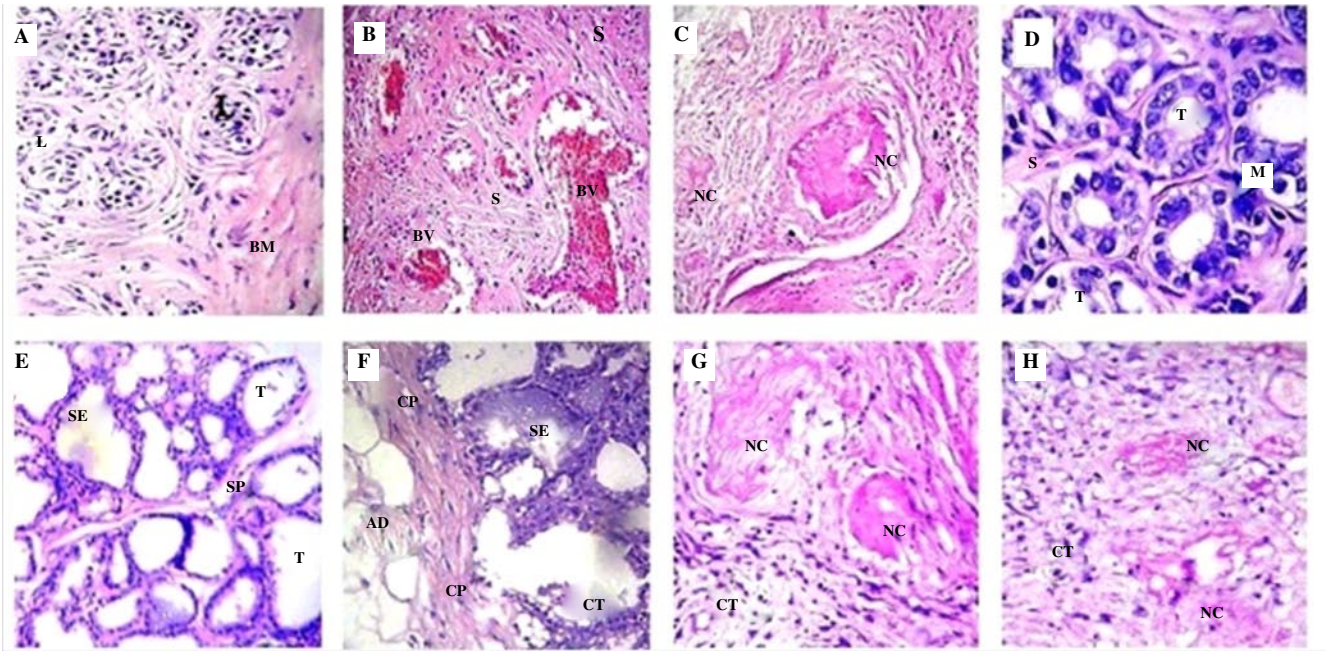

Figure 6. Histological study of tumors in hypophyllanthin and phyllanthin treated rats.

A: Normal mammary gland showing basement membrane (BM) and Lobules (L); B-F: Mammary tumors of untreated MNU group demonstrating stroma (S), congested blood vessels (BV), tumor tissue necrosis (NC), stromal proliferation (SP), tubules (T), mast cells (M), adipose tissue (AD), fibrous capsule (CP), cancerous tissue (CT) and tissue secretions (SE); G and H: Mammary tumors of hypophyllanthin and phyllanthin (10 mg/kg) treated groups (H&E, ×140).

4. Discussion

Breast cancer is one of the main life-threatening diseases[29]. Though different anticancer drugs were present in the market because of their serious adverse effects there is still need to identify potent anticancer molecules from natural origin. Herbal medicine has been regarded as one of the most visible fields for cancer chemoprevention and it constitutes the main source of effective new anticancer agents[30,31]. Plant lignans were found to have potent anticancer activity against breast cancer.

Aqueous and alcoholic extracts of *P. amarus* showed cytotoxicity against lung cancer (A549), breast cancer (MCF-7 and MDA-MB-435S) and leukemia (K-562; Lucena-1) cell lines. Cytotoxic activity against cancer lines has been reported for several members of lignans. Hypophyllanthin and phyllanthin exhibited *in vitro* cytotoxic activity against leukemia cells (K-562) and they also showed *in vivo* anticancer activity against Ehrlich ascites carcinoma (EAC) in Swiss albino mice[1,17]. This is the first study reporting the cytotoxic activity of hypophyllanthin and phyllanthin against MCF-7 and MDA-MB-231 breast cancer cell lines.

In the present research, *in vivo* anticancer activity of hypophyllanthin and phyllanthin against MNU induced mammary cancer in SD rats was reported for the first time. The present *in vitro* and *in vivo* anticancer activities exhibited by hypophyllanthin and phyllanthin might be allied with different mechanisms like direct free radical scavenging activity[19], inhibition of cytochrome P450 enzymes (Phase-I enzymes) which can cause specific mutations leading to carcinogenesis, and interference with estradiol biosynthesis by inhibition of key steroid enzymes like steroid dehydrogenase and aromatasewhich are previously reported for anticancer activity of lignans[13,32].

In conclusion, for the first time, the anticancer activities of hypophyllanthin and phyllanthin against human breast cancer lines *in vitro* and MNU induced mammary carcinoma *in vivo* were reported in this experimental study. The results demonstrated that hypophyllanthin and phyllanthin exhibited a strong inhibitory effect on the growth of MCF-7 and MDA-MB-231 human breast cancer cells *in vitro* and inhibited growth of mammary carcinoma *in vivo*. The results of this experimental study suggested that hypophyllanthin and phyllanthin would be useful as anticancer agents. The use of these lignans as applied therapeutics needs to be further investigated.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

This work was funded by National Agricultural Innovation Project and Indian Council for Agricultural Research, Government of India (NAIP/ICAR Project; Grant No. C4/40043101). The authors gratefully acknowledge the assistance from Prof. A. Annapurna, Department of Pharmacology and Dr. Balaji Path, Pathologist, King George Hospital, Visakhapatnam in carrying out the anticancer activity.

Comments

Background

Cancer disease is increasing day by day. Though several anticancer drugs are being used in the treatment, because of their side effects and chemoresistance of cancer cells to those drugs there is need to develop new therapies. The manuscript describes screening of isolated compounds, hypophyllanthin and phyllanthin against two different breast cancer cell lines (*in vitro*) using MTT assay and MNU induced mammary cancer model (*in vivo*) in rats. This manuscript will be helpful for developing new therapies in the field of breast cancer research.

Research frontiers

The author reported the efficacy of hypophyllanthin and phyllanthin for the inhibition of growth of human breast cancer cell lines (MCF-7 and MDA-MB-231) and inhibition of the mammary tumor growth in rats. Among the tested compounds, phyllanthin showed significant inhibition of both cell lines in *in vitro* studies and also significantly inhibited the growth of mammary tumors which was comparable to that of marketed drug tamoxifen.

Related reports

The natural products and isolated compounds are screened by using MTT assay (Mosmann T, 1983 and Muhammad Zia *et al*., 2013). The screening methodology was well defined in the manuscript and the results were presented in a good way. The results were further supported by previous anticancer reports on four plant species of *Phyllanthus* (Lee SH *et al*., 2011 and Sureban SM *et al.*, 2006).

The *in vivo* anticancer activity is peforemed for the first time and is more innovative.

Innovations & breakthroughs

The isolated lignans are having their structure related to gonadal harmones and by basing on this hypothesis the authors for the first time evaluated and reported the efficacy of hypophyllanthin and phyllanthin against breast cancer by *in vitro* and *in vivo* methods.

Applications

This study is applicable for developing new therapeutic agents used for breast cancer therapy.

Peer review

The manuscript is well documented and the results are discussed in a good way with suitable references. This research will be helpful for exploring highly potent new anticancer molecules for better therapy.

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