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EVALUATION OF ANTICANCER ACTIVITY OF FLAVONOID ISOLATED FROM THE ACETONE EXTRACT OF THE AERIAL PARTS OF PHYLLANTHUS *NIRURI AGAINST* HUMAN LUNG CANCER CELL LINE (A_{549})

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ABSTRACT

Phyllanthus niruri L. (Euphorbiaceae), known as "quebra-pedra" (Portuguese for "stonebreaker"), is an herb used for kidney disorders.Whole plants have been used in traditional medicine for treatment of jaundice, asthma, hepatitis and malaria. The present study was designed to determine the anticancer activity of flavonoid isolated from the acetone extract of the aerial part of *P. niruri* against human lung cancer cell line (A_{549}).MTT assay based cytotoxic activity study against human lung cancer cell line (A_{549}) was conducted to evaluate the potent activity of flavonoid isolated from *P. niruri.*TLC and GC- MS analysis were recorded to confirmthe presence of flavonoids in acetone extracts.Flavonoids isolated from the acetone extract of the aerial part of *P.niruri* showed cytotoxic activity against the human lung cancer cell line (A_{549}) and the inhibitory concentration at 50% growth (IC_{50}) was found to contain IC_{50} = 30.70±0.81µg/mL respectively. The results indicated that the flavonoids isolated from the *P.niruri* showed cytotoxic activity which can be effectively employed in anticancer treatment. Cytotoxic study suggested that flavonoid from the acetone extract of the aerial part of *P. niruri* was found to contain maximum inhibition of 66.6% at 7.8µg/mL concentration.

KEY WORDS*: Phyllanthusniruri*, flavonoids, MTT assay, cytotoxicity, GC-MS analysis.

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INTRODUCTION

Cancer is a major public health problem, causing approximately 7 million deaths every year worldwide[1]. More than 80% of cancer deaths are due to carcinoma in lung, breast, prostate, colorectal, and pancreas [2]. Lung cancer and colorectal cancers are responsible for the first and third most cancer related deaths in men and women. Breast cancer in women and prostate cancer in men rank second[3]. Cancer is largely environmentally determined, dietbeing a major variable. Dietary patterns, foods, nutrients and other dietary constituents are closely associated with the risk for several types of cancer and in this regard, it has been estimated that 35% of cancer deaths may be related to dietary factors. Recently, dietary polyphenols have received much attention for their anticancer properties[4-6]. Many studies in different cell lines, animal models and human epidemiological trials suggest a protective role of dietary polyphenols against different types of cancers[1]. Flavonoids are a group of more than 4,000 polyphenolic compounds that occur naturally in foods of plant origin and are categorized, according to chemical structure, into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidin and chalcones[7].Recently flavonoids have aroused considerable interest because of their potential beneficial effects on human health. They have been reported to have antiviral, anti-allergic, antiplatelet, anti-inflammatory, antitumor, antioxidant, antithrombotic, hypolipidemic and hypoglycemic activities[8-10]. *P.niruri (Euphorbiaceae)* originated in India and usually occurs as a winter weed throughout the hotter parts, contains over 600 species of shrubs, trees and annual or biennial herbs distributed throughout the tropical and subtropical areas. Whole plants have been used in traditional medicine for treatment of jaundice, asthma, hepatitis and malaria[11,12]. It has a potent free radical scavenging activity and could scavenge superoxides, hydroxyl radicals and can inhibit lipid peroxides[13].

MATERIALS AND METHODS

Plant Material

The plant material of aerial part of *Phyllanthus niruri* were collected from in and aroundthe districts of Kerala.It was identified using standard books. The aerial partswere shade dried and crushed into fine powder with electric blender. The powdered sample was sealed in polythene bags and was stored in desiccators until further uses.

Preparation of acetone extract

Dried and powdered aerial parts of *Phyllanthus niruri* (500 g) were extracted using soxhlet with 100% acetone (1:5 W/V) for about 72 hours. The extractwas removed and itwas concentrated to dryness in rotary vacuum evaporator below 50°C and stored until needed for the bioassays at -4 °C.

Test for Flavonoids

Three methods were used to determine the presence of flavonoids in the plant sample[14-17]. 5 mL of dilute ammonia solution was added to a portion of the aqueous filtrate of plant extract followed by addition of con. H_2SO_4. A yellow colouration observed in each extract indicated the presence of flavonoids. The yellow colouration disappeared on standing. Few drops of 1% aluminum solution were added to a portion of each filtrate. A yellow colouration was observed indicating the presence of flavonoids. A portion of the powdered plant sample was in each case heated with 10 mL ethyl acetate over a steam bath for 3 minutes. The mixture was filtered and 4 mL of the filtrate was shaken with 1 mL of dilute ammonia solution. A yellow colouration was observed indicating a positive test for flavonoids.

IDENTIFICATION OF FLAVONOIDS BY TLC

TLC was performed for the identification of flavonoids. The concentrated crude acetone extracts of aerial part of *P.niruri* were spotted on the lower side of the TLC plate (20× 20 cm) precoated with silica gel G. The diameter of each spot was about 5mm. Then TLC was allowed to run uni dimensionally in the mobile phase solvent (Toluene : Ethyl acetate, 7:3 ratio) and dried at room temperature. The developed plates were observed for visible spots and viewed under UV light. Then, the developed TLC plates were finally placed in iodine chamber[18,19]. The $R_f$values of the coloured spots were recorded.

$R_f$value = Distance travelled by the solute/Distance travelled by the solvent.

IN VITRO ANTICANCER ACTIVITY

Cell line and culture

Lung Cancer Cell line (A_{549}) was obtained from National centre for cell sciences Pune (NCCS). The cells were maintained in Minimal Essential Media supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 µg/mL) in a humidified atmosphere of 50 µg/mL CO_2 at 37 °C.

Reagents

MEM was purchased from Hi Media Laboratories Fetal bovine serum (FBS) waspurchased fromCistron laboratories Trypsin, methylthiazolyldiphenyl- tetrazolium bromide (MTT), and Dimethyl sulfoxide (DMSO)were purchased from (Sisco research laboratory chemicals Mumbai). All of other chemicals and reagents wereobtained from Sigma Aldrich Mumbai.

In vitro assay for Cytotoxicity activity (MTT assay).

The Cytotoxicity of samples on A_{549} was determined by the MTT assay[20]. Cells (1×10^5/well) were plated in 1mL of medium/well in 24-well plates (Costar Corning, Rochester,NY). After 48 hours incubation the cell reaches the confluence. Then, cells were incubated with the samples for 24 – 48hours at 37°C. After removal of the sample solution and washing with phosphate-buffered saline (pH 7.4), 100µl/well (5mg/mL) of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl--tetrazolium bromide cells(MTT) phosphate- buffered saline solution was added. After 4hours incubation, Viable cells were determined by the absorbance at 570nm. Measurements were performed and the concentration required for a 50% inhibition of viability (IC_{50}) was determined graphically. The absorbance at 570 nm was measured with a UV-Spectrophotometer using wells without sample containing cells as blanks. The effect of the samples on the proliferation of A_{549} was expressed as the % cell viability, using the following formula

% cell viability = $A_{570}$of treated cells / A_{570} of control cells × 100

Gas chromatography Mass Spectroscopy analysis acetone extracts of P. niruri(aerial part) .

INSTRUMENT	:	GC-MS-5975C [AGILENT]
GC CONDITION		
Column Oven Temperature	:	70˚C
Injector Temperature	:	250˚C
Injection Mode	:	Split (Split ratio :10)
Flow Control Mode	:	Linear Velocity
Column Flow	:	1.51 ml/min
Carrier Gas	:	Helium 99.9995% purity
Injection volume	:	1 microlitre
Column Oven Temperature Program		
Rate	Temperature (˚C)	Hold Time (min)
-	70.0	3.0
10	300	9.0 [34.0 mts total]
COLUMN	:	DB-5ms Agilent
Length	:	30.0 m
Diameter	:	0.25 mm
Film Thickness	:	0.25 µm
MS CONDITION		
Ion source temp	:	230 °C
Interface temp	:	240°C
Scan range	:	40-700 m/z
Solvent cut time	:	5 mins
MS start time	:	5 (min)
MS end time	:	35 (min)
Ionization	:	EI (-70ev)
Scan speed	:	2000
MS LIBRARY	:	**NIST- 11**

RESULTS

The acetone extracts of aerial part of *P. niruri* showed large number of compounds. For partial purification, the crude acetone extracts were subjected to TLC. R_f values were noted. A single visible spot showed the presence of flavonoid. The chromatogram was developed with a mixture of suitable solvent system (Toluene: Ethyl acetate) (7 : 3 ratio) .(Table: 2; Figure:2). The compounds present in the acetone extract of aerial part of *P. niruri* were identified by GC-MS (Figure:1). The active principles with their retention time (RT), molecular formula, molecular weight (MW) and percentage composition in the acetone extract of aerial parts of *P. niruri* is presented in (Table: 1). Nine (9) compounds were identified in the acetone extracts of aerial parts of *P. niruri*. The prevailing compounds were Bicyclo[3.1.1]heptane, 2,6,6-trime thyl-, (1.alpha.,2.beta.,5.alpha.) (0.68%), 4-Acetoxymethoxyacetophenone (32.04%), 4HImidazole-4-thione, 1,5-dihydro-1-methyl-2(methylthio)-5,5-diphenyl (2.94%), Indole-3-carboxylic acid, 5 methoxy-2-methyl-1-(3-methylphenyl), ethylester (4.24%), 1,2-Benzisothiazol3amine(1.01%),Propanoicacid, 2-[(5,7dimethyl[1,2,4]triazolo[1,5apyrimidin-2yl)thio], ethylester(1.43%), 3HImidazo[4,5b] pyridine, 2-(2- ethylhexylsulfanyl)(16.6%), 3H-Imidazo [4,5-b]pyridine, 2-(2-ethylhexylsulfanyl(11.48%), Phenol, 3-methoxy-2,4,6-trimethyl- (29.4%) Major phytocompounds obtained through the GC-MS study of aerial parts of *P.niruri* have been tabulated (Table:1). The activity of phytocomponents identified in *P.niruri* by GC-MS is based on Dr.Duke's phytochemical and ethnobotanical databases. The spectrum profile of GC-MS confirmed the presence of nine major components with the retention time 17.1, 26.1, 26.2, 26.2, 26.3, 26.4, 26.6, 27.2 and 27.3 respectively (Table:1) Visible spot of flavonoid was scrapped and dissolved in acetone. The extract of aerial part of *P. niruri* which showed the presence of flavonoid was further tested for *invitro* cytotoxicity against human lung cancer cell line (A_{549}). The inhibitory concentration at 50% growth value (IC_{50}) of aerial part of *P.niruri* was 30.70µg/mL respectively. Therefore the cytotoxic study suggested the extracted flavonoid of *P. niruri* had a maximum of 66.6% at 7.8µg/mL concentration. (Table: 3; Figure: 3)

DISCUSSION

The acetone extract of aerial part of *P. niruri* showed the presence of large number of bioactivecompounds. Gradient elution was conducted using solvent according to increase in their polarity. According to [21]the chromatograms of hydrolysed extracts of *P.niruri*(stem, leaves, root and callus) from Kimwenza (Km) presented yellow spot corresponding to control quercetin. In the present study, chromatogram was developed in a mixture of suitable solvent mixture of Toluene: Ethyl acetate in 7:3 ratio. A single visible yellow spot was developed corresponding to acetone extract of aerial part of *P.niruri.* The GC-MS spectral results and comparison of results with NIST- library search successfully enabled the identification of compounds with their retention time (RT), molecular formula, molecular weight (MW) and concentration (peak area%). According to [22].3, 5-di-t-butyl phenol is a phenolic compound and it maybe employed as an antioxidant, antimicrobial, antifungal and anti-inflammatory agents. Hexadecanoic acid (palmitic acid) is a fatty acid and it may be an active antimicrobial and antidiarrhoeal agent.2, 13- octatadecadiene-1-ol is suggested to be an aliphatic alcoholic compound and it employed as an antimicrobial agent. In the present study the chemical profile of *P. niruri* using GC-MS analysis was characterized. The chromatogram showed the relative concentration of various compounds getting eluted as a function of retention time. The heights of the peak indicate the relative concentrations of the components present in the plant. The mass spectrometer analyses of the compound eluted at different times were identified to analyse the nature and structure of the compounds. The large compound fragments into small compounds giving rise to appearance of peaks at different M/Z ratios. In addition to this the results of the GC-MS profile can be used as pharmacognostic tool for the identification of the plant. The present study supported and supplemented the

previousobservations[23-28]. GC-MS analysis showed the existence of various compounds with different chemical structures. The presence of various bioactive compounds confirms theapplication of *P.niruri*for various ailments by traditional practitioners. According to[29,30] the plant species of *Phyllanthussp.*(*P.amarus*, *P.niruri* and *P.watsonii*) possess selective antiproliferative effects on A_{549} cell line without cytotoxic effects on their normal cells. [29]reported thatmethanolic and aqueous extracts of *Phyllanthus* showed anti-metastatic effects on human lung (A_{549}) and breast (MCF-7) cancer cell lines. [31] reported that flavonoids isolated from *Aloe vera, Mimosa pudica* and *Phyllanthusniruri* showed cytotoxicity activity against human breast carcinoma cell line (MCF-7) and the inhibitory concentration at 50% growth (IC_{50}) was found to be, *Mimosa pudica* (IC_{50}= 35.52±0.50 µg/mL), *Aloe vera*(IC_{50}= 54.97±0.36µg/mL) and *Phyllanthus niruri* (IC_{50}= 84.88±0.87 µg/mL)[31]. In the present study the inhibitory concentration at (50% growth values) (IC_{50}) value of the aerial parts of *P. niruri* was found to be 30.70µg/mL respectively. The cytotoxic study revealed that flavonoids from aerial part of *P. niruri* was found to contain the maximum inhibition of 66.6%at 7.8µg/mL concentration.

Table 1
Components detected in acetone extracts of aerial parts of P. niruri

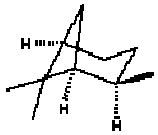
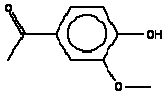
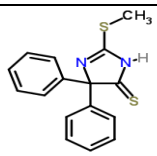
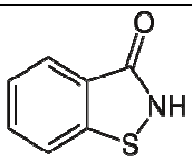
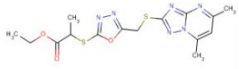
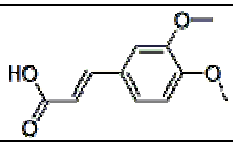
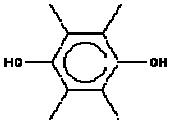
Name of the compounds	Retention time	Structure	Molecular formula	Molecular weight	Relative abundance
Bicyclo[3.1.1]heptane, 2,6,6-trime thyl-, (1.alpha,2.beta,5.alpha)	17.129		$C_{10}H_{18}$	138.24	0.68
4-Acetoxy-3 methoxyacetophenone	26.118		$C_9H_{10}O_3$	166.17	32.04
4H-Imidazole-4-thione, 1,5-dihydro -1-methyl-2-5,5-diphenyl-	26.207		$C_{16}H_{14}N_2S_{2z}$	298.42	2.98
Indole-3-carboxylic acid, 5-methoxy-2-methyl-1-(3-methylphenyl)-, ethyl ester	26.274		$C_{20}H_{21}NO_3$	323.38	4.24
1,2-Benzisothiazol-3-amine tbdms	26.378		C_7H_5NOS	151.19	1.01
Propanoic acid, 2-[(5,7-dimethyl[1 ,2,4]triazolo[1,5-a]pyrimidin-2-yl)thio]-, ethyl ester	26.452		$C_{15}H_{18}N_6O_3S_2$	394.47	1.43
3H-Imidazo[4,5-b]pyridine, 2-(2-ethylhexylsulfanyl)-	26.660	---		550.4	16.64
3H-Imidazo[4,5-b]pyridine, 2-(2-ethylhexylsulfanyl)-	27.210		$C_{11}H_{12}O_4$	208.21	11.48
Phenol, 3-methoxy-2,4,6-trimethyl-	27.329		$C_{10}H_{14}O_2$	166.21	29.49

Table 2

TLC profile of acetone extract of aerial parts of P. Niruri

Samples	No of spot	$R_f$value	
		Visible	UV
P. niruri(aerial part)	3	0.48,0.54, 0.72	0.48,0.54, 0.72

Table 3

Cytotoxicity of P.niruri of aerial parts on lung cancer (A_{549} cell line).

Concentration of acetone extracts (µg/mL)	Cell viability (%) *P. niruri* Aerial parts
1000	11.11±0.77
500	25.39±1.77
250	36.50±2.56
125	41.26±2.89
62.5	46.03±3.22
31.2	50.79±3.56
	58.73±4.11
7.8	66.66±4.67
Cell control	100±0.00
IC_{50}	30.70

Figure1

GC-MS chromatogram of acetone extract of aerial parts of P.niruri

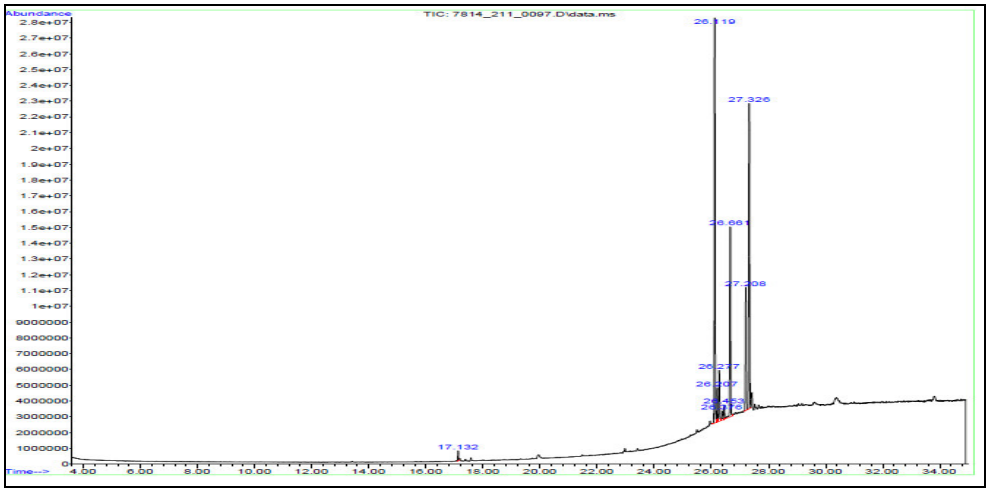


Figure2
TLC profile of acetone extract of aerial parts of P. niruri

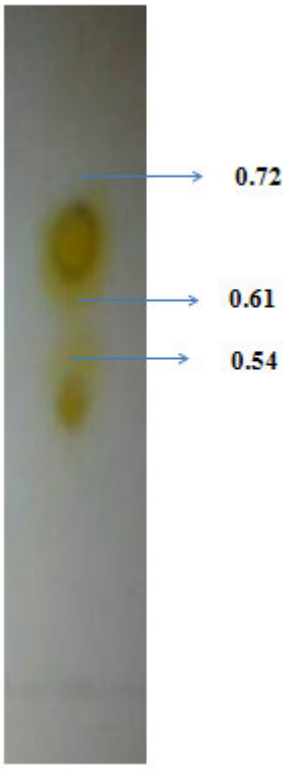
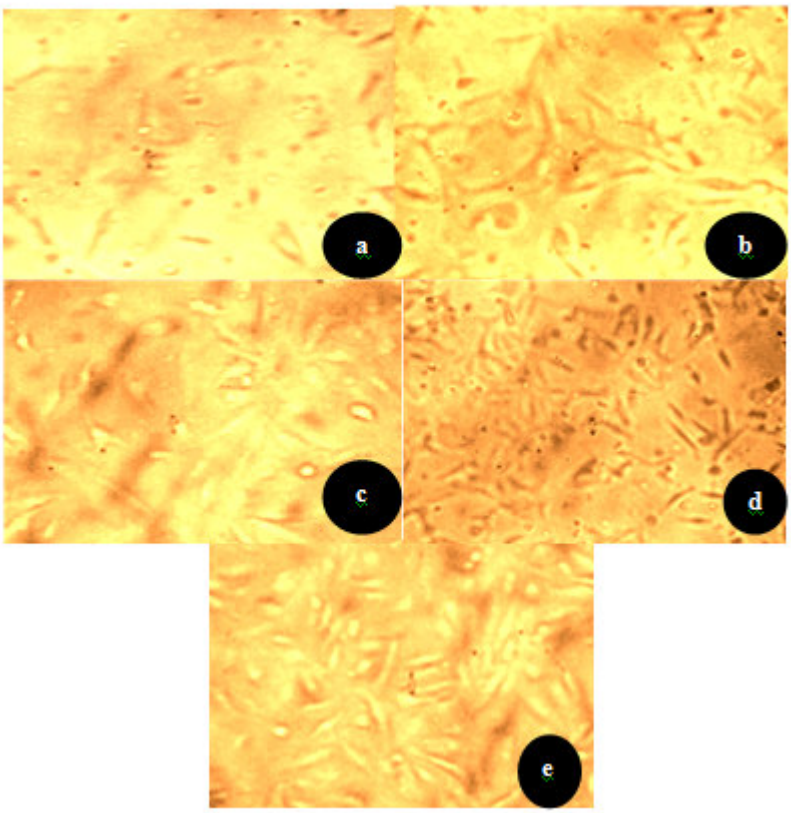


Figure 3
Anticancer activity from acetone extract of aerial parts of P.niruri



a. 1000µg/mL.b. 125µg/mL., c. 62.5µg/mL.d. 31.2µg/mL. e. Control.

CONCLUSION

The crude acetone extract isolated successfully from *P. niruri,* showed the presence of flavonoids as obtained from TLC and GC-MS analysis. The isolated flavonoids showed potential anticancer activity. Cytotoxic study suggested that flavonoid from *P.niruri* was found to contain the maximum inhibition of 66.6% at 7.8µg/mL concentration.So the findings of this study could be considered as a valuable information for the use of medicinal natural products in cancer treatment.

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