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PHYTOCHEMICAL SCREENING AND *IN VITRO* CYTOTOXICITY ANALYSIS OF UVARIA NARUM (DUNAL) WALL.

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ABSTRACT

Cytotoxic potential of the phytoconstituents extracted from leaves of *Uvaria narum* (Dunal) Wall. ex Hook.f. and Thoms was evaluated. Biochemical and thin layer chromatography (TLC) analysis revealed phytosterols, terpenoids, flavonoids, phenols and carbohydrates in various solvent extracts. Phytosterols and terpenoids were the major constituents in petroleum ether (PE) and chloroform (CHL) extracts. Cytotoxic effect of individual extract on Dalton's Lymphoma Ascites (DLA), Ehrlich's Ascites Carcinoma (EAC) and normal rat spleen cells were determined by trypan blue exclusion method. Towards these cells, PE, CHL, Acetone (ACT) and Methanol (MeOH) extracts showed significant toxic effect. However the aqueous extract was non toxic. The cytotoxicity displayed by PE extract was selective being toxic to cancer cells (IC_{50} for DLA was 19 ± 0.57 and that of EAC cells was 38 ± 0.74 μg/mL) while less toxic to normal spleen cells (Above 100 μg/mL). The study indicates the presence of cytotoxic phytochemicals in *U.narum* leaf with remarkable potential in the chemotherapeutic drug development.

KEYWORDS

Uvaria narum, Cytotoxicity, Phytochemicals, TLC analysis, Terpenes and Alkaloids

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INTRODUCTION

Phytochemicals are increasingly being used in the prevention and treatment of several human ailments including, cancer, heart diseases, diabetes, neuro degeneration and metabolic diseases[1]. Among the class of phytochemicals, polyphenols are known to possess chemopreventive activity[2] and terpenoids exhibit cytotoxicity against a variety of tumor cells as well as anticancer efficacy in pre-clinical animal models [3]. Recently, members of family Annonaceae have been investigated as potential sources of biologically active

acetogenins, a polyketide natural compound, which exhibited powerful anti-tumor activities [4]. The Uvaria species belonging to the same family exhibited anti-microbial and anti-helminthic activities in their individual screening and presence of acetogenins could be attributed to a great extent for the biocidal properties of *U. narum* Wall. and *U. hookeri* King. root extracts [5]. *Uvaria narum* (Dunal) Wall. ex Hook.f. and Thoms is widely distributed in the foot hills of Western Ghats, popularly used in ethnomedicine for the treatment of eczema and pityriasis [5]. The plant pacifies vitiated vata, pitta, eczema, itching, varicose vein, hemorrhoids, jaundice, inflammation and fever. The plant has been well recognized for its antibacterial, antifungal and antioxidant activities[5]. The hepatoprotective activity of the leaf extract of *U. narum* against thioacetamide induced hepatic injury has been reported[6]. There is no further information available on the phytochemical components and biological properties of *U. narum*. The present study was carried out to perform the phytochemical screening of various extracts of *U. narum* leaf and evaluate their cytotoxicity.

MATERIALS AND METHODS

Chemicals

Acetic anhydride, benzene, ferric chloride, ninhydrin, potassium iodide and vanillin were purchased from MERCK chemical Ltd. India. Anisaldehyde and bismuth nitrate were from Sisco Research Laboratories (SRL) Pvt. Ltd. India and 1-naphthol was purchased from SIGMA chemical Company Inc., India. All other chemicals and reagents were of analytical grade.

Animals

Swiss albino male mice (average body weight 25-35g) and male Wistar rats were purchased from the Small Animal Breeding Station (SABS), Agricultural University, Mannuthy, Kerala, India. The animals were maintained under standardized environmental conditions (22-28°C, 60-70% relative humidity, 12 h dark/light cycle) in the animal house of Amala Cancer Research Centre. The animals were provided with standard mouse chow (Sai Durga Feeds and Foods, Bengaluru, India) and water *ad libitum*. All animal experiments were conducted according to rules and regulations of CPCSEA, Government of India (No: 149/ 1999/ CPCSEA).

Cell lines

Dalton's Lymphoma Ascites (DLA) and Ehrlich's Ascites Carcinoma (EAC) cells maintained in the peritoneal cavity of Swiss albino mice.

Collection and preparation of plant samples

U. narum was collected locally from Thrissur, Kerala and authenticated by Dr. Sujanapal P, Taxonomist, Kerala Forest Research Institute, Peechi. A voucher specimen was maintained (Voucher Number. KFRI PS 19033). Approximately 30 g dried leaf powder was successively extracted using 10 volumes of solvents of increasing polarity such as petroleum ether (PE), chloroform (CHL), acetone (ACT), methanol (MeOH) and water for 24 h period. It was then centrifuged at 2500 rpm and the supernatant was collected and evaporated off the solvent. The residue was saved and stored in air tight bottles at 4ºC for various studies.

Phytochemical screening and TLC profiling of different extracts

Phytochemical screening of various extracts of *U. narum* leaves was carried out according to standard procedures [7, 8, 9]. One-dimensional TLC analysis was performed on 20 × 20 cm silica plates (Merck KGaA, Darmstadt, Germany) with different mobile phases for different extracts. N-hexane: diethyl ether: acetic acid (35:15:0.75 V/ V/ V) for PE, benzene: ethyl acetate (5:1 V/ V) for CHL, chloroform: methanol: water (16:5:0.8 V/ V/ V) for ACT and MeOH and, methanol: water: acetic acid (18:9:1 V/ V/ V) for water extracts were the mobile phases used. The Rf values of individual bands in each chromatogram were calculated and phytochemical classes were identified using general spraying reagents.

Short term *in vitro* cytotoxicity assay on various cell lines

Preparation of rat spleen cells: Male Sprague-Dawley rats weighing 250-300 g were anaesthetized with chloroform; the spleen was surgically removed and collected in a sterile nylon sieve over a petridish half filled with PBS. The spleen was gently pressed through the sieve using a plunger; PBS was then added to keep the cells moist. The dis-aggregated

splenocytes were transferred to a test tube and centrifuged at 1000 rpm for 5 minutes. Repeated the washing and centrifugation process until the suspension become free of debris. The cell pellet was re-suspended in PBS and counted using a haemocytometer.

In short term *in vitro* cytotoxicity analyses, cells (DLA, EAC and spleen) were suspended in 1 ml PBS and the cell number was adjusted to 1×10^6 cells/mL. Test materials were added at various concentrations (1-100 μg/mL) in minimum volume of vehicle and incubated for 3 hr at 37^{o}C. Cell death was checked using trypan blue exclusion method[10].

Statistical analysis

All *in vitro* assays were performed in triplicate and data represented are mean ± SD.

RESULTS

Phytochemical analysis

Qualitative phytochemical analysis of *U.narum* leaf extract revealed the presence of various classes of phytochemicals (Table No. 1). Terpenoids, steroids, flavonoids, tannins, alkaloids proteins, amino acids and carbohydrates were present in the extracts. In the aqueous extract however tannins and alkaloids were not detected.

Thin layer chromatography analysis

Thin layer chromatographic analysis of *U. narum* leaf PE extract resulted in the identification of five visible bands and under UV light (254nm) revealed two additional fluorescent bands. The bands with Rf values 0.32, 0.43, 0.57 and 0.97 were found positive for terpenoids class of compounds when sprayed with vanillin - sulphuric acid and anisaldehyde-sulphuric acid reagents (data not shown). In Liebermann - Burchard reagent, bands with R_f values 0.15 and 0.43 showed positive result indicating the presence of steroids.

Thin layer chromatography analysis of the chloroform extract revealed the presence of four visible bands, four additional fluorescent bands in UV light (254 nm) and two extra bands under iodine vapors. Among these, bands with Rf values 0.31and 0.64 were positive for terpenoids as observed with vanillin - sulphuric acid and anisaldehyde - sulphuric acid spray reagents. Liebermann-Burchard spray for steroids identified a band with Rf value 0.97.

A total of 17 bands were obtained in TLC analysis of acetone extract. Out of these, six were visible bands, nine were fluorescent bands under UV (254 nm) and two bands when exposed to iodine vapors were observed (Table No. 2). Compounds with Rf values 0.07, 0.32, 0.73 and 0.95 were obtained with solvent system chloroform: methanol: water (16:5:0.8 V/ V/ V) showed positive for terpenoids in vanillin - sulphuric acid and anisaldehyde- sulphuric acid spray reagents. Bands with Rf values 0.071 and 0.32 showed positive for steroids with Liebermann–Burchard spray reagent. Molecules with Rf values 0.15, 0.46, and 0.89 were positive for flavonoids in ammonia reagent and bands with Rf values 0.18, 0.33 and 0.94 were identified as anthraquinone positive under methanol-potassium hydroxide spraying reagent.

Thin layer chromatography of the methanolic extract revealed four visible, four fluorescent and two iodine positive bands. The chromatogram when sprayed with vanillin- sulphuric acid and anisaldehyde-sulphuric acid reagents, bands having Rf values 0.44, 0.54 and 0.91 were positive for terpenoids. In Liebermann–Burchard reagent spray, bands with Rf values 0.12 and 0.32 showed the presence of steroids. Compounds with Rf values 0.15, 0.46 and 0.89 positive for methanol- potassium hydroxide reagent were identified as anthraquinones. Ammonia reagent spray detected compounds with Rf values 0.18, 0.33 and 0.94 indicating the presence of flavonoids (Table No. 2 & Figure No. 1).

Short term *in vitro* cytotoxicity of different extracts on various cell lines

Petroleum ether extract at various concentrations (1 - 100 μg/mL) did not produce any appreciable cytotoxicity towards spleen cells (Figure No. 2c). Even upto 100 μg/mL, IC_{50} value was not detected. In case of DLA and EAC cells, there was a dose dependent increase in the cytotoxicity. The required PE extract concentration to produce half the cell death (IC_{50}) for DLA was found to be 19±0.57 μg/mL and that of EAC cells is 38± 0.74 μg/mL. Chloroform extract showed dose-dependent toxicity

towards all the three cell lines. The IC_{50} for CHL extract was 15±1.2, 17±0.99 and 9.1±0.36 μg/mL with EAC, DLA and splenocytes respectively (Figure No. 2). Acetone extract also showed significant cytotoxicity towards spleen cells, DLA and EAC cells with IC_{50} concentrations of 29± 3.21, 19±1.17 and 29±0.92 μg/mL respectively. Methanol extract showed high toxicity towards all the three cell lines in a dose dependent manner. The half maximum cell death for MeOH extract was observed at 1.9± 0.53, 3.6± 0.95 and 15± 1.56 μg/mL concentrations in EAC, DLA and splenocytes respectively (Figure No. 2). Aqueous extract however was less toxic to these cell lines.

DISCUSSION

In the present study, except aqueous extract, all other extracts of *U. narum* leaf show potential cytotoxicity. Compounds of PE extract show differential cytotoxicity, being toxic towards cancer cell lines while less toxic to normal spleen cells. In the phytochemical screening and TLC analysis of PE extract, terpenoids and phytosterols are detected as major phytoconstituents. Terpenoid and alkaloid class of compounds are known to have anti-tumor properties [11, 12, 13]. For instance, Vincristine and Vinblastine are alkaloids isolated from *Vinca rosea*, taxol is a triterpene obtained from bark of pacific yew tree (*Taxus brevifolia*) [14]. It is thus expected that the cytotoxic effect by PE extract may be due to its terpenoid or content. This necessitates further purification of these cytotoxic extract to identify cytotoxically active component. Many cancer chemotherapeutic drugs are showing side effect due to depletion of immune cells. Spleen is an important organ in the immune system towards which PE show less toxicity. This indicate that the active component likely have less side effects towards the body's immune system.

The CHL, ACT and MeOH and aqueous extracts are shown to contain various classes of phytochemicals such as alkaloids, terpenes, phytosterols, flavonoids, phenols and carbohydrates. The results obtained were comparable with earlier studies[15]. Potential cytotoxic effect has been shown by CHL, ACT and MeOH extracts and their cytotoxic potential towards cancer cells is equal in magnitude to that of normal cells. However further purification can yield better cytotoxic components from these extracts. Moreover the phytosterol, flavonoids and phenolic acid contents in these extracts may have other biological effect too. Many phytosterols are important physiological regulators that mainly act by eliciting hormonal signaling [16, 17, 18]. Flavonoids are important classes of antioxidant and anti-inflammatory molecules by way of which they produce known anticancer and chemopreventive properties[19]. Therefore it has been concluded that *U.narum* leaf is an important source of biologically relevant cytotoxic phytoconstituents.

Table No.1: Phytochemical screening of petroleum ether (PE), chloroform (CHL), acetone (ACT), methanol (MeOH) and aqueous extracts of *U. narum* leaf

S. No	Phyto Chemicals	Reactions	PE	CHL	ACT	MeOH	Water
1	Alkaloids	Mayers test	+++	+	+	-	-
		Wagners test	+	-	-	-	-
		Hagers test	+++	+	+	+	-
		Dragendorff's test	+	+	+	+	-
2	Saponins		-	-	-	+	+

3	Phytosterol and terpenoid	Salkowski Test	++	++	++	++	++
		Libermann-Burchard test	++	++	++	++	++
		Sulphuric acid test	++	++	++	++	++
4	Phenol and Flavanoid	Ferric chloride test	-	-	+	+	+
		Lead acetate test	+	+	+++	++	++
		NaOH test	++	++		++	+
5	Tannins	Gelatin	+	-	+	+	-
		Potassium hydroxide	_	-	-	-	-
6	Proteins and amino acids	Xanthoproteic test	+	+	+	+	+
		Ninhydrin test	-	-	-	+	-
		Biuret	+	+	+	+	-
7	Carbohydrates	Molischs test	+	+	+	+	+
		Benedicts test	+	+	+	+	+++
		Fehling's test	+	+	+	+	+
8	Anthraquinone		_	-	-	-	+
9	Glycoside	Borntragers test	_	-	-	_	-
10	Coumarin	NaOH test	+	+	+	-	-

Presence (+) and absence (–) indicated by the intensity of reaction.

Table No.2: TLC analysis of petroleum ether (PE), chloroform (CHL), acetone (ACT), methanol (MeOH) and aqueous extracts of *U. narum* leaf

S. No	Extract	Solvent system	Rf values			Phytochemicals detected
			Visible light	UV at 254nm	Iodine vapour	
1	PE	n-hexane: diethyl ether**:** glacial acetic acid (35:15: 0.75)	0.15, 0.32, 0.43, 0.50	0.67, 0.87	-	Terpenoids, Phytosterols
2	CHL	benzene: ethyl	0.066, 0.844,	0.311, 0.533,	0.22, 0.977	Terpenoids,

		acetate (5:1)	0.888, 0.97	0.644, 0.666		Phytosterols
3	ACT	chloroform: methanol: water (16:5:0.8)	0.071,0.18,0.32, 0.46, 0.63, 0.93	0.33,0.46, 0.57, 0.64, 0.73, 0.85, 0.89, 0.95	0.33, 0.94	Terpenoids, Phytosterol, Flavonoids , Anthraquinone
4	MeOH	chloroform: methanol: water (16:5:0.8)	0.035, 0.12, 0.32, 0.44	0.39, 0.54, 0.81, 0.9	0.26, 0.91	Terpenoids, Phytosterol, Anthraquinone Flavonoids
5	Water	methanol: water: acetic acid (18:9:1)	0.89	0.056, 0.83, 0.99	-	No appreciable positive response

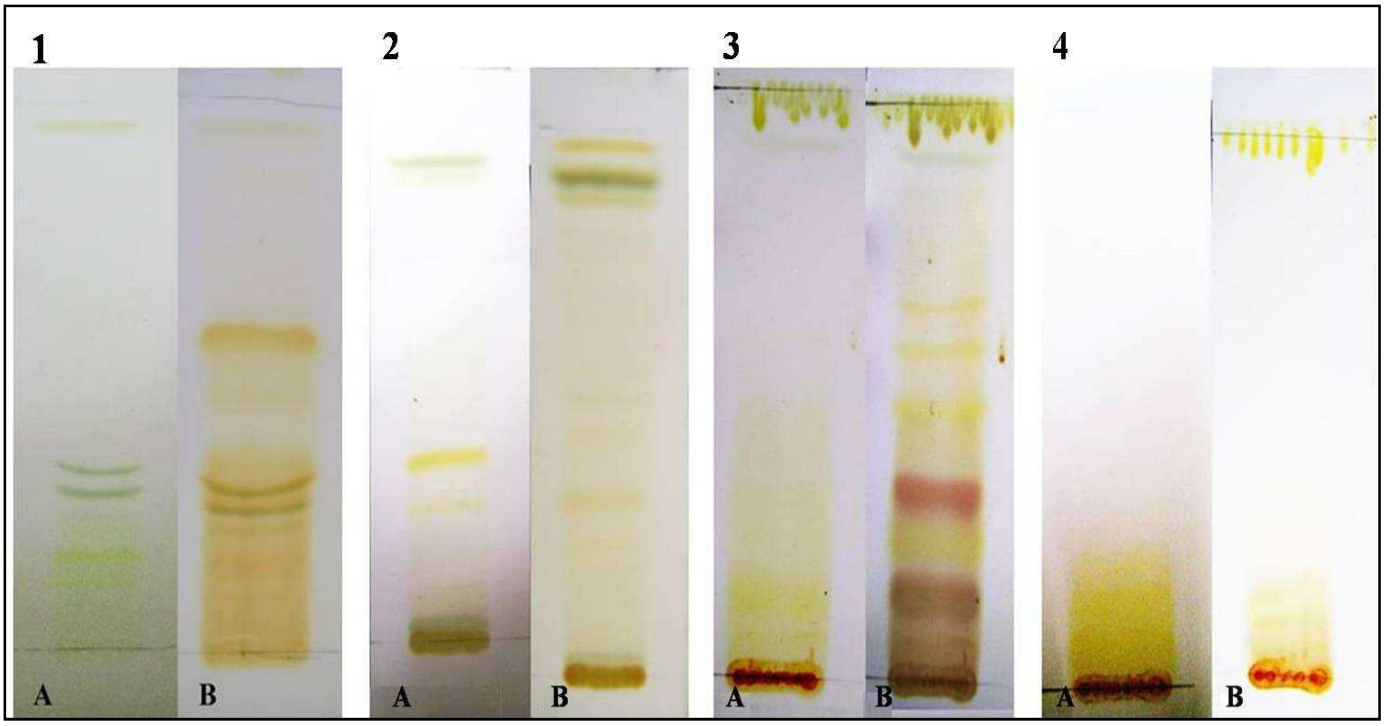

Figure No.1: TLC of the *U.narum* leaf PE (1), CHL (2), ACT (3), and MeOH (4) extracts. Extracts (10μg/mL) were developed using different mobile phases and observed under (A) visible light and (B) iodine vapour

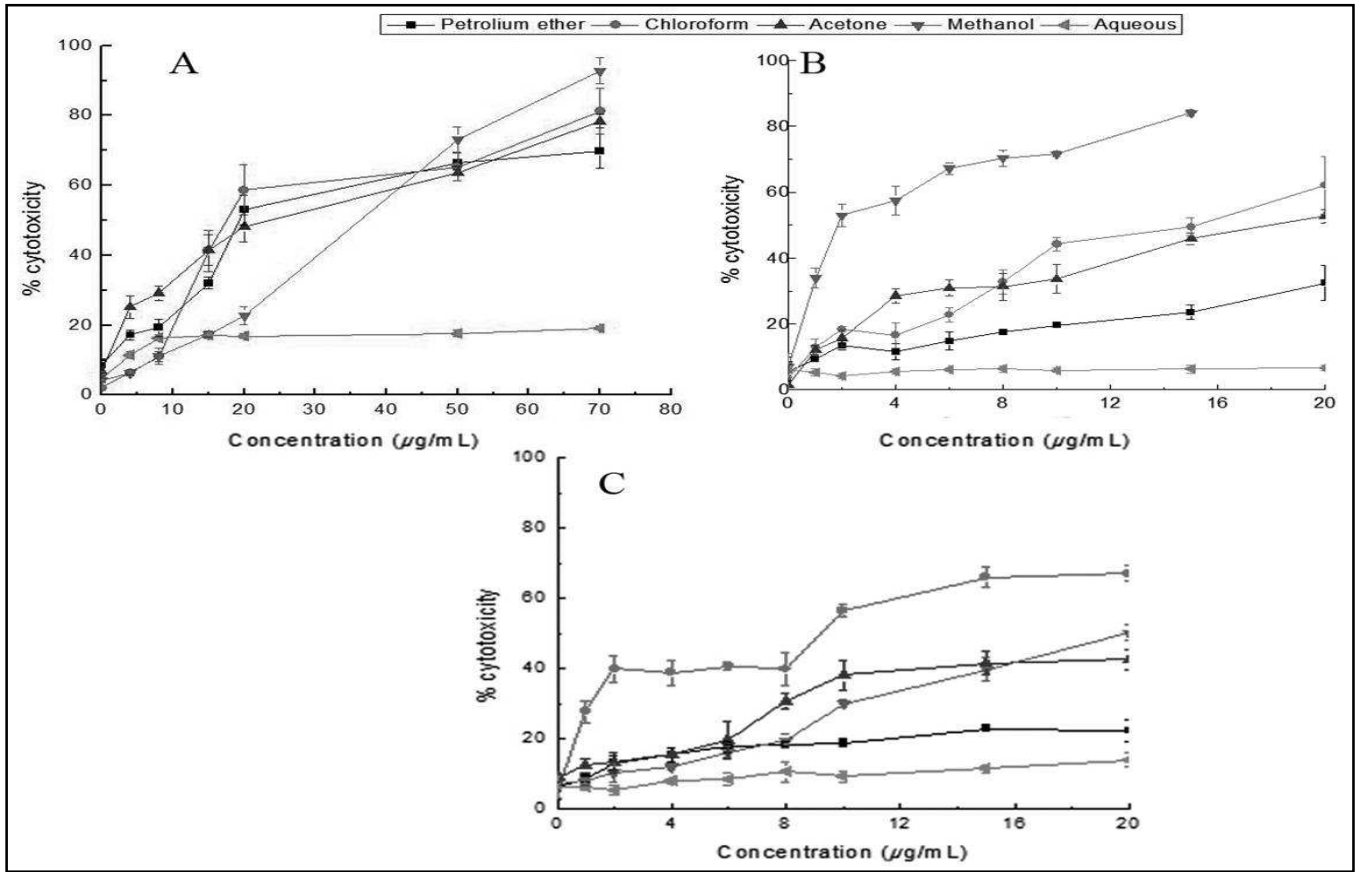

Figure No.2: *U.narum* leaf extracts exhibit cytotoxicity on (A) DLA, (B) EAC and (C) spleen cells. Various concentrations (10-100 μg/mL) of individual extracts were added to cell suspension in 1 mL PBS and incubated for 3h at 37 °C. The cell death was determined by Trypan blue method. Values are mean ± SD of three independent experiments.

CONCLUSION

Leaf of *U.narum* is rich source of terpenoids, phytosterols, flavonoids, phenols and carbohydrates. Compared with other solvent extracts, PE extract of *U. narum* leaves shows differential cytotoxicity. Terpenoids and phytosterols are the major phytoconstituents identified in this extract which may be responsible for its cytotoxic potential. The study reveals the potential of *U. narum* as a source of new effective cytotoxic compounds.

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