REPORT

Anticancer activity of the roots of *Ichnocarpus frutescens* R. Br. and isolated triterpenes

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Abstract: The roots of *Ichnocarpus frutescens* along with roots of *Cissampelos pareira*, *Bauhinia vahlii* and *Ardisia solanacea* are processed together and given orally to cure stomach cancer by the tribes of Chotanagpur and Santhal parganas of Bihar, India. *In vitro* anticancer activity of the residue from methanolic extract of roots of *I. frutescens* (MIF) and isolated triterpenes were evaluated by 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay using MCF-7, BEL-7402, SPC-A-1 and SGC-7901 cancer cell lines. MIF showed significant anticancer activity on four cancer cell lines with IC_{50} values 163.5±3.58, 156.3±2.95, 142.6±2.60 and 112.4±1.85 respectively as compared to vehicle treated control. Ursolic acid showed anticancer activity on four cancer cell lines with IC_{50} values 8.5±0.29, 9.9±0.12, 8.1±0.40 and 6.2±0.23 respectively, while IC_{50} values for α-amyrin on four cancer cell lines was found to be 7.2±0.12, 8.2±0.29, 7.6±0.06 and 5.0±0.12 respectively.

Keywords: *Ichnocarpus frutescens*; anticancer; polyvinylpolypyrrolidone; ursolic acid; α-amyrin.

INTRODUCTION

Ichnocarpus frutescens R. Br. belongs to family *Apocynaceae*, is a woody climber found almost in all part of India, having an altitude of 4000 ft. (Anonymous, 1987). *I. frutescens* is used by tribes as an alternative of Indian Sarsaparilla (*Hemidesmus indicus*), for the treatment of atrophy, convulsions, cough, delirium, dysentery, measles, splenomegaly, tuberculosis, abdominal and glandular tumors. Its roots are used as alterative, antidysentric, antipyretic, demulcent, diaphoretic and hypoglycemic (Chatterjee & Prakashi, 2003; Anonymous, 2002; Ambasta, 1999). *I. frutescens* is used in the indigenous system of medicine in the treatment of fever, gout, rheumatic arthritis, epilepsy, venereal diseases, herpes and skin diseases (Anonymous, 1959; Nandkarni, 1982). Decoction of roots is used as blood purifier (Goel & Mudgal, 1988). The Gond tribes of Patalkot and Tamiya, District Chhindwara, Madhya Pradesh use the roots of the plant as a remedy for jaundice (Rai, 1988). The roots of *I. frutescens* along with roots of *Cissampelos pareira*, *Bauhinia vahlii* and *Ardisia solanacea* are processed together and given orally to cure stomach cancer by the tribes of Chotanagpur and Santhal parganas of Bihar, India (Hembrom, 1991). Leaf of the plant is used by the tribes of Chitrakoot, Madhya Pradesh on cuts to stop bleeding (Sikarwar *et al*., 2008).

The present study involves the isolation and characterization of two triterpenes from the roots of *I. frutescens* and evaluation of *in vitro* anticancer activity of methanolic extract as well as isolated triterpenes using four different cancer cell lines.

MATERIALS AND METHODS

Cell lines and chemicals

MCF-7 (Human breast cancer cell line), BEL-7402 (Human hepatocellular carcinoma cell line), SPC-A-1 (Human lung cancer cell line) and SGC-7901 (Human gastric cancer cell line) obtained from the American Type Culture Collection (Manassas, USA). Penicillin and streptomycin were obtained from M P Biomedicals (India) Pvt. Ltd. MTT, polyvinylpolypyrrolidone (PVPP) and Cyclophosphamide monohydrate were obtained from Sigma-Aldrich Co. LLC. α-amyrin and ursolic acid used for anticancer activity were isolated from the residue of methanolic extract of roots of *I. frutescens*. All other chemicals of analytical grade were used in the present study.

Plant material

Plant material was collected from the medicinal garden of Rajiv Gandhi South Campus, Banaras Hindu University, Barkachha, Mirzapur during the month of December 2010. Plant material was identified by Prof. K. N. Dubey, Department of Botany, Faculty of Science, Banaras Hindu University, Varanasi. A voucher specimen (No. PRL-02) of the plant material has been deposited in the Department of Medicinal Chemistry, Faculty of Ayurveda, Institute of Medical Sciences, Banaras Hindu University, Varanasi for future reference.

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Extraction and isolation

Roots of *I. frutescens* were shade dried and pulverized in the form of coarse powder. Coarse powder (475g) of the roots of *I. frutescens* was subjected to maceration in methanol (2L). After 10 days solvent was filtered and concentrated in rotary evaporator at less than 60°C temperature. Methanolic extract (25 g) obtained was kept in vacuum desicator to remove solvent completely. Column chromatography was performed for residue obtained from methanolic extract using silica gel (60-120#) as an adsorbent. The gradient elutions were made by different ratio of petroleum ether: ethyl acetate and chloroform: methanol. Petroleum ether: ethyl acetate (20: 80) fraction yielded a brown color amorphous compound (1) which was crystallized in methanol. Compound has melting point 283ºC and R_f value 0.7 (hexane: ethyl acetate, 3: 1). Compound 1 was identified as ursolic acid on the basis of co-TLC with standard ursolic acid and its melting point.

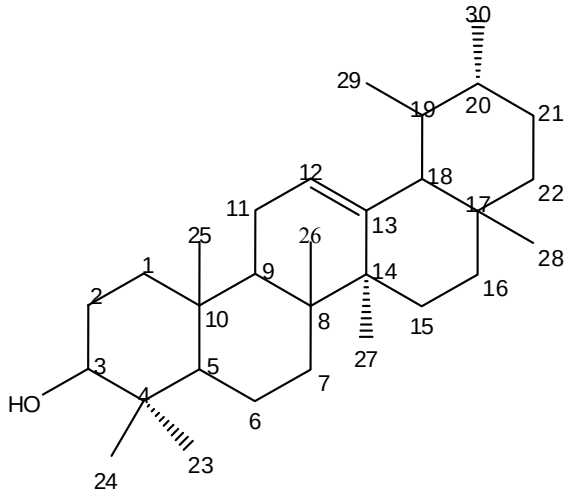

Fig. 1: Compound 2

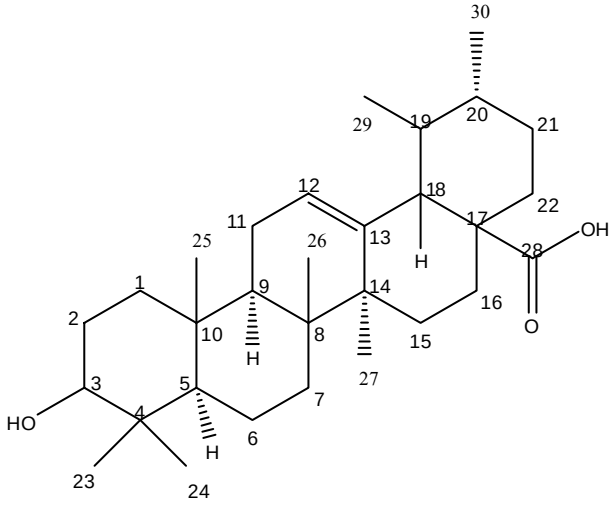

Fig. 2: Compound 1

The chloroform: methanol (90: 10) fraction yielded white color crystalline compound (**2**) which was crystallized in methanol. The physical and spectroscopic data of compound (**2**) are given below.

Compound (**2**): m. p. 178°C; R_f = 0.6 (chloroform: methanol, 9.5: 0.5); UV λ_{max} (MeOH) (log ε): 281(0.795) nm; IR bands (KBr): 3416, 2936, 2867, 1714, 1653, 1559, 1461, 1378, 1163, 1055, 671 cm^{-1}; ESI-MS (Positive ion mode) m/z (% rel. int.): 429 $[M+3]^+$ (100), 428 $[M+2]^+$ (8), 427 $[M+1]^+$ (40), 413 (35), 411(32), 391 (30), 387 (5), 293 (4), 240 (), 205 (5), 167 (3); ^{1}H-NMR (300 MHz, $CDCl_3$): δ: 5.11 (1H, t, 3Hz, H-12), 3.52 (1H, m, H-3), 0.96-1.08 (21H, m, 7 x Me); ^{13}C NMR (75 MHz, $CDCl_3$): 140.7 (C-13), 121.7 (C-12), 71.8 (C-3), 56.7 (C-18), 56.0 (C-5), 50.1 (C-9), 45.8 (C-14), 42.3 (C-22), 39.7 (C-8), 39.4 (C-20), 39.2 (C-19), 37.2 (C-4), 36.5 (C-1), 36.1(C-10), 33.9 (C-7), 31.9 (C-17), 31.6 (C-21), 28.2 (C-15), 26.0 (C-2), 24.2 (C-23), 24.0 (C-24), 23.0 (C-11), 21.0 (C-28), 19.8 (C-27), 19.3 (C-30), 19.0 (C-6), 18.7 (C-16), 11.9, (C-25), 11.8 (C-26), 11.4 (C-29). On the basis of physical properties and spectral data, compound 2 was identified as α-amyrin.

Preparation of plant extract

MIF (500 mg) was dissolved in 5 mL of distilled water. It was passed through PVPP column and eluted with distilled water (20 mL). The eluate obtained was freeze dried. Presence and absence of phenolic compounds in the untreated and PVPP treated extract were confirmed by the addition of 1 drop of 5% $FeCl_3$ solution (Tunon, 1997).

Quantitative HPTLC of methanolic extract of the roots of I. frutescens

HPTLC was performed on plates (20 cm × 10 cm) coated with silica gel 60G F_{254} (Merck, Mumbai, India). Camag (Muttenz, Switzerland) Linomat V sample applicator equipped with a 100µL Hamilton (USA) syringe was used for making band on the HPTLC plates. Standard solution of α-amyrin and ursolic acid (40µg/mL, each) and methanolic extract (1.25mg/mL) were applied to the plates for making the band of 8.0 mm wide, 30.0 mm apart from each other and 10 mm from the bottom edge of the plates. Chromatographic plates were developed in ascending order up to a height of 80 mm at room temperature (28±2°C), using chloroform: methanol, 9.5: 0.5 (v/v), as mobile phase in Camag glass twin-trough chamber. Plates were dried after development and scanned at 510 nm using Camag TLC Scanner with deuterium lamp and WINCAT software.

MTT assay

In vitro anticancer activity was performed on four different cancer cell lines, MCF-7, BEL-7402, SPC-A-1 and SGC-7901 by MTT assay (Mosmann, 1983). Cell lines were obtained from the American Type Culture Collection (Manassas, USA), were cultured in RPMI

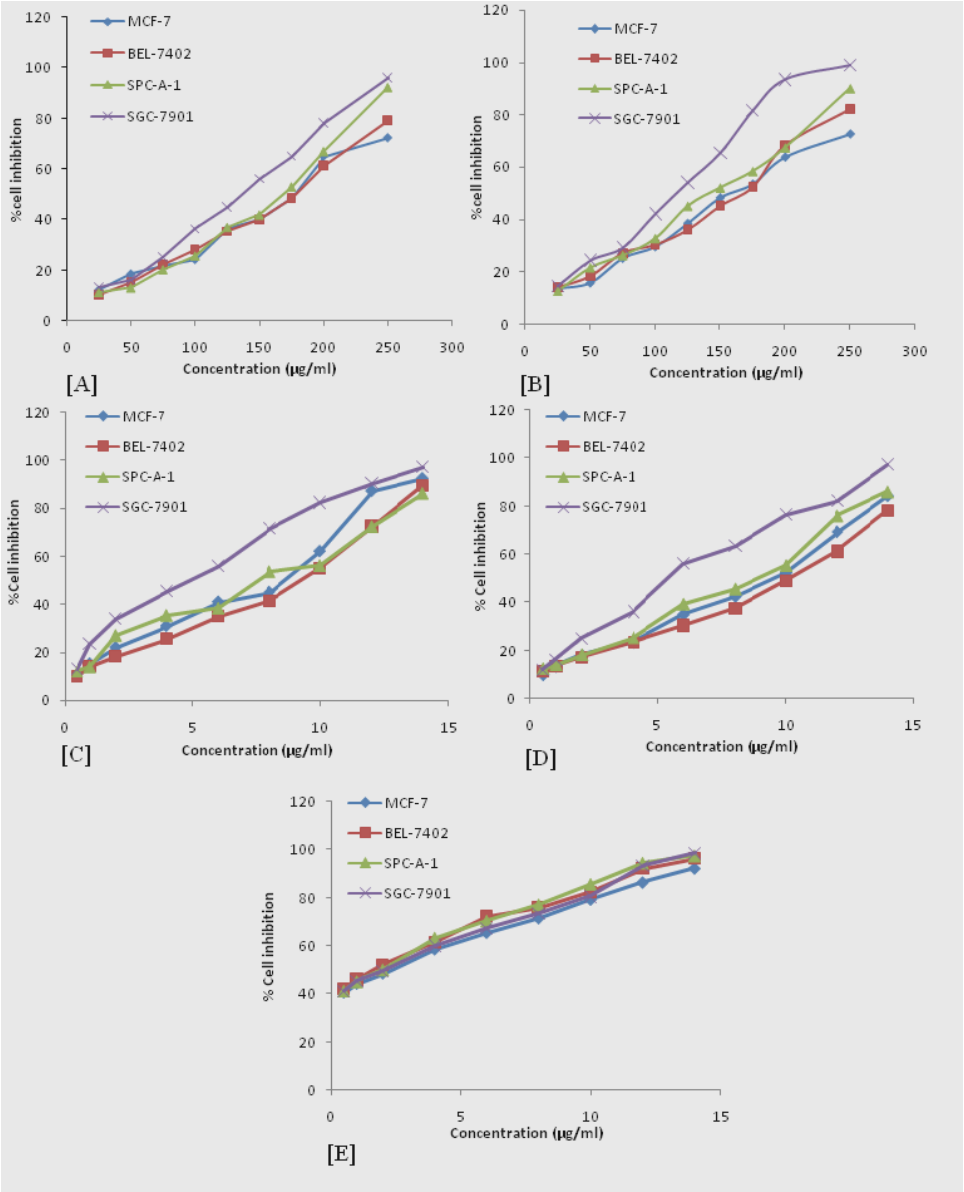

Fig. 3: Dose response curve of (A) PVPP treated MIF (B) MIF (C) α-amyrin (D) Ursolic acid (E) Cyclophosphamide monohydrate on four different cancer cell lines.

1640 (Gibco, Grand Island, NY) cell culture medium containing 1% (w/v) penicillin-streptomycin and 10% (v/v) fetal bovine serum (Gibco) at 37°C in a humidified and 5% CO_2–95% air atmosphere. Cells were incubated at a density of 1×10^4 cells/well in 96-well plates for 12 h. 5 mg of each, MIF and PVPP treated MIF were dissolved in 200 µL of DMSO (dimethyl sulphoxide). 100 µL of this solution was diluted with 10 mL of culture media. Various concentrations of MIF and PVPP treated MIF (25, 50, 75, 100, 125, 150, 175, 200, and 250 µg/mL) were prepared from the stock solution. 1 mg of each, α-amyrin, ursolic acid and cyclophosphamide monohydrate were dissolved in 100 µL of DMSO and diluted with 10 mL of culture media. Various concentrations of α-amyrin, ursolic acid and cyclophosphamide monohydrate (0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10, 12, and 14µg/mL) were prepared from stock solution. Cells were incubated in presence of 100 µL of test and standard solutions of various concentrations for 4 h. After treatment, 20 µL MTT solution was introduced to each incubated plate at a concentration of 5 mg/mL and incubated for another 4h. The purple formazan crystal formed was dissolved in 200 µL DMSO and the optical density of each well was measured with ELISA reader at 570 nm. The effect of α-amyrin, ursolic acid and MIF on cell proliferation was measured in terms of percentage, relative to vehicle treated control, by which the IC_{50} values were calculated.

STATISTICAL ANALYSIS

Mean optical density (OD ± SEM) for each concentration from the three replicate wells in a single plate was calculated. IC_{50} concentrations were calculated from linear regression analysis. Data was accessed by one way ANOVA using Graph prism software. Differences were considered significant at $p<0.05$. The mean IC_{50} ± SEM from three separate plates for each test substance is given in (table 1).

RESULTS

MIF showed significant anticancer activity on four cancer cell lines with IC_{50} values 163.5±3.58, 156.3±2.95, 142.6±2.60 and 112.4±1.85 respectively as compared to vehicle treated control. MIF after removal of phenolics with PVPP showed anticancer activity on four cancer cell lines with IC_{50} values 172.2± 3.87, 167.2±3.23, 155.3±4.10 and 131.7±1.45 respectively. Cyclophosphamide monohydrate used as positive control and IC_{50} values for four cell lines were found to be 2.3±0.23, 1.6±0.06, 1.8±0.29 and 2.1±0.06 respectively. The IC_{50} of ursolic acid on four cancer cell lines were 8.5±0.29, 9.9±0.12, 8.1±0.40 and 6.2±0.23 respectively and for α-amyrin 7.2±0.12, 8.2±0.29, 7.6±0.06 and 5.0±0.12 respectively.

Table 1: IC_{50} values of methanolic extract of the roots of *I. frutescens* and isolated triterpenes.

	IC_{50} (µg/mL)*			
	MCF-7	BEL-7402	SPC-A-1	SGC-7901
α-amyrin	7.2± 0.12	8.2± 0.29	7.6± 0.06	5.0± 0.12
Ursolic acid	8.5± 0.29	9.9± 0.12	8.1± 0.40	6.2± 0.23
Plant extract without PVPP treatment	163.5± 3.58	156.3 ±2.95	142.6± 2.60	112.4 ±1.85
Plant extract after PVPP treatment	172.2± 3.87	167.2± 3.23	155.3 ±4.10	131.7 ±1.45
Cyclophosphamide Monohydrate#	2.3± 0.23	1.6± 0.06	1.8± 0.29	2.1± 0.06

*IC_{50} ± SEM expressed as mean from three separate plates for each test substance. #Positive control.

DISCUSSION

The roots of *I. frutescens* along with roots of *Cissampelos pareira*, *Bauhinia vahlii* and *Ardisia solanacea* are processed together and given orally to cure stomach cancer (Hembrom, 1991). Polyphenolic extract of leaves of *I. frutescens* reported to have antitumor activity (Kumarappan and Mandal, 2007). Phytochemical investigation reveals that triterpenes are the major constituents and only trace amount of phenolics present in the roots of *I. frutescens* was observed. We have isolated two triterpenes α-amyrin and ursolic acid from the roots of *I. frutescens*. This is the very first report for the isolation of α-amyrin from the roots of *I. frutescens* to the best of our knowledge. Both triterpenes are well known to have anticancer activity (Neto, 2007). Phenolic compounds are considered not of great interest due to their non specificity as therapeutic agents. Also phenolic compounds in the plants may cause interference in many biological assays (Well *et al*., 1996). Phenolics may be removed from the plant extract by passing them from PVPP column (Tunon, 1997).

In the present study, MTT assay was performed for the evaluation of *in vitro* anticancer activity of MIF and isolated compounds on four different cancer cell lines (fig. 3). The IC_{50} values obtained are shown in (table 1). In the present study, an attempt was made to find out the fact whether anticancer activity of the roots of *I. frutescens* is due the presence of phenolic constituents or triterpenes. MIF showed significant anticancer activity on four cancer cell lines with IC_{50} values 163.5±3.58, 156.3±2.95, 142.6±2.60 and 112.4±1.85 respectively as compared to vehicle treated control. MIF after removal of phenolics with PVPP showed anticancer activity on four cancer cell lines with IC_{50} values 172.2± 3.87, 167.2±3.23, 155.3±4.10 and 131.7±1.45 respectively, indicates that phenolic constituents have no much effect on anticancer activity. Cyclophosphamide monohydrate used as positive control and IC_{50} values for four cell lines were found to be 2.3±0.23, 1.6±0.06, 1.8±0.29 and 2.1±0.06 respectively. The content of ursolic acid and α-amyrin in the MIF were quantified by quantitative HPTLC. The amount of ursolic acid was found approximately 2% w/w in the methanolic extract and approximately 0.1% w/w in the dried plant material. For α-amyrin, amount was approximately 1.2% w/w in the methanolic extract and approximately 0.06% w/w in the dried plant material. The IC_{50} of ursolic acid on four cancer cell lines were 8.5±0.29, 9.9±0.12, 8.1±0.40 and 6.2±0.23 respectively and for α-amyrin 7.2±0.12, 8.2±0.29, 7.6±0.06 and 5.0±0.12 respectively. The IC_{50} of ursolic acid and α-amyrin were approximately of a similar order to their concentration in the MIF. This indicates that the anticancer activity of the MIF may be due to the presence of these two triterpenes.

CONCLUSION

The present study report the isolation, characterization of ursolic acid and α-amyrin from the roots of *I. frutescens* and significant anticancer activity due presence of two triterpenes. The results support the use of roots of *I. frutescens* and its constituent triterpenes as anticancer agent.

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