ABSTRACT

TITLE: ISOLATION AND CHARACTERIZATION OF ANTICANCER PRINCIPLES FROM ZANTHOXYLUM ALATUM ROXB.

INTRODUCTION
Cancer is a multistage process in which the uncontrolled growth of cells results into accumulation of lumps of cells in a particular tissue which may further metastasize. Cancer is the second cause of mortality after cardiovascular diseases. There are various synthetic drugs which are known to have potent anticancer activity but they have various side effects. So, there is requirement of those drugs which shows good anticancer activity but with lesser side effects. Plants have a long history of use in the treatment of cancer. Naturally occurring drugs that are accepted internationally includes; vinca alkaloids, taxanes, anthracyclines, podophyllotoxin, camptothecin and its derivatives. There are large numbers of molecules that are still remains to be explored by the medicinal chemists. Today much attention has been paid to natural antioxidant and their association with health benefits. Plants are the potential source of natural antioxidants. Apoptosis is major cell death pathway for removing unwanted and harmful cells. Most anticancer therapies rely on the activation of apoptotic pathways. Defective apoptosis represents a major causative factor in the development and progression of cancer. Zanthoxylum alatum (Rutaceae) is a small tree upto 6 m. in height with dense glabrous foliage and straight prickles on stem. It is distributed in Himalayas from Kashmir to Bhutan upto 2100 m and in Khasia hills upto 1350 m. Zanthoxylum alatum has been used traditionally for number of disorders like cholera, diabetes, cough, diarrhoea, fever, headache, microbial infections, toothache, inflammation and cancer. So, the present study was carried out to isolate active principles from Zanthoxylum alatum which can be used for the treatment of cancer.

AIM AND OBJECTIVE
To scientifically evaluate the Zanthoxylum alatum Roxb. stem bark for its antioxidant and anticancer potential and to isolate, purify and characterize the compound(s)
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responsible for anticancer activity. The identified most potent compound will further subjected to apoptosis study.

MATERIAL AND METHODS

The stem bark of Zanthoxylum alatum was collected from the local areas of Tehri (Garwal), Uttarakhand, India and authenticated by Dr. Sunita Garg from NISCAIR, New Delhi (Ref. NISCAIR/RHMD/Consult/2013/2233/14). Plant drug was shade dried (< 40°C), coarsely powdered and stored in air tight container till further use. The physiochemical parameters such as ash and extractive values were determined according to the procedure given in WHO guidelines. Successive solvent extraction scheme was followed for the preparation of different extracts (Petroleum ether, chloroform, ethyl acetate, methanol extracts were prepared). All extracts were screened phytochemically to confirm the type of constituents present in the plant. Extracts were subjected to cytotoxic assay (MTT assay) to select the most potent extract for the isolation of active constituents. The compounds isolated from most potent extract(s) were also subjected to cytotoxic assay. The extract(s) having cytotoxic potential were tested for their antioxidant property using different established in vitro models such as DPPH, NO radical scavenging activity and reducing power assay. All experiments were performed thrice and results were expressed in IC50 values. Petroleum ether and ethyl acetate extracts were subjected to column chromatography for the isolation of active compound as they both showed significant antioxidant and cytotoxic activity as compared to other extracts. Four compounds A, B, C, D were isolated from petroleum ether and two compounds E and F were isolated from ethyl acetate extract. Compounds were purified using recrystallization technique. Structure elucidation was done by various spectrophotometric techniques like UV, IR, 1H NMR, 13CNMR and Mass spectroscopy. The type of cell death caused by most active
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Compound D was explored by fluorescence microscopy using acridine orange/ethidium bromide (AO/EB) double staining assay.

RESULTS

The percentage ash values (w/w) such as total ash, acid insoluble ash and water soluble ash were found to be 8.5 ± 0.53, 2.0 ± 0.67 and 3.5 ± 1.01 respectively. The percentage extractive values (w/w) in hot and cold were 19.2 ± 1.04, 17.4 ± 2.76 in ethanol; 14.1 ± 1.39, 12.4 ± 2.51 in water. The percentage yields of extracts were as petroleum ether (5.2% w/w), chloroform (1.8% w/w), ethyl acetate (6.5% w/w) and methanol (2.8% w/w). The plant has shown the presence of glycoside, steroids, phenolic compounds, flavonoids and alkaloids.

The petroleum ether and ethyl acetate extracts had shown more cytotoxic potential as compared to other extracts. Results were expressed in IC$_{50}$ values. The IC$_{50}$ values of petroleum ether, ethyl acetate, methanol, chloroform extract and standard docetaxel were found to be 63.1 ± 1.15, 78.0 ± 1.63, 92.43 ± 1.52, 136.58 ± 2.88 and 11.12 ± 2.20 in case of MIA-PaCa; 68.13 ± 1.20, 85.33 ± 1.52, 118.0 ± 1.73, 141.34 ±2.05, 12.01 ± 2.10 in case of A-549; 96.66 ± 1.53, 113.66 ± 2.08, 142.34 ± 3.21, 153.77 ± 2.51, 19.67 ± 1.15 in case of MCF-7 and 133.33 ± 3.21, 138.0 ± 2.64, 172.34 ± 2.30, 186.94 ± 1.53, 8.89 ± 1.52 in case of CaCo-2 cancer cell lines. As petroleum ether and ethyl acetate extract showed most promising results in MIA-PaCa and A-549 cancer cell lines; moreover, both these extracts has shown significant antioxidant activity. So, petroleum ether and ethyl acetate extracts were subjected to silica gel column chromatography for isolation of bioactive compounds.

The structures of all isolates were elucidated through analysis of their spectroscopic data and comparison of the data with the reported values. One steroid; β-sitosterol (A) and three lignans; sesamin (B), kobusin (C) and 4’O demethyl magnolin (D) were isolated from petroleum ether and two flavonoids; apigenin (E) and kaempferol-7-O-glucoside (F) were isolated from ethylacetate extract. Compounds (B-D) belongs to
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tetrahydrofuran class of lignans and the spectras of compounds E and F supported the flavones class of flavonoids.
Isolated compounds were also subjected to MIA-PaCa and A-549 cancer cell lines for checking their cytotoxic potential. Compounds had shown cytotoxic potential in different ranges but the most potent inhibition of cell proliferation was observed with lignan 4’O demethyl magnolin. The IC_{50} value of β-sitosterol, sesamin, kobisin, 4’O demethyl magnolin, apigenin and kaempferol-7-O-glucoside were found to be 112.33 ± 3.78, 37.46 ± 1.09, 34.71 ± 2.33, 26.47 ± 1.87, 53.66 ± 4.72, 35.34 ± 3.51 in case of A-549 and 138.00 ± 2.64, 34.04 ± 1.76, 32.86 ± 2.02, 21.72 ± 1.50, 44.33 ± 1.52, 48.43 ± 2.08 in case of MIA-PaCa cell line respectively.
In course of our research to discover anticancer principles, the chromatographic separation of petroleum ether extract resulted in the isolation and identification of a novel tetrahydrofuran lignan, 4’O demethyl magnolin. This compound appears to be first report from natural source. In apoptosis study, treatment caused typical apoptotic morphological changes. 4’O demethyl magnolin enhances the apoptosis at IC_{50} dose (21.72 µg/ml), however showing necrotic cell death at higher dose after 24 h on MIA-PaCa cell lines.

CONCLUSION
The results of the present study support and justify the traditional and folklore therapeutic claims attributed to *Zanthoxylum alatum* in the treatment of tumour. The conducted studies indicated that petroleum ether and ethyl acetate extracts of the plant has cytotoxic potential. Thus, traditional value of the plant has been scientifically proved by MTT and AO/EB assay on human cancer cell lines. It has been proved that the cytotoxic potential of plant may be attributed to the combined effects of the phenolic compounds particularly, lignans and flavonoids. *Zanthoxylum alatum* has excellent antioxidant activity supporting the use of the plant as a natural source of phenolic for antioxidant and anticancer benifits.

*Isolation and Characterization of Anticancer Principles from Zanthoxylum alatum Roxb.*