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## Document heading

Isolation and bioactivity evaluation of two metabolites from the methanolic extract of *Oroxylum indicum* stem bark

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## ABSTRACT

**Objective:** To isolate metabolites from the methanolic extract of *Oroxylum indicum* (*O. indicum*) stem bark and test their bioactivities. **Methods:** Silica gel column chromatography was employed to isolate bioactive metabolites. The isolated metabolites were confirmed by C18 column isocratic and gradient HPLC. The antioxidant activity of the isolated compounds was tested by DPPH radical scavenging assay and their cytotoxicity were tested on cancerous cells (MDA-MB-435S) and normal cells (WRL-68) by the XTT assay. **Results:** HPLC confirmed the presence of single/pure metabolites. Bioactivity of the two isolated compounds confirmed that the Compound-1 has cytotoxic potential. The cytotoxicity of Compound-1 on cancer cells was found to be 1.5 times higher than in normal cells. Compound-2 on the other hand exhibited good antioxidant property. **Conclusions:** The isolated compounds hold promise to serve as a medicine against diseases involving metabolic stress, genotoxicity/cytotoxicity and cancer.

## 1. Introduction

A great deal of research has been done all over the world to isolate antitumor metabolites from natural resources. Drug discovery from natural sources is an area pertinent to complementary and alternative medicine and natural sources such as plants, animals and microorganisms provide a basis for the isolation of unique and potentially effective bioactive compounds. Medicinal plants have been harvested from the wild since ancient times and are traditionally recognized as primary health care system in many rural communities because of its effectiveness, lack of modern medical alternatives and cultural preferences[1,2]. Over 60% of the World's population and around 80% in developing countries, depend directly on plants for their medical purposes. Medicinal plants have occupied conspicuous limelight in contemporary time due to their overwhelming potential to treat cancer[3–6].

Isolation of pure, pharmacologically active constituents from plants remains a long and tedious process. It is necessary to have methods available for efficient separation from plant extracts, which are typically mixtures of thousands of different molecules[7]. There are several ways to identify these molecules from these extracts. The common approach is to set up a fractionation scheme and to screen the fractions for the presence of the desired bioactive properties. Active fractions are subfractionated and tested, until the molecules responsible for the bioactivity can be identified. Thin-layer chromatography (TLC) and column chromatography are the simplest and cheapest method of detecting plant constituents because the method is easy to run, reproducible and requires little equipment[8–11]. However, for efficient separation of metabolites, good selectivity and sensitivity of detection, hyphenated high performance liquid chromatographic (HPLC) techniques are preferred[12,13].

The present study was focused to isolate active metabolites from the methanolic extract of *Oroxylum indicum* (*O. indicum*) by silica gel column chromatography (60–120 mesh) with varying solvent polarities and check their bioactivities after purity confirmation by HPLC.

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## 2. Materials and methods

### 2.1. Chemicals

Silica gel (60–120 mesh), 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and phenazine methosulfate (PMS) (also known as N-methylphenazonium methosulfate) were purchased from Himedia Laboratories Pvt. Ltd. (India). XTT {2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide} was obtained from Sigma Chemical Co. (St. Louis, MO, USA). The remaining chemicals and solvents used were of standard analytical grade and HPLC grade respectively. MDA-MB-435S (human breast carcinoma) and WRL-68 (normal human hepatic cells) cell lines were obtained from National Centre for Cell Science (Pune, India).

### 2.2. Plant material

*O. indicum* stem bark was collected in December, 2007, from their natural habitat in the Mundoor forest range near Kanjikode (10° 47' North, 76° 47' East; 120 m above sea level), Palakkad district, Kerala, India. The plant was identified by Prof. R. V. Nair, Senior Botanist, Centre for Indian Medical Heritage (CIMH), Kanjikode, Palakkad, Kerala, India (Ref: CIMH/MP/2019/2007). Vouchers specimens are maintained in our laboratory for future reference. The collected specimens were shade dried, powdered and extracted using a soxhlet apparatus using methanol as solvent (1:6; powder: solvent ratio).

### 2.3. Isolation and purity confirmation of isolated compounds

#### 2.3.1. Thin layer chromatography (TLC)

TLC is one of the most widely used and potent techniques to resolve mixture of plant compounds. The TLC plate supplied by Merck, Germany (TLC Silica gel 60 F<sub>254</sub>) was used to observe the separation of individual compounds as a single spot from the selected crude extract employing varying solvent polarities such as Ethyl acetate in Hexane; Chloroform in ethyl acetate; chloroform in Methanol and Water in Methanol. The developed TLC plate after visualizing with different staining procedures (Iodine, UV, Ninhydrin and KMnO<sub>4</sub>), serves as a reference to identify and confirm the compounds eluted through column chromatography.

#### 2.3.2. Column chromatography

The column chromatography (Length: 450 mm; Bore: 30 mm) was performed using 60–120 mesh silica gel to elute out individual components from the crude plant extract. The column was rinsed with hexane and completely dried before use. The column was filled 3/4th with hexane and the silica gel was packed approximately 2/3rd of the column length with simultaneous draining of the solvent to aid proper packing. The packing was performed after activating the silica gel at 100 °C for 1 h and gently poured on the top of the column with constant tapping to avoid air bubbles and cracks after mixing with hexane. The column was run with varying solvent polarities (Hexane in Ethyl Acetate) after loading with

the crude plant extract (3–5 g) mixed with 10–20 g of activated silica gel. The flow rate was maintained at 1 mL per min and the fractions collected were tested by TLC for single spot. Fractions with identical spots and  $R_f$  were pooled together and preceded for further analysis.

### 2.4. C18 column isocratic and gradient HPLC of isolated compounds

The fractions obtained from column chromatography were analyzed for compound purity by isocratic and gradient High Performance Liquid Chromatography (HPLC) using a Waters 2487 HPLC system consisting of a dual detector, a Waters 1525 binary pump, and equipped with a Waters Symmetry C18 column (5 mm, 4.615 0 mm) and Waters Sentry™ universal guard column (5 mm, 4.620 mm) (Waters Corporation, Milford, MA, USA). Methanol and ethyl acetate were used as mobile phases and the analysis was performed at 280 nm. The isocratic HPLC was performed employing 40% methanol in 60% ethyl acetate as mobile phase. The gradient HPLC was performed with varying ratios of methanol and ethyl acetate (Table 1).

**Table 1**

Gradient C18 column HPLC program

Time (min)	Solvent A (%) <sup>*</sup>	Solvent B (%) <sup>**</sup>
5	100	0
10	80	20
20	60	40
30	40	60
45	20	80
60	0	100

<sup>\*</sup>Methanol, <sup>\*\*</sup>Ethyl acetate.

### 2.5. Bioactivity of isolated compounds

#### 2.5.1. DPPH radical scavenging activity

DPPH radical scavenging ability of the isolated compounds was tested by DPPH radical scavenging assay (DRSA) as described by Blois[14]. Different concentrations of the compounds were taken in test tubes and made up to 0.5 mL with the respective solvents. An amount of 3 mL 0.1 mM DPPH<sup>•</sup> in ethanol was added to each tube and incubated in dark at room temperature for 30 min. Absorbance was read at 517 nm using a Cary 50 UV-Vis Spectrophotometer (Varian Inc., Australia). Percentage DPPH radical scavenging activity (% DRSA) was calculated using the formula,

$$\% \text{ DPPH}^{\bullet} \text{ radical scavenging (\%DRSA)} = [(A_c - A)/A_c] \times 100$$

where  $A_c$  is the absorbance of the control and  $A$  is the absorbance of the extract.

#### 2.5.2. Cytotoxicity assay in MDA-MB-435S and WRL-68 cell lines

Cytotoxicity of the compounds isolated was tested by the method of XTT-formazan dye formation[15]. Cells were seeded in a 96-well microtiter plate at a concentration of

$6 \times 10^3$  cells/200  $\mu$ L of growth medium (MEM culture medium for WRL-68 and L-15 culture medium for MDA-MB-435S) and incubated for 24 h at 37 °C with/without 5% CO<sub>2</sub>. The cells were then treated with 200  $\mu$ L of different compound concentrations prepared in respective culture medium after dissolving them in DMSO. DMSO controls were also maintained for the assay. Compound-treated cells were re-incubated for 24 h maintaining the same conditions. After incubation, medium containing the isolated compounds was substituted by 200  $\mu$ L of fresh medium followed by the addition of 50  $\mu$ L of XTT (0.6 mg/mL in medium) containing 25  $\mu$ M PMS. The plate was further incubated for 4 h. Absorbance was measured at 450 nm (with a 630 nm reference filter) in a Dynex Opsys MRtm Microplate Reader (Dynex Technologies, VA, USA).

Percentage cytotoxicity was calculated by the following formula:

$$\% \text{Cytotoxicity} = [(A_c - A_t)/A_c] \times 100$$

where A<sub>c</sub> and A<sub>t</sub> denote the absorbance of the control and test wells respectively.

## 2.6. Statistical analysis

All analyses were carried out in triplicates. Data were presented as mean  $\pm$  standard deviation (SD). Statistical analyses were performed by one-way ANOVA. Significant differences between groups were determined at  $P < 0.05$ . To evaluate relationships between experimental parameters, results were analyzed for correlation and regression and tested for significance by Student's t-test ( $P < 0.05$ ). MATLAB ver. 7.0 (Natick, MA, USA), GraphPad Prism 5.0 (San Diego, CA, USA) and Microsoft Excel 2007 (Roselle, IL, USA) were used for the statistical and graphical evaluations.

## 3. Results

### 3.1. Yield of extract

50 g of bark powder yielded 1.92 g of crude methanolic extract.

### 3.2. Isolation and purity confirmation of compounds from *O. indicum* methanolic extract

Column chromatography, performed with varying solvent polarities using hexane and ethyl acetate yielded two compounds, Compound 1 and 2 (97.8 mg and 90 mg from 5 g of crude extract respectively). The chromatograms obtained from Isocratic and Gradient HPLC for Compound-1 showed the presence of doublet peak which might be due to the presence of a racemic mixture while the chromatograms for Compound-2 showed a single peak. Figure 1 and 2 depicts the HPLC chromatograms of the isolated compounds.

### 3.3. Bioactivity of isolated compounds

The isolated compounds (Compound-1 and Compound-2)

have revealed differential DPPH• radical scavenging abilities quantitatively in a concentration dependent manner. Figure 3 illustrate the mean ( $\pm$  SD at  $P < 0.05$ ) values of percentage DPPH• radical-scavenging exhibited by the compounds. Compound-2 was the most efficient antioxidant displaying very high scavenging activity nearing 100% ( $94.37\% \pm 0.29\%$ ) even at a lowest concentration of 0.5 mg/mL when compared to Compound-1 ( $27.6\% \pm 0.96\%$ ).

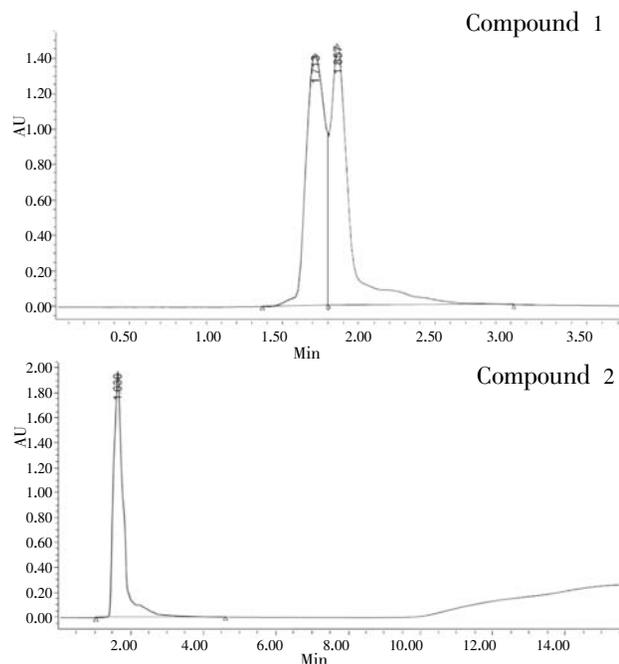


Figure 1. Isocratic HPLC chromatograms of Compound-1 and 2.

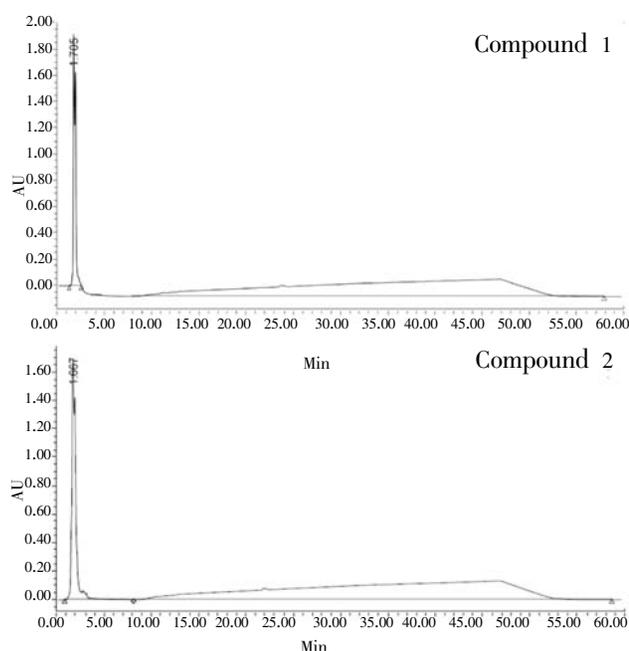
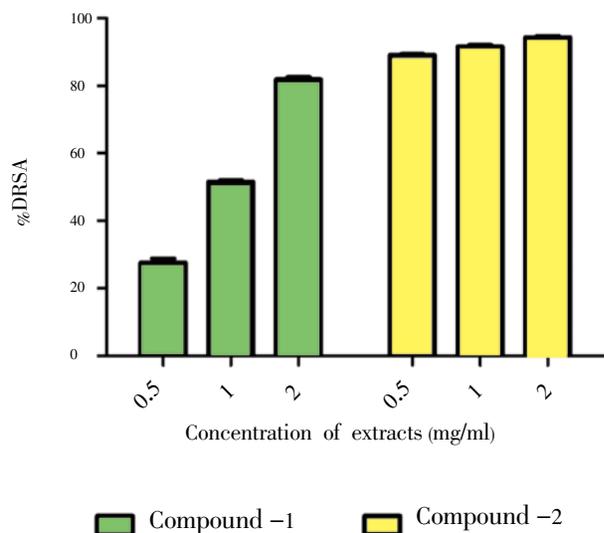


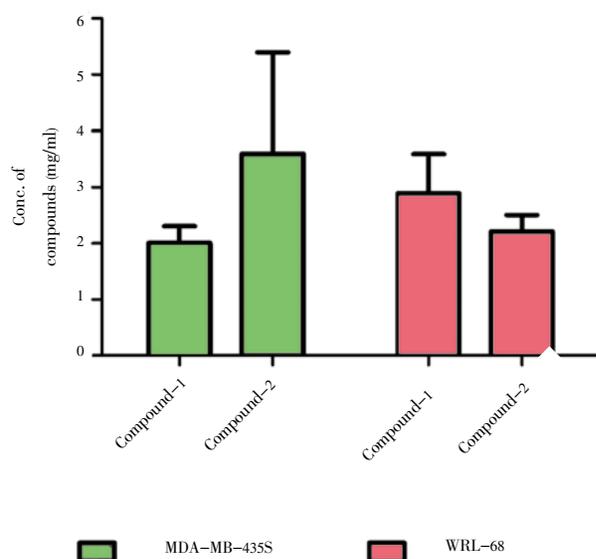
Figure 2. Gradient HPLC chromatograms of Compound-1 and 2.

The cytotoxicity of the isolated compounds was tested on MDA-MB-435S (cancer cell) and WRL-68 (normal cell) cell lines. Compound-1 was observed to be more

cytotoxic when compared to Compound-2. Furthermore, the cytotoxicity of Compound-1 on cancer cells was found to be 1.5 times higher than in normal cells. There was no difference in the cytotoxicity exhibited by Compound-2 on both the cell lines. The  $IC_{50}$  cytotoxicity dose of Compound-1 was  $(2.01 \pm 1.30)$  mg/mL in MDA-MB-435S cells and  $(2.89 \pm 1.70)$  mg/mL in WRL-68 cells whereas the  $IC_{50}$  dose of Compound-2 was  $(3.60 \pm 2.80)$  mg/mL in MDA-MB-435S cells and  $(2.21 \pm 1.30)$  mg/mL in WRL-68 cells. The cytotoxic  $IC_{50}$  dose of Compound-1 and Compound-2 on MDA-MB-435S and WRL-68 cell lines is shown in Figure 4.



**Figure 3.** %DRSA of Compound-1 and 2. Data expressed as mean  $\pm$  SD of  $n = 3$  samples ( $P < 0.05$ ).



**Figure 4.**  $IC_{50}$  cytotoxic dose of Compound-1 and 2.

#### 4. Discussion

Medicinal plants are reported to possess a wide

range of properties including anti-carcinogenic, anti-inflammatory, antioxidant, antitumor, antimicrobial, immunomodulatory, antihelminthic, antiviral, and antibiotic activities[16,17]. According to WHO, medicinal plants would be the best source to obtain a variety of drugs for cancer, since cancer cells develop resistance for current cancer therapies.

Plants are known to possess many active metabolites responsible for most of the observed properties. Since plant based treatments are considered as an alternative for chemoprevention and possible cure for cancer, plant components possessing antioxidant and cytotoxic activities have been identified and their possible use for cancer treatments are studied[16,18]. The traditionally used *Hygrophila auriculata*, extracted with various solvents have demonstrated antioxidant, anticancer, hepatoprotective and antitumour activities[19-21]. Pereira *et al*[22] reported the antioxidant potential of *Catharanthus roseus* while Siddiqui *et al*[23] reported its cytotoxicity. On a similar account, the antioxidant and cytotoxicity of *O. indicum* has been previously reported[24]. The antioxidant and the cytotoxic nature of several plant extracts and compounds isolated from these plants have been reported by many others[19,25,26].

All the compounds present in a crude plant extract might not be extracted using a single solvent. Different compounds according to their polarity elute out in different solvents[16,18]. In the present study, column chromatography and TLC eluted two compounds, Compound-1 and Compound-2. Bioactivity of the two isolated compounds confirmed that Compound-1 has cytotoxic potential and Compound-2 has good antioxidant property. According to Ghani (2003) an ideal anticancer compound should possess low toxicity to normal cells while exhibiting selective cytotoxicity to cancer cells. Accordingly, Compound-1 was observed to be 1.5 times specific to cancerous cells than normal cells (WRL-68). Both isolated compounds thus exhibited promising bioactivities that render them ideal candidates for drug development.

Natural products discovered from medicinal plants have played an important role in combating cancer irrespective of their multifactorial origin. The current study provides useful insights to research in isolation and identification of potential anticancer chemopreventive metabolites from the traditionally known/used medicinal plants. From the bioactivities exhibited by the isolated metabolites (Compound-1

and 2), *O. indicum* methanolic extract could serve as a potential source for anticancer metabolites. It can also be inferred that the compounds isolated in this study might be used as precursors for designing effective antioxidant and anticancer drugs.

### Conflict of interest statement

We declare that we have no conflict of interest.

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