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A promising study on cytotoxicity and *in vitro* anti-inflammatory activity of *Heracleum candolleanum* (Wight et. Arn.) gamble

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Abstract
The methanol extracts of roots, seeds, and leaves of *Heracleum candolleanum* (Wight et. Arn.) Gamble were subjected to *in vitro* anti-inflammatory and anticancer studies. The anti-inflammatory studies were carried out using HRBC membrane stabilization, inhibition of protein denaturation, and proteinase inhibitory methods. Cytotoxicity of the extracts was evaluated on cancer cells of various origins, namely, A431, A375, MDA-MB-231, U937, and HeLa, representing non-melanoma, melanoma, breast, acute myeloid leukemia, and cervical cancer cell lines respectively. From the present study it can be concluded that methanol extract of all the three parts of *H. candolleanum* may have significant anti-inflammatory activity against inflammatory based diseases or allied conditions. The study also revealed that the roots, leaves and seed extract possessed selective *in vitro* cytotoxicity, against five different human malignant cell lines.

Keywords: *H. candolleanum*, Methanol extract, *in vitro* anti-inflammatory activity, cytotoxicity.

Introduction
Heracleum candolleanum (Wight et Arn.) Gamble of Apiaceae is an ethno-medicinal plant endemic to the Western Ghats. Decoction of the whole plant is widely administered by the Kani tribes internally as a nerve tonic and for inflammatory conditions (Saradamma *et al*., 1990) [10]. A few furanocoumarins and two monoterpenoids have been reported from this plant (Susan *et al*., 2001) [14]. Methanol extract of the root possesses antioxidant and antimicrobial activity (Hemand & Rajesh, 2012) [5]. Furanocoumarins from its seeds exhibit antioxidant activity. There are reports on the isolation of monoterpenoids 2-exo, 3-endo-camphanediol and 2-pinene-4, 10-diol from the seeds of *H. candolleanum* (Susan *et al*., 2000) [13]. The presence of furanocoumarins has also been reported in the genus Heracleum (Doi *et al*., 2004) [3]. *H. Persicum* is reported to have anti-inflammatory activity (Valiollah *et al*., 2009) [15]. Reports suggest that drugs which inhibit inflammation induce apoptosis in some cancerous cells and have preventive as well as therapeutic values (Sergei *et al*., 2012) [11]. *Heracleum rigens* found in the Western Ghats of India is known to possess anti-inflammatory and anticancer properties (Nataraj *et al*., 2012) [7]. The current study was conducted with the objective to rationalize the *in vitro* anti-inflammatory activity and to screen the methanol extract of *Heracleum candolleanum* for its cytotoxicity on cancer cells of different origins.

Materials and Methods
H. candolleanum was collected from the Vagamon Hills, Kerala, India. The plant was identified and a voucher specimen has been kept in the herbarium of Navajyothi Sree Karunakara Guru Research Center for Ayurveda and Siddha, Uzhavoor, Kerala, India for future reference. Approximately 50g dried powder of leaves, roots, and seeds were taken and extracted using methanol in a Soxhlet apparatus. The filtrate was dried using a rotavapor, and the extract was used for *in vitro* anti-inflammatory and cytotoxicity studies. The methanol extracts were dissolved in a minimum amount of methanol and made up to the required volume in 1% gum acacia (Guruvayoorappan *et al*., 2006) [4].

Inhibition of Albumin Denaturation
The method of Mizushima and Kobayashi (1968) [6] was followed with minor modifications. The reaction mixture consisted of the test extract at different concentrations and 1% aqueous solution of bovine albumin fraction; the pH of the reaction mixture was adjusted to 6.4 using a small amount of 1N HCL.

The samples were incubated at 37 ºC for 20 minutes and then heated at 57 ºC for 20 minutes. After cooling, the samples, the turbidity was measured spectrophotometrically at 660 nm. The experiment was performed in triplicate. Percent inhibition of protein denaturation was calculated as follows:
% inhibition = (Absorbance of control-Absorbance of sample) X 100 / Absorbance of control

HRBC Membrane Stabilization Test

Preparation of Human Red Blood Cells (HRBC's) Suspension

Fresh whole human blood (10 ml) was collected and transferred to heparinized centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10 minutes and were washed three times with equal volume of normal saline. The volume of the blood was measured and reconstituted as a 10% v/v suspension with normal saline (Sadique *et al*., 1989) [9].

Heat-Induced Hemolysis

The reaction mixture (2 ml) consisted of 1 ml of test drug solution and 1 ml of 10% HRBC's suspension. Instead of the drug, saline was added to the control test tube. Aspirin was taken as the standard drug. All the centrifuge tubes containing the reaction mixture were incubated in a water bath at 56ºC for 30 minutes. At the end of the incubation, the tubes were cooled under running tap water. The reaction mixture was centrifuged at 2500 rpm for 5 minutes, and the absorbance of the supernatants was taken at 560 nm (Shinde *et al*., 1999) [12]. The experiment was performed in triplicate. Percent membrane stabilization activity was calculated by the formula mentioned above.

Proteinase Inhibitory Action

The test was performed according to the modified method of Oyedepo and Femurewa (1995) [8]. The reaction mixture (2 ml) contained 0.06 mg trypsin, 1 ml 20 mM Tris HCl buffer (pH 7.4), and 1 ml test sample of different concentrations. The mixture was incubated at 37ºC for 5 minutes, and then 1 ml of 0.8% (w/v) casein was added. The mixture was incubated for an additional 20 minutes. 2 ml of 70% perchloric acid was added to terminate the reaction. Cloudy suspension was centrifuged, and the absorbance of the supernatant was read at 210 nm against buffer as blank. The experiment was performed in triplicate. The percent inhibition of proteinase inhibitory activity was calculated.

Evaluation of anticancer activity

Methanol extract of the seeds, roots, and leaves were investigated for cytotoxicity towards cancer cells of various origins. Epithelial carcinoma cell line A431, breast adenocarcinoma cell line MDA-MB-231, histiocytic lymphoma cell line U937, cervical cancer cell line HeLa, and human malignant melanoma cell line A375 were used for the study. The cell lines were obtained from NCCS, Pune, India, and were maintained in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, along with 100 units/ml penicillin and 50 µg/ml streptomycin. Cells were maintained at 37 ºC in a humidified atmosphere of 5% CO2. For the experiments, cells were counted and seeded in 96-well micro titre plates (3000 cells/well). After overnight incubation, various concentrations of the drugs (10-100 µg/ml) were added and the cells were allowed to grow for 72 hours followed by MTT assay. For MTT assay, after removing the drug-containing medium, 0.1 ml of 20% MTT solution (5 mg/ml in PBS) in complete medium was added to each of the wells and incubated for 2 hours. At the end of incubation, 0.1 ml MTT lysis buffer (20% sodium dodecyl sulphate in 50% dimethyl formamide) was added to the wells and incubated for another 1 hour at 37ºC, after which the optical densities at 570 nm were measured using a plate reader (Bio-Rad). The relative cell viability in percentage was calculated as: (A570 of treated samples / A570 of untreated samples) x 100.

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is based on the ability of mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and to form dark blue formazan crystals, which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. The number of surviving cells is directly proportional to the level of the formazan product formed.

Statistical Analysis

The results are expressed as the mean± SD for three replicates. Linear regression analysis was used to calculate IC50 value.

Results and Discussion

The methanol extract of the roots showed significant inhibition of albumin denaturation in a concentration-dependent manner when compared to the seed and leaf extract. The percentage of inhibition was 67% for the root extract at a concentration of 500 µg/ml, whereas it was 59% and 43% for seed and leaf extract respectively at the same concentration (Table 1). The methanol extract of the roots showed a significant protection of HRBC membrane at different concentrations when compared to the standard drug. The percentage inhibition of hemolysis was 71% for the root extract at a concentration of 500 µg/ml, whereas it was 63% and 48% for seed and leaf extract respectively at the same concentration (Table 2). The methanol extract of the roots showed a significant inhibition of proteinase activity in a concentration-dependent manner when compared to the seed and leaf extract. The percentage inhibition was 65% for the root extract at a concentration of 500 µg/ml, whereas it was 57% and 41% for seed and leaf extract respectively at the same concentration (Table 3).

Cytotoxic activity of methanol extract from the leaves, roots and seeds of the plant was investigated towards cancer cell lines of various origins. Among the five cancer cell lines studied, the leaf extract showed maximum activity towards the non-melanoma epithelial cancer cell line, A431 (IC_{50} 43.95 µg/ml) followed by the breast cancer cell line, MDA-MB-231 (IC_{50} 54.78 µg/ml) (Fig 1). The human malignant melanoma cell line A375 was found to be the most resistant to the leaf extract (IC_{50} 167.16 µg/ml) followed by U937 and HeLa cell line (IC_{50} 117.6 µg/ml and 100.82 µg/ml). The seed methanol extract fraction, showed maximum cytotoxicity to histiocytic lymphoma cell line U937 (IC_{50} 44.50 µg/ml), followed by MDA-MB-231 (IC_{50} 48.69 µg/ml), HeLa (IC_{50} 50.02 µg/ml), A431 (IC_{50} 63.22 µg/ml) respectively (Fig 2). A375 was found to be resistant to seed extract (IC_{50} 127.68 µg/ml). The methanol extract of roots showed maximum cytotoxicity to A431 cell line (IC_{50} 45.93 µg/ml) followed by U937 (IC_{50} 48.81 µg/ml), HeLa (54.57 µg/ml) and MDA-MB-231(IC_{50} 58.96 µg/ml) (Fig 3). As in the case of leaf extract and seed extract, methanol extract of roots was less cytotoxic towards A375 cell line (IC_{50} 102.6 µg/ml). The methanol extract of the roots showed significant cytotoxicity towards the human cancer cell lines tested. The IC50 values for the

root extract were found to be 45 µg/ml, 38 µg/ml, 52 µg/ml, 49 µg/ml, and 41 µg/ml for A431, A375, MDA-MB-231, U937, and HeLa cells respectively. The seed and leaf extracts also showed cytotoxicity but to a lesser extent when compared to the root extract .

Table 1: Protein denaturation activity of methanol extract of leaves, roots and seeds

Concentration (µg/ml)	Protein denaturation activity (% Prevention of denaturation)			
	Methanol extract of Leaves	Methanol extract Roots	Methanol extract Seeds	Aspirin
50	40.69±0.84	3.04±0.12	44.83±1.46	0.51±0.70
100	51.73±1.02	3.04±0.07	48.28±1.36	0.51±0.65
150	62.07±0.83	3.54±0.24	57.94±0.97	1.02±1.01
250	69.66±0.82	22.61±1.02	62.07± 1.65	16.58±1.35
500	79.32±1.07	58.09±1.17	92.42±1.29	50.51±1.49

Table 2: HRBC membrane stabilization activity of the methanol extract of leaves, roots and seeds

Concentration (µg/ml)	HRBC membrane stabilization activity (% Prevention of lysis)			
	Methanol extract of Leaves	Methanol extract Roots	Methanol extract Seeds	Aspirin
50	23.65±0.89	20.94±1.46	38.76±0.73	41.09±1.18
100	42.25±0.95	41.87±1.51	44.19±0.75	48.84±1.26
200	43.80±0.79	44.58±1.26	50.00±0.97	52.33±1.55
400	54.24±1.08	86.68±1.47	54.5±1.21	53.88±1.16
800	68.61±1.75	89.54±1.02	61.09±1.72	76.90±1.80

Table 3: Proteinase inhibitory activity of methanol extract of leaves, roots and seeds

Concentration (µg/ml)	Proteinase inhibitory activity (Prevention of inhibition %)			
	Methanol extract of Leaves	Methanol extract Roots	Methanol extract Seeds	Aspirin
50	36.38±0.25	10.41±0.76	40.21±0.26	15.85±0.75
100	40.40±0.31	22.34±0.45	43.83±0.14	32.66±1.06
200	42.59±0.21	36.67±0.50	44.31±0.32	40.87±0.97
400	45.55±0.30	40.11±0.33	50.08±0.24	44.69±0.53
800	50.04±0.45	49.66±0.33	57.72±0.52	47.37±0.44

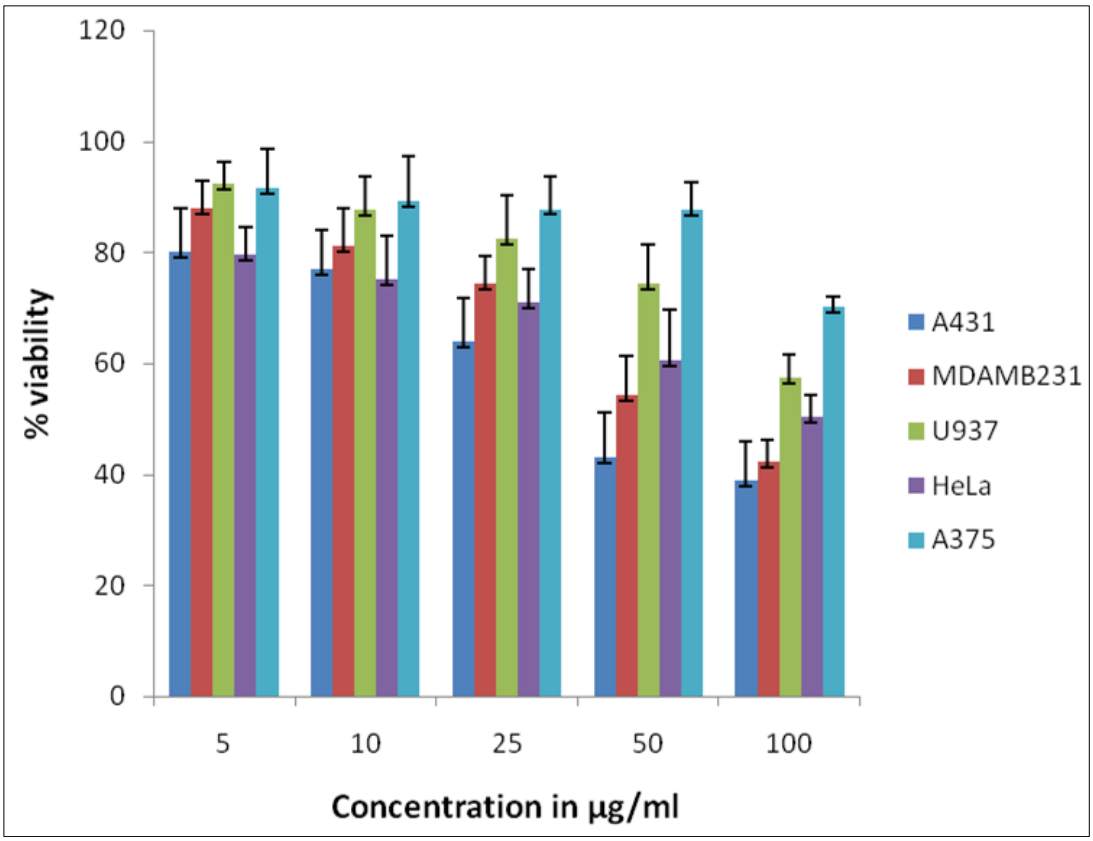

Fig 1: Percent of cell viability methanol extract of *H. candolleanum* leaves

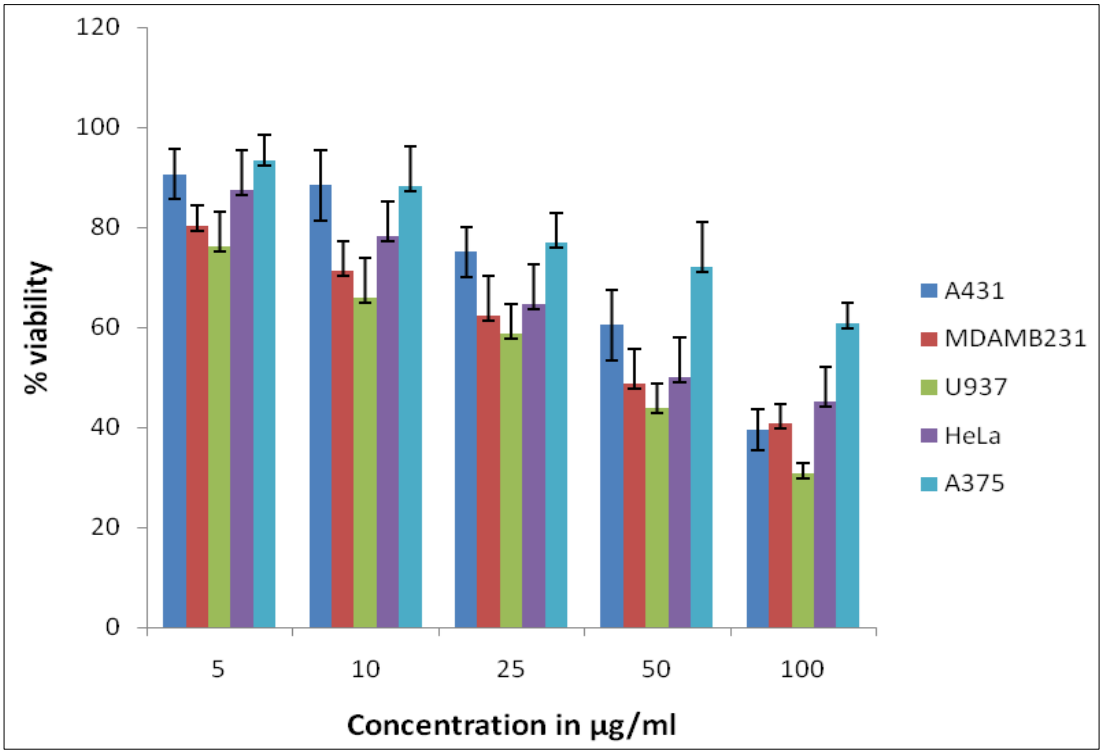

Fig 2: Percent of cell viability methanol extract of *H. candolleanum* seeds

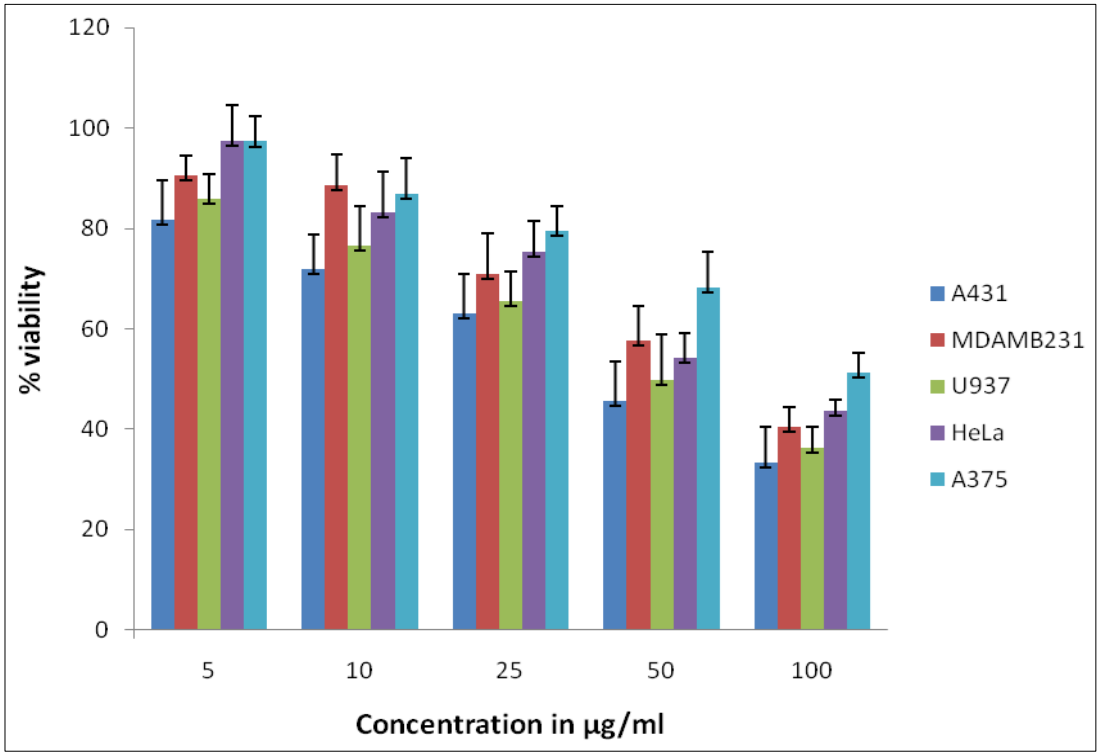

Fig 3: Percent of cell viability methanol extract of *H. candolleanum* roots

Conclusion

The results indicate that the methanol extracts of *H. candolleanum* roots possess significant anti-inflammatory and cytotoxic activities. Further studies are warranted to isolate and characterize the active compounds responsible for these activities.

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