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Evaluation of anti-lipid peroxidation and cytotoxic activity in the methanolic extracts of Hibiscus and Amla

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Abstract
Hibiscus and Amla are the most locally available medicinal plants in India. Hibiscus flowers and leaves are used as skin and hair products, and Amla fruit has been operating as a traditional medicine to treat many diseases. Both plant materials have an excellent value in the field of ayurvedic medicines in India. In this present study, Hibiscus and Amla were tested for anti-lipid peroxidation and cytotoxic activity. The extracts were prepared by the solvent extraction method, and Methanol was used as the solvent. The egg yolk was used to evaluate the anti-lipid peroxidation, TBARS assay, and the *in vitro* cytotoxic study performed against UMR 106 cell lines, and MTT was used to estimate the cell growth. The result of the TBARS assay for the Amla and Hibiscus methanolic extracts had shown IC50 values of 3.97µg/ml and 7.03µg/ml, respectively, in which Amla has shown significant results when compared with Hibiscus. In the development of MTT assay, sample Hibiscus showed an EC50 value of 64.74% proliferation in UMR106 cells, whereas Sample Amla did not show significant proliferation; hence EC50 was not calculated. The result concludes that the Amla has good anti-lipid peroxidation activity, whereas the Hibiscus flower has less cytotoxic property.

Keywords: Hibiscus, Amla, Medicinal plant, TBARS assay, Cytotoxicity, MTT assay

Introduction
From past generations, humans have to depend on Nature for their daily needs and survival. In India, the use of medicinal plants to cure some common diseases is practicing from the age of Rigveda, and Ayurveda is the Indian local system of medicine from the post-Vedic period. The complete design of old Indian medicine is based on the relationship between man and Nature. With the gradual development of science, scientists have introduced many new drugs with the help of the pharmaceutical industry; nowadays, the practice of medicinal plants is reduced due to these synthetic drugs [1].

The Hibiscus (*Rosa Sinensis*) and Amla (*Emblica Officinalis*) are the plants that come under Malvaceae and Euphorbiaceae family, respectively, and these are readily available medicinal plants in almost all parts of India. Both plants have excellent therapeutic value and help treat many common diseases, and these plants have been using in the ayurvedic medicinal system from past generations [6]. Hibiscus is a natural remedy for some illnesses and painful symptoms and is also used as an analgesic, antipyretic, anti-asthmatic, and anti-inflammatory agent. Many kinds of research have been done so far to know the antioxidant, anti-fungal, and antimicrobial properties in flowers of Hibiscus Rosa-Sinensis [2]. Amla is used to treat the common cold, scurvy, cancer, heart diseases, diabetes, cardiovascular disease, ulcers, anemia, and hair fall problems [3].

Both Hibiscus and Amla contain various phytochemicals, which effectively help to treat many common diseases. The effectiveness of the plants can be measured by using multiple assays; in this study, the MTT and TBARS assay is performed to determine the cytotoxicity and anti-lipid peroxidation activity, respectively. The MTT system measures the cytotoxic activity of the living cells via mitochondrial dehydrogenases [4]. Traditionally, the *in vitro* determinations of toxic effects of unknown compounds have been performed by counting viable cells after staining with a vital dye [5]. As far as much research is held on these samples, the studies on anti-lipid peroxidation and cytotoxic studies are less. So the present research is undertaken to fill this research gap by analyzing the cytotoxic activity using UMR 106 cell lines (rat osteosarcoma; the cell responsive in bone reabsorbing and having more similarities with human bone cells) anti-lipid peroxidation using egg yolk as lipid medium.

Materials and Methods

Collection of plant material

The Hibiscus flower and Fruit of Amla were collected from the healthy plants located in the gardens.

Extraction and phytochemical analysis

20g of dried sample powder (Hibiscus flower and Fruit of Amla) were dissolved in 100ml of Methanol. Then the beaker was kept in a hot water bath at 50º C for 4 hours, covering with aluminum paper. Then extract was filtered with Whatman filter paper, the filtrate was taken for further use. Then filtrates were kept at 50ºC until the extract got dried entirely and turned into a semisolid form; the samples were stored in a refrigerator when not in use. The phytochemical estimation of the plants analyzed using standard protocols explained by (RNS Yadav and Munin Agarwala 2011).

TBARS Assay

This assay was performed using a modified thiobarbituric acid-reactive species (TBARS) to measure the lipid peroxidation using egg yolk homogenate as a lipid-rich medium (Ohkowa *et al*., 1979). Egg homogenate (0.5ml of 10% v/v) and 0.1ml of extract were added to a Eppendorf and made up to 1ml with distilled water. 0.005ml of FeSO4 (0.07M) was added to induce lipid peroxidation and incubated for 30 min. Then 1.5ml of 20% acetic acid (pH adjusted to 3.5 with NaOH) and 1.5ml of 0.8% (w/v) TBA solution in 1.1% sodium dodecyl sulfate and 0.5ml 20% TCA have added the resulting mixture was vortexed and then heated at 95 ºC for 60 min. After cooling, 5.0ml of butanol was added to all tubes and centrifuged at 3000 rpm for 10 minutes. The absorbance of the supernatant was observed at 532nm. The incubation percentage of lipid peroxidation with the extract was calculated according to [(1-E/C) x 100. C is the absorbance value of the control, and E is (Abs532+TBA –Abs532-TBA)].

Calculating percentage growth inhibition:

$$\% \text{ Inhibition} = \frac{(\text{OD of Control} - \text{OD of Sample})}{\text{OD of Control}} \times 100$$

MTT assay

Preparation of test solutions

For cytotoxicity studies, 32mg/ml stocks were prepared using DMSO. Serial two-fold dilutions were prepared from 320µg/ml to 10µg/ml using DMEM plain media for treatment.

Cell lines and culture medium

All cell lines were procured from ATCC, and stock cells were cultured in DMEM supplemented with 10% inactivated Foetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) in a humidified atmosphere of 5% CO_2 at 37 ºC until confluent. The cells were dissociated with cell dissociating solution (0.02 % EDTA, 0.05 % glucose in PBS, 0.2 % trypsin,) and the viability of the cells is checked and centrifuged. Further, 50,000 cells /well were seeded in a 96 well plate and incubated for 24 hrs at 37 ºC, 5 % CO_2 incubator.

Procedure

The monolayer cell culture was separated from the culture tube using trypsin, and the cell count maintained to 1.0x105 cells/ml using suitable media containing 10% FBS. To 96 well plates, 100 µl of the diluted cell suspension (50,000 cells/well) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium, and 100 µl of different test concentrations of test drugs were added on to the partial monolayer in microtiter plates. The plate was incubated at 37 ºC for 24hrs in a 5% CO_2 atmosphere. After incubation, the test solutions in the wells were discarded, and 100 µl of MTT (5 mg/10 ml of MTT in PBS) was added to each well. The cell plates were incubated for four hours at 37 ºC in a 5% CO_2 atmosphere. The obtained supernatant was removed, and 100 µl of DMSO was added, and the cell plates were gently shaken to dissolve the formed for mazan. The absorbance was measured using a digital spectrophotometer at the wavelength of 590 nm. The percentage growth proliferation was calculated using the following formula % proliferation = [(OD of sample/OD of Control) x 100]-100.

Statistical analysis

EC50 values for cytotoxicity tests were derived from nonlinear regression analysis (curve fit) based on sigmoid dose-response curve (variable) and computed using Graph Pad Prism 6 (Graph pad, San Diego, CA, USA).

Results and Discussion

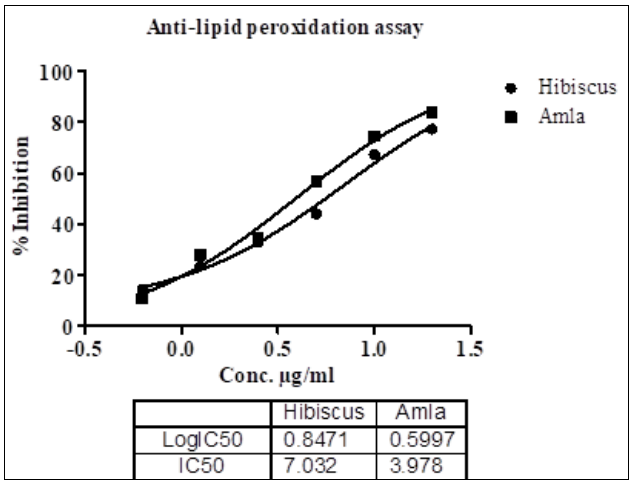
The collected samples of Hibiscus and Amla were subjected to phytochemical screening to determine the various phytochemicals present. Screening of the phytochemicals performed using phytochemicals analysis includes Alkaloids, Carbohydrates, Tannin, Terpenoid, Glycine, Steroid, Saponin, Flavonoids, Proteins, Glycoproteins, Volatile oils. As a result, the Hibiscus contains the Alkaloid, Tannin, Steroid, and Flavonoid, and in Amla alkaloid, Terpenoids and Flavonoids are noticed.

Anti-lipid peroxidation activity is determined using TBARS assay over methanolic extract of Hibiscus flower and Amla fruit. The results expressed in (Graph 1), the lipid peroxidation activity in Hibiscus showed with the IC50 value of 7.032µg/ml, and Amla showed 3.978µg/ml respectively. Amla has shown significant results with less IC50 value of 3.97 µg/ml when compared with Hibiscus. Therefore, intake of Amla fruits helps reduce reactive oxygen species (ROS), and that could be helpful to extend the responses and injury caused by the ROS.

Table 1: Anti-lipid peroxidation activity in Hibiscus and Amla.

Sample Name	Concentration (µg/ml)	OD_{+TBA} with TBA	Lipid peroxidation Inhibition (%)	IC_{50} Value
Control	0	0.2428	0.00	
Hibiscus	0.625	0.2074	14.58	7.032µg/ml
	1.25	0.1854	23.64	
	2.5	0.1626	33.03	
	5	0.1358	44.07	
	10	0.0794	67.30	
	20	0.0552	77.24	
Amla	0.625	0.2159	11.08	3.978µg/ml
	1.25	0.1748	28.01	

	2.5	0.1583	34.80	
	5	0.1054	56.59	
	10	0.0619	74.51	
	20	0.0389	83.98	

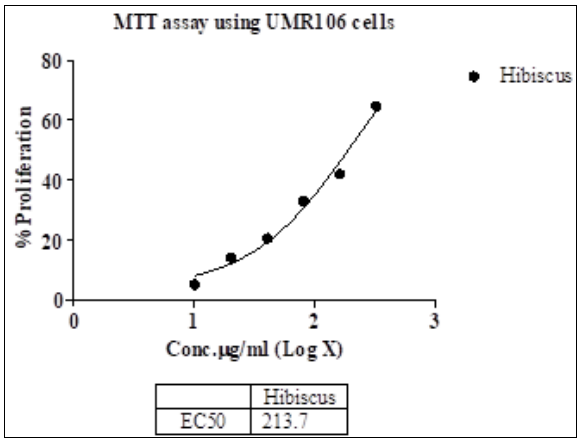


Graph 1: The graph showing the anti-lipid peroxidation activity of the Hibiscus and Amla

For the MTT assay, the critical component used is (3- [4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) or MTT, a water-soluble tetrazolium salt yielding a yellowish solution when prepared in media or salt solutions lacking phenol red. The MTT assay is performed using the UMR 106 cells using Hibiscus flower and Amla fruit extracts. The result obtained showed that Sample Hibiscus showed an EC50 value of 64.74% proliferation in UMR106 cells, whereas Sample Amla did not shown significant proliferation; hence EC50 was not calculated, the cytotoxic activity is significantly less in the methanolic extract of Hibiscus flower. This property of the Hibiscus could help reduce osteoporosis activity in the bone cells, which keep the bones healthy.

Table 2: UMR106 cell proliferative activity in Hibiscus and Amla

UMR106				
Compound name	**Conc. µg/ml**	**OD at 590nm**	**% proliferation**	**EC50 µg/ml**
Control	0	0.536	0.00	213.70
Hibiscus	10	0.564	5.22	
	20	0.612	14.24	
	40	0.646	20.60	
	80	0.713	33.02	
	160	0.761	42.00	
	320	0.883	64.74	
Amla	10	0.556	3.79	EC50 is not calculated due to lesser proliferation
	20	0.566	5.53	
	40	0.605	12.95	
	80	0.625	16.51	
	160	0.657	22.57	
	320	0.700	30.65	



Graph 2: Graph showing the proliferation of the UMR 106 cells in the Hibiscus sample.

Conclusion

This current study successfully screened the anti-lipid peroxidation and cytotoxic activity in methanolic extract of Hibiscus flower and fruit of Amla. The fruit extract of the Amla fruit has shown dramatically inhibited the lipid peroxidation activity compared to the Hibiscus. However, only the Hibiscus extract has shown high cell proliferation; thus, it has a less cytotoxic effect. The MTT analysis revealed a solid constructive between cytotoxic activity and flavonoid content but a destructive correlation between anti-lipid peroxidation activities. This property may effectively help to reduce osteoporosis diseases, so future studies will evaluate and investigate the action of Hibiscus flower extract against UMR 106 rat osteosarcoma cells in greater detail and identify the possible bioactive compound that contributes anti-osteoporotic activity of the extract.

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