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In-Vitro Evaluation of Anti Proliferative and Haemolysis Activity of Selected Plant Extracts on Lung Carcinoma (A549)

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Abstract

Plants are important source for screening and development of herbal medicines, herbs have been on the forefront whenever we talk about cancer cure and herbal medicines have a vital role in the prevention and treatment of cancer. In the present investigation, an attempt was made to exploit bioactive compounds of three medicinal plants viz. *Curcuma longa* L., *Clitoria ternatea* L. *and Aloe vera* (L.) Burm. f., investigated their anti-proliferative properties *in-vitro* on lung carcinoma and evaluated the haemolytic activity. Methanolic extract of *Curcuma longa* L. showed inhibition (IC 50=207.3 µg/ml) of growth against human lung carcinoma cell line (A549) and inhibition was not observed for *Clitoria ternatea* L. and aqueous extract of *Aloe vera* (L.) Burm.f. while all three plants found to be non haemolytic. Our studies infer that the bioactive compounds of *Curcuma longa* L. are a potent medicinal source for human lung cancer as it showed a significant inhibition of growth of cancer cells and non haemolytic in nature.

Keywords

Curcuma longa L., Anti-Proliferative, *In-Vitro* Cytotoxicity, Haemolytic Activity, Human Lung Carcinoma, A549

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1. Introduction

Cancer is a condition caused by the uncontrolled growth of normal cells and the different kinds of cancer are named based on the type of cell in a tissue behave in an uncontrolled way. Cancer can arise from nearly any part of the body and there are more than 125 different types of cancers [1]. Lung cancer is found to be common worldwide and accounts for major death annually and responsible for 17.8% of all cancer deaths. Cancer rates could further increase by 50% to mark 15 million new cases in the year 2020. However, the report also provides clear evidence that the healthy lifestyles, public health action by governments and health practitioners could stem this trend, thus prevent as many as one third of cancers worldwide [2]

Cervical cancer was the number one cause of cancer death in India; in males, oral, lung and stomach cancers was the three most common cancer incidence whereas in females cervical, breast and oral cancers are cause of death [2].

Plants have been a major source of medicine for thousands of years and even today, the World Health Organization estimates that up to 80 percent of people still rely mainly on alternative and traditional remedies such as herbs as source for their medicines [3]. Especially, plants growing at high altitude in Himalayan pastures are time-honored sources of health and general well-being of local inhabitants. As of today, Himalayan plants are a major contributor to the herbal pharmaceutical industry in India and other countries [4]. The search for anti-cancer agents from plant sources started in the

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early 1950s with the discovery and development of the vinca alkaloids, vinblastine and vincristine. They were the first agents to advance into clinical use, isolated from the Madagascar periwinkle, *Catharanthus roseus* (L.) G.Don. These agents are primarily used in combination with other cancer chemotherapeutic drugs for the treatment of a variety of cancers, including leukemias, lymphomas, advanced testicular cancer, breast and lung cancers and Kaposi's sarcoma [5].

Medicinal herb *Curcuma longa* L. which yields curcumin, is a small perennial herb native to India bearing many rhizomes on its root system. Curcumin has been shown to exhibit antioxidant, anti-inflammatory, antiviral, antibacterial, antifungal, and anticancer activities and thus has a potential against various malignant diseases, diabetes, allergies, arthritis, Alzheimer's disease and other chronic illnesses [6]. *Clitoria ternatea* L. is a vine or creeper, perennial, herbaceous plant, with elliptic, obtuse leaves, grow well in moist and neutral soil. The fruits are 5 – 7 cm long, flat pods with 6 to 10 seeds in each pod and they are edible when tender [7]. In *Clitoria ternatea* L. flavonoids, saponins, alkaloids, carbohydrate, protein, steroids and terpenoids are reported [8-9] and various parts of *Clitoria ternatea* L. have been reported to have tranquilizing property, anti-inflammatory, analgesic activity, antipyretic, and immune modulatory activities. Also it has antibacterial, hepato protective and anti hyperlipidemic activities [7, 10]. Bioactive compounds from *Aloe vera* (L.) Burm.f. are very effective in various treatments, such as burns, allergic reactions, rheumatoid arthritis, rheumatic fever, acid indigestion, ulcers, diabetes, skin diseases, dysentery, diarrhoea, piles and inflammatory conditions of the digestive system and other internal organs, including the stomach, small intestine, liver, kidney and pancreas. The active ingredients have been shown to have analgesic, anti-inflammatory, antioxidant and anticancer property. Besides this, it also has hypoglycemic and hypolipidemic activity, wound healing activity, antimutagenic, immunomodulatory, gastroprotective and antifungal activity [11-12]. In the present investigation, an attempt was made to screen the anti proliferative property of *Curcuma longa* L., *Clitoria ternatea* L. and *Aloe vera* (L.) Burm.f. which are commonly used in traditional medicinal herb. Our study presents the potential anti cancerous herb from the investigated plants based on their anti proliferative property and non haemolytic nature.

2. Materials and Methods

2.1. Plant Materials

Healthy rhizome of *Curcuma longa* L. was collected from local market, Bangalore, Karnataka, India and healthy leaves of *Aloe vera* (L.) Burm.f. and seeds of *Clitoria ternatea* L. were collected from GKVK, Bangalore, Karnataka, India. The plant materials were brought to the laboratory and washed several times with tap water and once with distilled water and allowed to dry in shade at room temperature. The plant materials were authenticated form Department of Botany, Bangalore University, Bangalore and herbarium was maintained in CMR Institute of Management Studies, OMBR Banaswadi Layout, Bangalore, Karnataka, India.

2.2. Preparation of Plant Extracts

Aqueous Extract: 50 g of thoroughly washed leaves of *Aloe vera* (L.) Burm.f. were macerated with 250 ml of sterile distilled water in a Waring blender for 10 min. The macerate was first filtered through double-layered muslin cloth and then centrifuged at 4000 g for 30 minutes. The supernatant was filtered through Whatman filter paper No.1 and sterilized at 120° C for 15 minutes. The extract was preserved aseptically until further use.

Solvent Extract: 10g dried powder was used for extraction as described in Shruti et al. 2015 [13]. Total yield of the crude extract obtained for plants *Curcuma longa* L., *Clitoria ternatea* L. was 0.727g and 3.018g respectively. 32 mg of semi-solid crude extract was taken from the total yield and dissolved in 1 ml of DMSO in an eppendorf tube to make it 32 mg/ml stock solution. Then the tube was kept on hot water bath at 60ºC for 1 hour for proper dissolution of the pellet in DMSO. The working concentrations of the test sample i.e. 0, 10, 20, 40, 80, 160 and 320µg/ml were prepared from the stock solution.

2.3. Cell Culture

Human lung carcinoma (A549) cell line was obtained from the American Type Culture Collection (ATCC). A549 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were incubated at 37°C, pH 7.4 in a 5% CO_2 humidified incubator.

2.4. Thawing and Revival

Cryo-vials containing the frozen cells from liquid nitrogen storage were quickly thawed as described in Shruti et al. 2015 [13]. Thawed cells were re suspended in complete growth medium and transferred to T-25 flask under the recommended culture environment (5% CO_2 at 37°C). Growth of viable cells was monitored, were trypsinized and sub cultured once they reached a confluence of 70-80%.

3. Anti Proliferation Activity Against A549 Cell Line

3.1. In Vitro Assay for Cytotoxic Activity

The A549 cells at 5.0 X 10^4 are plated in 96 well plates containing DMEM with plant extracts at 10, 20, 40, 80, 160 and 320µg/ml and are incubated in 5 % CO_2 incubator at 37°C. After incubation with plant extracts, the media is removed from the wells and 100 µl / well MTT reagent was added and incubate again for 3-4 hrs. MTT reagent was drawn and 100 µL of DMSO was added to each well and gently shaken. Plant extracts treated cells are compared to untreated cell control wells and measure the absorbance at 570 nm using a microplate reader (ELx 800, Bio-Tek Instruments Inc., Winooski, VT, USA). The percentage inhibition was determined using the formula: Inhibition (%) = 100 - (optical density of sample/optical density of control) × 100. In this study, IC50 values were calculated as the concentrations that show 50% inhibition of proliferation on any plant extract treated cell Shruti et al. 2015 [13].

3.2. Haemolytic Activity

Blood sample (5 ml) was collected from healthy volunteers in the tubes containing 5.4 mg of EDTA to prevent coagulation and the erythrocytes separation were performed according to the methods described by Shruti et al. 2015 [13].

Haemolytic activity is a method to study the interactions between blood and biomaterials that may induce erythrocyte lysis. 50 µl of 10 dilution (100µl Erythrocytes suspension: 900µl 1X PBS) of erythrocytes suspension was taken into 2 ml of new eppendorf tube and incubated with 100 µl of different concentration of plant extracts (10, 20, 40, 80, 160 and 320 µg/ml) at 37^0C water bath for 60-90 min. Here we are using 100 µl of 1X PBS as negative control and 100 µl of 1% SDS as positive controls. Then volume of reaction mixture was adjusted to 1 ml by adding 850 µl of 1X PBS. Finally centrifuged at 3000 rpm for 3 min and the resulting hemoglobin in supernatant was measured at 540 nm by Tecan micro plate reader, Magellan ™ Data Analysis Software to determine the concentration of haemoglobin. The haemolysis caused by 100 µl of 1% SDS was taken as 100 % haemolysis and the percentage haemolysis was calculated by the equation; % Hemolysis = [(Control - Sample)/ Control] x 100.

3.3. Statistical Analysis

IC_{50} values for cytotoxicity tests were derived from a nonlinear regression analysis (curve fit) based on sigmoidal dose response curve (variable) and computed using Graph Pad Prism 5 (Graphpad, San Diego, CA, USA)

Table 1. Evaluation of methanolic extracts of study plants by cytotoxic (MTT) assay on A549 cell line and haemolysis assay on human erythrocytes.

Cytotoxic (MTT) assay					Haemolytic assay		
Plant Material /	**Concn. (µg/ml)**	**OD at 590 nm**	**Inhibition (%)**	**IC 50 (µg/ml)**	**Concn. (µg/ml)**	**OD at 540 nm**	**Haemolysis (%)**
Control		0.6279	0	-	Control	0.2971	0
Vehicle		0.6319	0		1% SDS	0.071	76.04
Curcuma longa L.	10	0.61	3.89	207.3	10	0.30	0.00
	20	0.55	13.74		20	0.30	0.00
	40	0.47	25.62		40	0.30	0.00
	80	0.37	40.81		80	0.27	8.01
	160	0.32	49.79		160	0.24	20.73
	320	0.12	81.55		320	0.23	23.76
Clitoria ternatea L.	10	0.66	-4.48	-	10	0.30	0.00
	20	0.56	11.20		20	0.30	0.00
	40	0.64	-0.78		40	0.25	16.49
	80	0.69	-8.42		80	0.23	21.17
	160	0.61	3.88		160	0.23	23.39
	320	0.59	5.98		320	0.22	26.69
Aloe vera (L.) Burm.f.	10	0.75	-19.31	-	10	0.30	0.00
	20	0.78	-23.83		20	0.30	0.00
	40	0.91	-44.79		40	0.30	-0.64
	80	0.80	-25.84		80	0.30	-0.64
	160	0.80	-26.70		160	0.29	3.06
	320	0.70	-11.06		320	0.28	6.09

4. Result and Discussion

4.1. The Inhibitory and Ant Proliferative Effects of Methanolic Extract of *Curcuma longa* L., *Clitoria ternatea* L. and Aqueous Extract of *Aloe vera* (L.) Burm. f. on Human Cancer Cells (A549)

To verify the possible anti-proliferative effect of above plant extracts, as a first step toward the development of novel putative anticancer agents, we tested methanol extract of *Curcuma longa* L. - (Rhizome), *Clitoria ternatea* L. - (Seeds) and aqueous extract of *Aloe vera* (L.) Burm. f. - (Leaves) on inhibition and proliferation of A549 cancer cell lines at concentration of 10-320 µg/ml. Among these plants, proliferation of these cells was significantly inhibited by methanolic extracts of *Curcuma longa* L. in a concentration-dependent manner for 24 h. (Table 1, Figure 1)

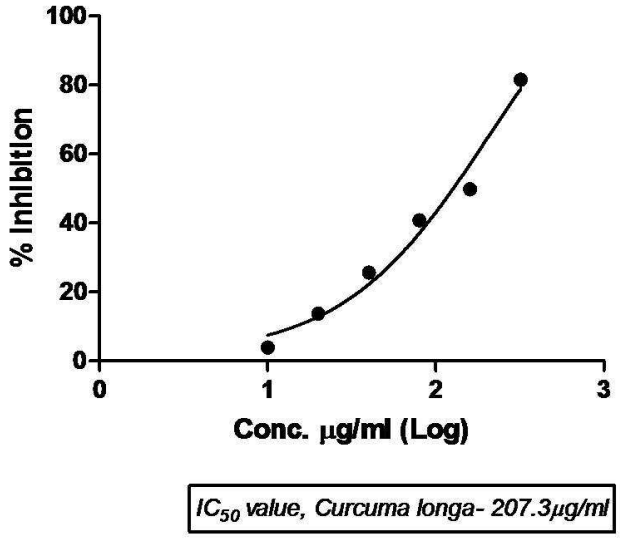

Figure 1. Cytotoxic effect of *Curcuma longa* L. on human lung carcinoma (A549).

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is based on the conversion of MTT into formazan crystals by living cells, which determines mitochondrial activity. Since for most cell populations the total mitochondrial activity is related to the number of viable cells, this assay is broadly used to measure the *in-vitro* cytotoxic effects of drugs on cell lines or primary patient cells. The present study designed with possible inputs for the identification of suitable plant source with bioactive compounds for combating human lung cancer. For the cytotoxic assay, the A549 cells were treated and observed for 3-4 hours with methanol extracts of *Curcuma longa* L., *Clitoria ternatea* L. and aqueous extract of *Aloe vera* (L.) Burm.f. in a concentration range of (0-320µg/mL). *Curcuma longa* L. showed a highest inhibition (IC 50 (µg/ml) = 207.3) as compared to other two extracts in which there was no inhibitory activity and not detectable. This observation support the anti-cancerous or anti inhibitory or anti proliferative activity of the rhizomes of *Curcuma longa* L. for human lung carcinoma. The qualitative analysis reported by earlier researchers suggests the presence of phytochemical constituents such as curcumin-sulphate, curcumin-glucuronide, vanillic acid and ferulic acid in the rhizome of *Curcuma longa* L. which would be the inhibiting factor in the growth of A549 cells *in vitro*. Radhakrishna Pillai et al. 2004 [14] investigated the anti-inflammatory, antioxidant and anti-cancer activities of *Curcuma longa* L. and found that the chemo-preventive action of curcumin might be due to its ability to induce apoptosis to arrest cell cycle. They also found the cellular and molecular changes induced by curcumin leading to the induction of apoptosis in human lung cancer cell lines-A549 and H1299. Pourhassan Mohammad et al. 2010 [15] reported that telomerase can be reactivated in lung cancer cells but not normal cells. Hence they targeted lung cancer cells with natural compound curcumin from *Curcuma longa* L. and found the anti-cancer activities like cytotoxic and telomerase-inhibitory effect on cell line A549. They inferred that *Curcuma longa* L. can be exploited as potential source for developing novel drugs against lung cancer.

4.2. The Effects of Methanolic Extract of *Curcuma longa* L., *Clitoria ternatea* L. and Aqueous Extract of *Aloe vera* (L.) Burm. f. on Human Erythrocytes

Haemolytic assay method is appropriate to evaluate the haemocompatibility of biomaterials and medical devices according to the international standard ISO 10993-4:2002. *Curcuma longa* L. showed anticancer activity in A549 human cancer cells and hence we have subjected the methanolic extracts (0-320 µg/ml) of *Curcuma longa* L., *Clitoria ternatea* L., and *Aloe vera* (L.) Burm.f. extracts for haemolysis test using normal human erythrocytes. None of the concentrations of the methanolic extracts showed any visible haemolysis activity (Table 1). Since ancient time, plants products have been utilized for the treatment of various health problems. Plants are one of the most important sources of drug discovery and development. The plants used in this study have been excessively used in traditional medicine to cure a variety of diseases. Haemolytic activity of the plant is expressed in percentage haemolysis and noted the intact RBCs without lysis. The presence of cardiac glycosides, alkaloids, tannins are responsible for the lysis of erythrocytes and hence the study plants did not have phytochemicals that induce lysis of erythrocytes. Thus,

Curcuma longa L. has both anti proliferative property and not induce haemolysis in normal human erythrocytes. Rupachandra and Sarada (2014) [16] demonstrated using the cytotoxic activity against lung (A549) and cervical (HeLa) cancer cells exhibited by a protein (F3) of *Borreria hispida* in a dose-dependent manner at concentrations ranging from 10 μg to 1000 μg/mL. This study is the first report of a protein from the seeds of *Borreria hispida* with antiproliferative and apoptotic activity in lung (A549) and cervical (HeLa) cancer cells [16]. Strategies adopted and the inferences in the present investigation coincide with the earlier *in-vitro* studies [13-16]. The cytotoxic affects and haemolysis activity of numerous anticancer agents are mysterious and doubtful and understanding of fundamental role of medicinal herbal extracts made from plants will support and has found to play an essential role in future for the development of herbal medicines to treat different type cancer.

5. Conclusion

Of the investigated plants, *Curcuma longa* L. has cytotoxic activity on A549 human lung carcinoma cells and no haemolysis activity on normal human erythrocytes. This points up a conclusion that *Curcuma longa* L. is a potential herb and has bioactive compounds for human lung cancer cure. Our key findings demonstrate the hope for the plant drug source for human lung cancer as they are non haemolytic in nature. Further studies on understanding the chemical nature of the bioactive compound and molecular mechanism of interaction against human lung cancer activity will pave a way for novel drug molecules that combat the dreadful disease.

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