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Research Article

EVALUATION OF PLANT EXTRACTS AGAINST LUNG CANCER USING H460 CELL LINE

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ABSTRACT

Objectives: In the present study, we endeavored to exploit bioactive compounds of two plants viz. *Limonia acidissima* and *Syzygium cumini* investigated their anti-proliferative properties for lung cancer and evaluated the hemolytic activity.

Methods: Methanol extracts of these plants experimented for cytotoxic activity and hemolytic assay against H460 cell proliferation.

Results: Methanol extracts of Limonia acidissima and Syzygium cumini were found to selectively inhibit H460 cell proliferation and non-hemolytic.

Conclusion: Our study deduces that the potential bioactive compounds of these plants have vibrant chance to fight lung cancer as were seen to be nonhemolytic to blood cells, and they are very much recorded as a traditional medicine for various therapeutic uses.

Keywords: *Limonia acidissima*, *Syzygium cumini*, H460 cell line, Cytotoxic assay, Flavonoids, Anti-proliferative activity, Hemolytic activity.

INTRODUCTION

Natural products, including plants, animals, and minerals, have been the basis of treatment of human diseases [1,2]. History of medicine dates back practically to the existence of human civilization. The current accepted modern medicine or allopathy has gradually developed over the years by scientific and observational efforts of scientists. However, the basis of its development remains rooted in traditional medicine and therapies. The history of medicine includes many ludicrous therapies. Nevertheless, ancient wisdom has been the basis of modern medicine and will remain as one important source of future medicine and therapeutics [3,4].

Limonia acidissima Linn., syn. *Feronia limonia* (Rutaceae) is a moderate-sized deciduous tree grown throughout India. The fruits are woody, rough, and used as a substitute for bael in diarrhea, dysentery [5,6], and for breast cancer and immunomodulatory activity [7-9]. The bark and leaves of the plant are used for vitiated conditions of vata and pita while the fruits are used for treating tumors, asthma, wounds, cardiac debility, and hepatitis. The fruit contains flavonoids, glycosides, saponins, and tannins [10]. Some coumarins [11] and tyramine derivatives [12] have also been isolated from the fruits of Limonia. The leaves are reported to possess hepatoprotective activity [11] while the fruit shells contain antifungal compounds, namely, psoralene, xanthotoxin, 2, 6-dimethoxybenzoquinone, and osthenol [13].

Syzygium cumini (Family-Myrtaceae) is native to India and East Indies. It is commonly called as Jamu Koli in Odia; Black Plum, Java Plum in English, and Jamun in Hindi. The plant possesses acetyl oleanolic acid, triterpenoids, ellagic acid, isoquercitin, quercetin, kaempferol, and myricetin in different concentrations [14]. Most of these compounds have been reported to possess antioxidant and free radical scavenging activities [15]. The fruit has been also reported to possess anti breast cancer activity [16].

In the present investigation, an attempt is made to inhibit potential lung cancer properties of *S. cumini* and *L. acidissima* and this approach laid the foundation on route to identify plants as an effective treatment for lung cancer.

METHODS

Collection of plant materials and extraction

Ripe fruits of *L. acidissima* and *S. cumini* were collected from the forest part of Bhubaneswar hill area situated in the eastern part of India in the month of May and identified by Dr. S K Sahu, a taxonomist at Utkal University, Vani Vihar, Odisha, and Dr. S P Panda, Herbarium Keeper, Regional Plant Research Center, Bhubaneswar. Voucher specimens were deposited in the herbarium of the Department of Botany, Utkal University. The fruits were cut into small pieces, and the pulp was separated from the seeds. The pulp was shade-dried and milled. The coarsely powdered, shade dried fruit pulp of both the plants was first defatted with petroleum ether using Soxhlet apparatus. The extracts were concentrated using a rotary evaporator to get the solid residue. The marc from the central compartment was removed, dried, and extracted by exhaustive extraction with a series of solvents of increasing polarity with Soxhlet extractor was done [17]. The weight of the residue extracts was recorded and percent yield calculated. Solvents used with increasing polarity are petroleum ether, ethyl acetate, and methanol.

Preliminary phytochemical screening

The percentage yield of other extracts except methanolic extract was negligible. So, the methanolic extract was taken for further experimental work. The prepared methanolic extract was subjected to routine phytochemical analysis [18] to identify the presence of various phytochemicals such as carbohydrates, alkaloids, glycosides, saponins, flavonoids, tannins, sterols, and phenols.

Cell culture

Human lung cancer (H460) cell line obtained from the American Tissue Culture Collection was used for the *in vitro* assay and grown in RPMI-1640 supplemented with 2 g of sodium bicarbonate. The pH 7.4 was maintained, and the cells were incubated at 37°C with 5% CO_2 in humidified incubator.

Thawing and revival

The frozen cells in cryovials were removed from liquid nitrogen storage and quickly thawed (<1 minute) in 37°C water bath by gently swirling the vial. In a sterile tube, the thawed cells were transferred with a required amount of media and were mixed for uniform distribution.

Centrifugation of the cell suspension was carried out at 1200 g for 5 minutes. For visibility of the complete pellet, the clear supernatant was checked and again resuspended in complete growth medium. The cells were then transferred to the T-25 flask under the recommended culture environment (5% CO_2 at 37°C). The cell growth was monitored for a confluence of 70-80% and as they attain they were trypsinized and subcultured.

Anti-carcinogenic activity against H460 cell line

Cytotoxic assay

H460 cells (5.0×10^4) were plated in 96-well plates with serum-free RPMI-1640 media aliquots with plant extracts at 0, 2, 4, 8, 16, 32, 64, and 128 µg/ml concentration in triplicates and incubated for 24 hrs at 37°C in a 5% CO_2 incubator. Then, the media was removed, and 100 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent was added to each well and incubated again for 3-4 hrs. Before adding 100 µl dimethyl sulfoxide to each well, MTT reagent was removed and gently shaken [19]. The untreated cells were compared to plant extract-treated cells. The absorbance was measured at 570 nm using a microplate reader. The percentage inhibition was determined using a formula:

(% Inhibition = 100−(OD of sample/OD of control) × 100)

Inhibitory concentration 50% (IC_{50}) values were calculated as the concentrations that show 50% inhibition of proliferation on any tested cell.

Hemolytic assay

Erythrocytes were isolated from 5 ml of blood collected in a tube containing 5.4 mg of ethylenediaminetetraacetic acid from healthy volunteers, centrifuged at 1000 rpm for 10 minutes at 40°C. Plasma and the white buffy layer were removed. The erythrocytes were then washed thrice with ×1 phosphate buffered saline (PBS), pH 7.4 and used within 6 hrs for the hemolysis assay [20,21]. The erythrocyte suspension was incubated for 1 hr with test material in incubated shaker at 37°C. After incubation, samples are collected and centrifuged to obtain supernatant containing free hemoglobin, and the hemoglobin concentration was determined with a spectrophotometer (540 nm). Test samples were compared to reference materials (1% Tritonx-100 and 1% sodium dodecyl sulfate [SDS]). 50 µl of 10 aliquots of erythrocytes suspension was taken and incubated with 100 µl of different concentration of plant extracts (0, 2, 4, 8, 16, 32, 64, and 128 µg/ml) at 37°C water bath for 60-90 minutes. Here, 100 µl of ×1 PBS served as negative control and 100 µl of 1% SDS as a positive control. Then, the volumes of reaction mixture were adjusted to 1 ml using ×1 PBS. Finally, centrifuged at 3000 rpm for 3 minutes and the resulting hemoglobin in the supernatant was measured at 540 nm by microplate reader and determined the concentration of hemoglobin using Magellan - Data analysis software. The hemolysis caused by 100 µl of 1% SDS was taken as 100% hemolysis and the percentage hemolysis was calculated: (% Hemolysis = [{control – sample}/control] × 100).

Statistical analysis

IC_{50} values for cytotoxicity tests were derived from non-linear regression (curve fit) based sigmoidal dose response curve (variable) and computed using Graph Pad Prism 5 (Graph pad, San Diegro, CA, USA).

RESULTS AND DISCUSSION

Cytotoxic assay and hemolysis activity for methanolic extracts of *L. acidissima* and *S. cumini*

Lung cancer remains the most common malignancy worldwide with approximately 1.3 million new cases and 300,000 deaths each year estimated by the World Health Organization. Cancer is affecting great many individuals consistently, and our study is to explore potential plant sources and to develop a novel anticancer candidate that can combat lung cancer in a better way. Medicinal plants have been in use from time immemorial, and their utility has been increasing day by day in the present world. Naturally obtained compounds are considered safer and easily biodegradable than synthetic compounds and the problem of drug resistance observed in synthetic drugs is also reduced [22]. Plants represent a source of leads for many pharmaceutical compounds and the phytochemical compounds, and secondary metabolites present in plants have been used in treating a number of human ailments. The MTT 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 effect of test molecules on cell proliferation and their cytotoxic effects were investigated using tetrazolium reduction and other assays like resazurin reduction and protease activity assays as viability indicators [23]. Among the study both the plants, methanol extracts of *Limonia acidissima* and *S. cumini* have an IC_{50} of 46.21% (µg/ml) and 35.21% (µg/ml), respectively (Table 1 and Fig. 1). Interestingly, methanol extracts of both the plants studied have not shown hemolysis on human erythrocytes between 40 and 160 µg/ml with the 1% SDS as a positive control. The present breakthrough in this study thereby gives enough scope to use these

Table 1: Evaluation of methanol extracts of *L. acidissima* and *S. cumini* by MTT assay on cell line and hemolysis assay on human erythrocytes

Cytotoxic (MTT) assay					Hemolysis assay		
Plant extract	**Concentrated (µg/ml)**	**OD at 590 nm**	**Inhibition (%)**	**IC_{50} (µg/ml)**	**Concentrated (µg/ml)**	**OD at 540 nm**	**Hemolysis (%)**
Control		0.44	0	-	Control	0.38	0.00
Vehicle		0.51	0	-	1% SDS	0.10	74.38
L. acidissima	10	0.47	07.96	46.21	40	0.93	−145.78
	20	0.42	17.82		80	0.70	−83.32
	40	0.35	31.72		160	0.69	−82.00
	80	0.30	41.24				
	160	0.25	50.64				
	320	0.20	61.74				
S. cumini	10	0.48	07.24	35.21	40	0.66	−73.59
	20	0.42	18.34		80	0.78	−104.45
	40	0.33	35.07		160	0.97	−156.41
	80	0.28	45.56				
	160	0.23	54.34				
	320	0.18	65.19				

Values are mean of three triplicates. *L. acidissima: Limonia acidissima, S. cumini: Syzygium cumini,* OD: Optical density, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

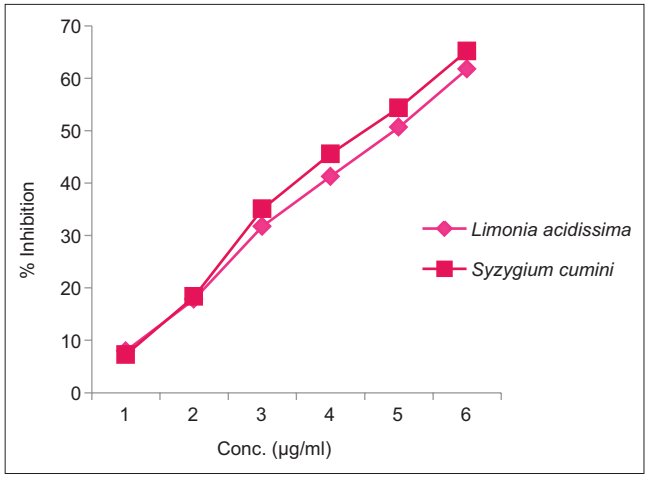

Fig. 1: Cytotoxic effect of *Limonia acidissima* and *Syzygium cumini* on H460 cell line

plant extracts for further investigation at the molecular level to identify the compound accountable for the treatment of lung cancer activity. In the present investigation, the focus was limited to plant extracts that caused substantial growth inhibition in a given cell line within the test concentration range of <320 µg/ml although such plants may likely demonstrate greater cytotoxicity at higher concentrations. Various parts of *S. cumini* have been used to study its effectiveness on different types of cancer, and the leaves extract has shown a protective effect against DNA damage and biochemical changes in mice caused due to atrazine. The qualitative tests used by researchers have identified phytochemical constituents of the *L. acidissima* and *S. cumini* [7,8] and showed the presence of contains flavonoids, glycosides, saponins and tannins, which may help to inhibit the growth of the cells *in vitro*.

***In vitro* erythrocyte hemolysis inhibition assay**

Treatment of various health problems has been encountered with various plant products since long. Plants are one of the most important sources of drug discovery and development. In this study, hemolytic activity of the methanol extract of fruit pulp of *L. acidissima* and *S. cumini* against normal human erythrocytes lysis. Hemolytic activity of the plant is expressed in percentage hemolysis and it was found that none of the extract of study plants showed any lysis in the human blood red blood cells, and thus these plants did not contain cardiac glycosides, alkaloids, saponins, and phlobatannins as which are responsible for the lysis of the erythrocytes [24].

CONCLUSION

Based on the inferences from the cytotoxic and erythrocyte hemolytic assay of the methanol extracts of fruit pulp of *L. acidissima* and *S. cumini*, the present study draws a conclusion that the above-mentioned two plants are suitable candidature for the treatment of lung cancer. Major findings of the present study show hopeful plant source for lung cancer and their non-hemolytic activity. Further studies may attribute to elucidate the nature of the chemical and molecular mechanism of interaction for the said activity.

Although the clinical efficacy and extent of toxicity of numerous anticancer agents are unknown and uncertain, understanding the fundamental role of herbal extracts made from plants has found to play an essential role in the development of herbal drugs and use for treatment of cancer.

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