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IN VITRO STUDIES ON THE EVALUATION OF SELECTED MEDICINAL PLANTS FOR LUNG CARCINOMA

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ABSTRACT
The present study describes the anticancer, hemolysis and antimicrobial and DPPH assay, NOS assay, MTT assay of Neem, lemon, cinnamon extracts were prepared from these plants and their hemolytic, anticancer and antimicrobial activities were evaluated. First the hemolytic activities of the plants were tested on human erythrocytes so as to check the toxicity of the plants selected. The results showed that the percentage of hemolysis of all the samples were less so they are considered as non toxic. The antiproliferative activity of the plants was studied on non-small lung cancer cell line A549 and cell viability was assessed by MTT assay and non linear regression graph was made by using graph pad prism. The result showed a dose dependent behavior of the extract *cinnamon* and it exhibited an IC_{50} value of 21.9 µg / ml towards the lung cancer cell line A549. The antibacterial activity and antifungal activity was measured by disc diffusion method on *S.Pyagenes* and *Alricanes*. There is no zone of inhibition was observed against the bacterial pathogen *S.Mutans* when it compared with the stranded fluconazole, ciproflaxacin antibiotics. These obtained results have showed that *cinnamona* can be considered as a promising chemotherapeutic agent that can be used for lung cancer treatment.

KEYWORDS: Cinnamon, Lemon, Neem, DPPH, NOS, MTT Assays.

INTRODUCTION
Lung cancer is the most common cancer and it cause leading cancer deaths worldwide. The prognosis of the patients can be improved by effective treatments, but the 5-year survival rate of the patients with advanced lung cancer is only about 16%.[1] Various novel therapeutic cytotoxic drugs are currently available in clinical use, but it is limited due to intrinsic or acquired resistant and toxicity.[2] The majority cases of the patients with lung cancer presents with locally advanced inoperable or metastatic disease.[3] Cell migration is a biological process that crucially develops into a variety of physiological, wound healing and the inflammatory reaction. In addition, cell migration is also responsible for the malignance of cancer disease as it allows tumor cells to invade into the surrounding tissues, thereby forming metastases.[4] Cancer cells are characterized by the accelerated proliferative capacity and resistance to apoptosis. In lung cancer cases about 85% of patients are diagnosed with non-small cell lung cancer (NSCLC) and by using the aggressive radio or chemotherapy, fewer than 20% of such patients reach to a 5 year survival rate.[5] This is mainly due to significant resistance of NSCLC to such conventional cytotoxic drugs. Therefore, there is an urgent need to identify a new possible effective prevention drugs and also therapies to treat NSCLC.

Cinnamon is a most common spice that used in the different cultures around the world for several centuries. It is obtained from the genus *Cinnamomum*, a tropical evergreen plant that has only two main varieties; *Cinnamon cassia* and *Cinnamomum zeylanicum*. In addition to its biological uses, in native Ayurvedic medicine it is considered to be a remedy for digestive respiratory and gynaecological ailments. Almost all the parts of the Cinnamon tree (bark, leaves, flowers, fruits and roots) have some medicinal properties or culinary use. The volatile oils that obtained from the leaf, bark and root barks vary somewhat in chemical composition, so it suggests that they might be vary in their pharmacological effects as well.[6]

Lemon is a most important medicinal plant belongs to the family *Rutaceae*. It is mainly cultivated for its alkaloids, which are having anticancer and the

antibacterial activities.[7] Citrus flavonoid possesses a large spectrum of biological activities like antifungal, antidiabetic, antibacterial, anticancer and antiviral activities.[8-9]

Azadirachta indica is also called as "Neem" and it belongs to the family *Meliaceae*. It is a very important medicinal plant that is traditionally used for treating different diseases. It is widely distributed in the tropical and sub-tropical regions. Its crude extracts from bark and leaves have been used in the folk medicine to control the diseases such as respiratory system, intestinal helminthiasis and leprosy.[10]

In addition, earlier studies have been reported that neem possesses antipyretic, antiarthritic hypoglycemic, anti-inflammatory, antibacterial, antigastric ulcer, antifungal, and antitumor activities.[11-15]

MATERIALS AND METHODS

Phytochemical Analysis (Qualitative)

Preparation of Extract

20g of sample was extracted by the method of crude extraction using 100mL of Methanol. Through whatmann No. 1 filter paper the methanolic extract was filtered and the obtained filtrate was used for phytochemical analysis.

Procedure

Test for Alkaloids: 3 ml of 1% HCl was stirred with the 3 ml of aqueous extract on the steam bath. Then to the mixture Mayer and/or Dragondroff's reagent was added. Turbidity of resulting precipitate was taken as an evidence for the presence of alkaloid.

Test for Tannins

$FeCl_3$ Test: 2 ml of distilled water was stirred with 2 ml of the aqueous extract and to it few drops of FeCl3 solution were added. Formation of the green precipitate was indication of presence of tannins.

Gelatin test: To each extract 1% gelatin solution containing 10% sodium chloride were added. Formation of precipitate indicated the presence of tannins and phenolic compounds.

Test for Saponins: In a test tube 5 ml of aqueous extract was shaken vigorously with 5 ml of distilled water and warmed. The stable foam was formed and that taken as an indication of the presence of saponins.

Test for Phlobatannins: About 2 ml of 1% HCl was added to 2 ml of aqueous extract and the mixture was boiled. Deposition of a red precipitate was taken as an evidence for the presence of phlobatannins.

Test for Flavonoids

Alkaline reagent test: To 1 ml of 10% lead acetate solution 1 ml of aqueous extract was added. The formation of a yellow precipitate was taken as a positive test for flavonoids.

Shinoda Test: To leaf and bark (mixture) extracts, separately 5ml of 95% ethanol was added. Each mixture was treated with 0.5g of magnesium turnings and a few drops of concentrated HCL. Pink colour, if produced, may confirm the presence of flavonoids.

Test for Terpenoids

Salkowski's Test: The extracts was treated with chloroform and to it few drops of concentrated sulphuric acid added, shaken well and allowed to stand for some time; formation of the yellow colored lower layer indicated the presence of terpenoids.

Tests for glycosides

Liebermann's Test: In 2 ml of chloroform, 2 ml of the organic extract was dissolved and then to it 2 ml of acetic acid was added. The solution was cooled well in ice and sulphuric acid was then added carefully. A colour change was observed from violet to blue to green that indicates the presence of a steroidal nucleus (that is, a glycone portion of glycoside).

Tests for steroids: A red colour is produced in the upper layer when 2 ml of organic extract was dissolved in 2 ml of chloroform and to it 2 ml concentrated sulphuric acid was added, that indicates the presence of steroids.

DPPH Assay

DPPH (EEC No. 217-591-8, Sigma, USA), store at less than 0°C. Methanol, HPLC grade (Ranbaxy Chemicals).

Inhibitor (reference standard)

Gallic acid [3, 4, 5-Trihydroxy benzoic acid] (EEC No. 205-749-9, Sigma, USA), store at room temperature.

Preparation of working solutions.

DPPH: 1.3 mg/ml in HPLC grade methanol. Gallic acid: 5 mg dissolved in 100 ml methanol.

Assay Procedure

DPPH assay is carried out as per the method of Vani.et. al.[16] In brief, 90µl of DPPH solution is treated with 180µl of various concentrations of test solution& standard. The different concentrations tested for reference standard are 0.3, 0.6, 1.2, 2.5, 5, 10µg/ml. The reaction mixture is mixed and incubated at 25°C for 15 minutes. The absorbance is measured at 510 nm using Plate reader. A control reaction is carried out without the test sample.

Determination of % Inhibition

% Inhibition = (Control – Sample/ Control) x 100

Nitric Oxide Scavenging Assay

Preparation of working solutions

Phosphate buffered saline pH 7.0

Weigh 8 g of NaCl, 1.21g of K_2HPO_4 and 0.34g of KH_2PO_4 in 1000 mL distilled water.

Preparation of sodium nitroprusside (10mM)
29.79 mg is dissolved in 10 mL phosphate buffered saline pH 7.0.

Preparation Griess reagent
1 g sulfanilamide dissolved in 5% H_3PO_4 solution (5 mL of H_3PO_4 in 100mL de-ionized water).
0.1% N – napthyl ethylene diamine - 10 mg is dissolved in 10 mL de-ionized water.
Mix both A & B in equal proportion (1:1) before using the reagent. Reference standard (Curcuminoids). 4 mg is dissolved in 10 mL methanol (HPLC grade).

Assay Procedure
Nitric oxide scavenging assay (NOS) is carried out as per the method of Sreejayan and Rao et. al. [17]. 200 µl of 10 mM sodium nitroprusside and 200 µl of test solution / reference standard of various concentrations are incubated at room temperature for 150 minutes.

Add 500 µl Griess reagent and incubate for 10 minutes at room temperature. Measure the absorbance at 546 nm. Test substances are replaced by buffer solution for a control.

Calculations: The percentage inhibition of Nitric Oxide is calculated as follows.

$$\% \text{ inhibition} = \frac{\text{Absorbance (control)} - \text{Absorbance (test sample)}}{\text{Absorbance (control)}} \times 100$$

Cytotoxicity Studies using A549 cell line by MTT Assay
MTT Powder (the solution is filtered through a 0.2 µ m filter and then solution is stored at 2–8 °C for frequent use or frozen for extended periods).

Procedure
The cells were collected when they reach about 70-80% confluency. Check for the viability and centrifuge the cells. About 50,000 cells / well were seeded in a 96 well plate and incubate for 24 hrs at 37°C, 5 % CO_2 incubator.

Add samples to be tested from 0-320µg/ml (2 fold variation) concentration in RPMI without FBS & are incubated for 24 hr. After incubation with test samples, add 100 µl/well (50 µg /well) of the MTT (5 mg/10 ml of MTT in 1X PBS) was added to the respective wells and incubated for 3 to 4 hours. After incubation with MTT reagent, discard the MTT reagent by pipetting without disturbing cells and add 100 µl of DMSO to rapidly solubilize the formazan. Measure the Absorbance at 590 nm.

Calculating Inhibition
% Inhibition = 100 – (OD of sample/OD of Control) x 100.

Hemolytic Assay
Isolation of erythrocytes
5 ml of blood was collected from healthy volunteers in the tubes containing 5.4 mg of EDTA to prevent coagulation and then it is centrifuged at 1000 rpm for 10 min at 4^0C. Carefully plasma was removed and the white buffy layer was removed completely through aspiration with a pipette with utmost care.

The erythrocytes were then washed for additional three times with 1X PBS at pH 7.4. Washed erythrocytes were then stored at 4°C and used within 6 h for the haemolysis assay.

Protocol
Take 50 µl of 10 dilution (100 µl Erythrocytes suspension : 900 µl 1XPBS) of erythrocytes suspension into 1.5 ml of new eppendorf tube and add 100 µl of test samples (plant extracts, compounds, etc), 100 of 1XPBS as negative control, and 100 µl of 1% SDS as positive controls. Reaction mixture is incubating at 37^0C water bath for 60 min. Adjust the volume of reaction mixture to 1 ml by adding 850 µl of 1XPBS. Finally centrifuge at 300 rpm for 3 min and the obtained hemoglobin in supernatant was measured at 540 nm to determine the concentration of hemoglobin.

Calculating Inhibition
% Hemolysis = (Control - Sample)/ (Control) x 100

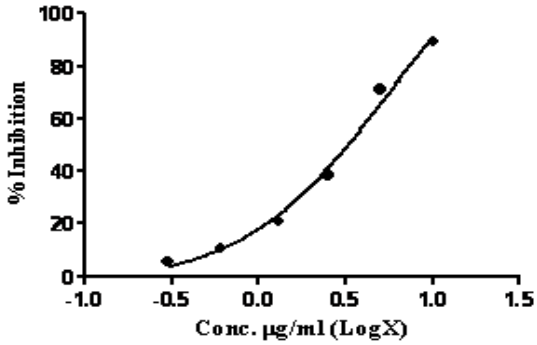
RESULTS
Phytochemical constitute of the methanolic extract of Poly Herbal Formulation.
Table – 1 Phytochemical constitute of the methanolic extract of Poly Herbal Formulation.

Sl. No.	Phytochemical	Neem	Lemon	Cinnamon
1	Carbohydrates	+	+	+
2	Steroid	+	+	-
3	Alkaloids	+	+	+
4	Tannins	+	+	_
5	Phlobatannins	-	-	-
6	Saponins	+	+	-
7	Flavonoids	+	+	+
8	Terpenoids	+	+	-
9	Glycosides	+	+	-

Statistical Analysis of DPPH Assay
IC50 values for DPPH radical scavenging activity of test compounds is derived from a nonlinear regression analysis (curve fit)based on sigmoidal dose response curve (variable) and computed using Graph Pad Prism 5 (Graph pad, SanDiego, CA, USA)

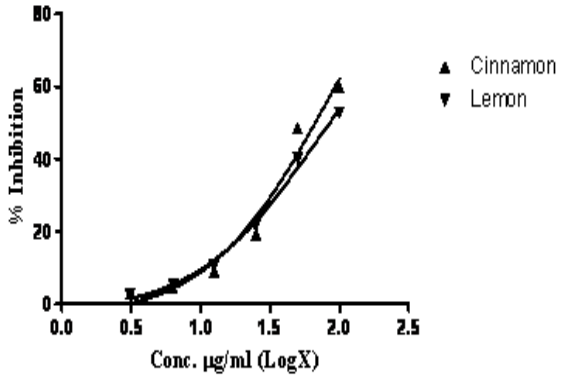
Table – 2: Statistical Analysis of DPPH Assay.

Plants Name	Concentration (µg/ml)	Absorbance	% Inhibition	IC_{50}
Control	0.0	0.481	0.00	
Standard (Quercitin)	0.3	0.455	5.50	5.879µg/ml
	0.6	0.429	10.79	
	1.3	0.380	20.92	
	2.5	0.295	38.57	
	5.0	0.138	71.24	
	10.0	0.052	89.19	
Neem	0.0	0.481	0.00	NA
	3.1	0.474	1.44	
	6.3	0.462	3.87	
	12.5	0.447	7.04	
	25.0	0.413	14.12	
	50.0	0.344	28.58	
	100.0	0.254	47.16	
Cinnamon	0.0	0.481	0.00	82.18µg/ml
	3.1	0.468	2.77	
	6.3	0.459	4.50	
	12.5	0.438	8.95	
	25.0	0.389	19.11	
	50.0	0.248	48.47	
	100.0	0.193	59.91	
Lemon	0.0	0.481	0.00	66.57µg/ml
	3.1	0.469	2.50	
	6.3	0.456	5.21	
	12.5	0.430	10.68	
	25.0	0.377	21.55	
	50.0	0.288	40.12	
	100.0	0.227	52.75	



	Quercetin
log(inhibitor) vs. response	
Best-fit values	
BOTTOM	147.1
TOP	-3.569
LOGIC50	0.7693
IC50	5.879

Figure – 1 DPPH assay – Quercetin



	Cinnamon	Lemon
log(inhibitor) vs. response		
Best-fit values		
BOTTOM	116.9	91.34
TOP	-4.327	-2.844
LOGIC50	1.915	1.823
IC50	82.18	66.57

Figure – 2 DPPH assay – Cinnamon and Lemon

Statistical Analysis of Nitric Oxide Scavenging Assay.

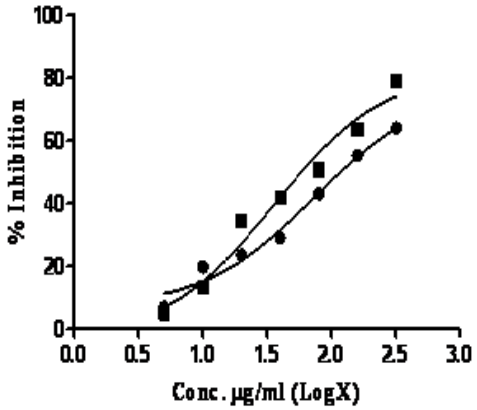
Table – 3 Statistical Analysis of Nitric Oxide Scavenging Assay.

Plants Name	Concentration (µg/ml)	Absorbance	% Inhibition	IC_{50}
Control	0.0	0.536	0.00	
Standard (Curcumin)	2.5	0.501	6.53	31.63 µg/ml
	5	0.479	10.63	
	10	0.435	18.84	
	20	0.332	38.06	
	40	0.239	55.41	
	80	0.166	69.03	
Neem	0.0	0.457	14.74	NA
	3.1	0.535	0.21	
	6.3	0.519	3.15	
	12.5	0.504	6.02	
	25.0	0.467	12.89	
	50.0	0.415	22.56	
	100.0	0.321	40.08	
Cinnamon	0.0	0.457	14.74	NA
	3.1	0.514	4.13	
	6.3	0.479	10.55	
	12.5	0.435	18.92	
	25.0	0.366	31.76	
	50.0	0.278	48.11	
	100.0	0.436	18.72	
Lemon	0.0	0.457	14.74	72.46µg/ml
	3.1	0.525	2.13	
	6.3	0.514	4.09	
	12.5	0.476	11.22	
	25.0	0.424	20.85	
	50.0	0.326	39.17	
	100.0	0.250	53.44	

Statistical Analysis of Cytotoxicity studies using A549 cell line by MTT Assay.

Table – 4: Statistical Analysis of Cytotoxicity studies using A549 cell line by MTT Assay.

A549				
Plants name	**Conc. µg/ml**	**OD at 540 nm**	**% Inhibition**	**IC50**
	Control	0.5278	0.00	
Cinnamon	5	0.4528	14.21	NA
	10	0.4246	19.55	
	20	0.3894	26.22	
	40	0.3682	30.24	
	80	0.3427	35.07	
	160	0.3195	39.47	
	320	0.2753	47.84	
Lemon	5	0.4913	6.92	73.35
	10	0.4232	19.82	
	20	0.4037	23.51	
	40	0.3745	29.05	
	80	0.3008	43.01	
	160	0.2366	55.17	
	320	0.1897	64.06	
Neem	5	0.5015	4.98	35.65
	10	0.4575	13.32	
	20	0.3453	34.58	
	40	0.3057	42.08	
	80	0.2603	50.68	
	160	0.1923	63.57	
	320	0.1102	79.12	



	Lemon	*Neem*
log(inhibitor) vs. response		
Best-fit values		
BOTTOM	76.99	82.83
TOP	6.657	-3.908
LOGIC50	1.865	1.552
IC50	73.35	35.65

Figure – 3 Cytotoxicity studies using A549 cell line by MTT Assay

Statistical Analysis of Hemolytic Assay.

Sample	Treatment	Absorbance	Absorbance	Average	% Inhibition
Control	PBS	0.8927	0.8821	0.8874	0.00
Positive control	1% SDS	0.1824	0.1791	0.1808	79.63

Table – 5: Statistical Analysis of Hemolytic Assay

Sample	Conc. µg/ml	Absorbance	% Inhibition
Control	0.00	0.8874	0.00
Cinnamon	40.00	0.8525	3.93
	80.00	0.8138	8.29
	160.00	0.7827	11.80
	320.00	0.7358	17.08
Lemon	40.00	0.8596	3.13
	80.00	0.8015	9.68
	160.00	0.7593	14.44
	320.00	0.7176	19.13
Neem	40.00	0.8413	5.19
	80.00	0.8075	9.00
	160.00	0.7652	13.77
	320.00	0.7283	17.93

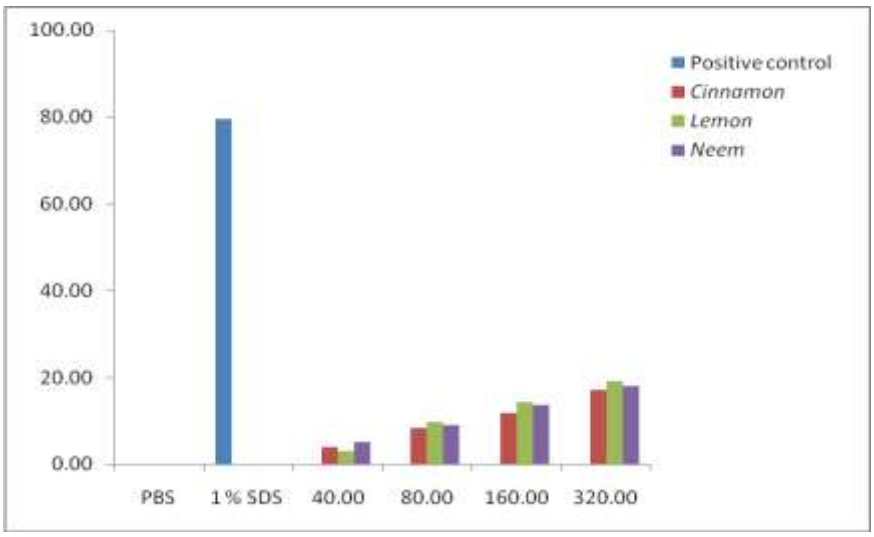


Figure – 4 Statistical Analysis of Hemolytic Assay

DISCUSSION

Different phytochemical assays were performed where plant extracts showed some positive results and negative results. Among them, Neem showed positive results for carbohydrates, steroid, alkaloids, tannins, Saponins, flavonoids, terpenoids and glycosides. Lemon showed

positive results for carbohydrates, steroids, alkaloids, tannins, Saponins, flavonoids, terpenoids and glycosides. Cinnamon showed positive results for carbohydrates, alkaloids and flavonoids.

The antioxidant properties of three plants were studied using DPPH assay. In DPPH assay from Table 2 and Fig. 2, Cinnamon and lemon showed IC50 values of 82.18 µg/ml and 66.57µg/ml respectively as compared to standard (Quercetin) which showed 5.879 µg/ml (Fig.1). Neem was seen to be inactive in DPPH assay.

The antioxidant properties of three plants were studied using Nitric oxide scavenging assay. In NOS assay from Table 3, lemon showed IC50 values of 72.46 µg/ml respectively as compared to standard (Curcumin) which showed 31. 63 µg/ml. Neem and Cinnamon were seen to be inactive in NOS assay.

In the MTT assay from Table 4 and Fig. 3, the three methanolic plant extracts were treated on non-small cell lung carcinoma cell line and their cytotoxic properties were examined. Among the tested plants, Neem showed highest cytotoxic activity than lemon and showed IC50 values of 35.65µg/ml and 73.35 µg/ml respectively. The plant cinnamon was seen to be inactive on the A549 cell line.

The plants extracts were tested for their hemolytic activities using normal human erythrocytes by hemolysis assay. From Table 5 and Fig. 4, it was seen that as compared to the control used, all the three plant extracts showed no hemolytic activity at lower concentrations where as they were observed to be partially hemolytic at higher concentrations.

CONCLUSION

Cancer is the most dreaded disease by humans; the present need is to develop drugs that potentially target cancer cells alone by means of their inherent difference to normal cells. Plants extracts or its constituents which are known to inhibit the lung cancer cells without affecting the normal cell certainly has the potential to be used in cancer therapy. The development of such specific drugs with differential action will be valuable in lung cancer chemotherapy without the observed side effects. The methodology involves the use of cancer cell lines to test the efficacy of the plant extracts Invitro.

The present study describes the anticancer, antioxidant, hemolytic and antimicrobial activity of Lemon, neem and cinnamon on non-small cell lung carcinoma (A549) cell line. Lemon and Neem shows a strong evidence to become a potent natural remedy against lung cancer. The strong activities witnessed in these plants are due to the presence of certain phytochemical compounds present in them. Hence from these studies, we can conclude that lemon and neem can play important roles as anti-cancerous agents.

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