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INVITRO STUDIES ON THE EFFECT OF *LANTANA CAMARA LINN*. IN LIVER CANCER HEPG2 CELL LINES

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ABSTRACT

The leaves of *Lantana Camara* L. was regarded both as a notorious weed and a popular ornamental garden plant that are commonly used in traditional medicine by many Asian and Middle Eastern Countries to treat wound, skin allergy, ulcer and many other diseases. This leaves extracts are found to have antioxidant and anticancer activities, Triterpenes is the active principle which is responsible for these beneficial effects. The aim of the present study was to check the *in vitro* antioxidant and antitumor activity of *Lantana Camara* Linn leaves on Liver cancer HepG2 cell lines. We found Methanol extracts of *Lantana Camara* L. leaves have phytochemical compounds that are inhibitorier, scavenging free radicals with less anticancer activities.

KEYWORDS: *Lantana Camara* L. Triterpenes, HepG2 cell line.

INTRODUCTION

Cancer is the fastest growing problem, which is about 6 million cases new incidence per year. It is the second major cause of deaths after cardiovascular diseases that may affect different parts of body. Cancer is characterized by a rapid and uncontrolled formation of abnormal cells. Many factors are known to accelerate the risk of cancer including tobacco use, dietary factors, certain infections, exposure to radiation, lack of physical activity, obesity, environmental pollutants and other things. These factors can directly damage genes or combine with existing genetic faults within cells to cause cancerous mutations. Approximately 5–10% of cancers are traced directly due to inherited genetic defects.[1]

Hepatocellular carcinoma (HCC), a primary liver cancer, is one of the most frequent tumors representing the fifth commonest malignancy worldwide and the third cause of mortality from cancer. Unfortunately, the liver cancer is resistance to chemotherapeutic drugs and metastasis to other organs; hence treatment is unsatisfactory which are mainly due to late diagnosis and poor treatment efficacy.[2] There is a necessity to find new compound for cytotoxic activity to cancer treatment. The drugs which are available now are often unsatisfactory due to many problems like causing cytotoxicity to normal cell along with cancer cell. Plants are considered as the valuable sources of bioactive compounds with antioxidant activity. They produce certain substances which has the capacity to stimulate apoptosis in cancer cells.

HepG2 cell line

Organism : *Homo sapiens*, human
Tissue : Liver
Disease : Hepatocellular carcinoma
Age : 15 years adolescent
Gender : Male
Morphology : Epithelial
Growth Properties: Adherent

The HepG2 cell line was originally established in 1979 by Barbara Knowles and colleagues, and mistakenly reported as a hepatocellular carcinoma. A "Human hepatoma-derived cell line" patent was filed in 1980 by investigators at The Wistar Institute in Philadelphia. Since then, HepG2 has been listed on the ATCC repository (American Type Culture Collection, Rockville, MD, USA) as a human cell line (HB 8065) "derived from the liver tissue of a 15-year-old white male with a well-differentiated hepatocellular carcinoma." The mistaken classification of the HepG2 tumor of origin has created confusion between investigators and a divided body of scientific literature. With the help of all previous work performed by various histopathologist, the background of the tumor from which HepG2 was originated excised by extended lobectomy from the liver of a 15-year-old white male. The tumor is a classic example of an epithelial hepatoblastoma.[3]

Lantana Camara

Lantana Camara is a tropical origin plant and native to Central and Northern South America. *L. Camara* is now spreaded to nearly 60 countries viz, New Zealand, Mexico, Florida, Trinidad, Jamaica, Brazil and India. It is also reported in many African countries including Kenya, Uganda, Tanzania and South Africa.

Plant description

Morphology of *L. Camara* is a subscandent vigorous shrub or low erect having tetrangular stem, stout recurved pickles and a strong odour of black currents. Plant grows up to 1 to 3 meters and it can spread to 2.5 meter in width. Leaves are ovate or ovate oblong, crenate serrate, acute or sub acute, rugose above, scabrid on both sides. The leaves are 3-8 cm long by 3-6 cm wide and green in color. Leaves and stem are covered with rough hairs. Small flower held in clusters (called umbels). Generally it will be in orange, sometime varying from white to red in various shades and the flower usually changes there colours as they ages. Flowers are having a yellow throat, in axillary head. The calyx is small, corolla tube is slender, limb spreading is around 6 to 7 mm wide and divided in to unequal lobes. Stemen four in two pairs, included and ovary two celled, two ovuled. Inflorescences are produced in pairs in the axils of opposite leaves and it is compact, dome shaped 2-3 cm across and contain 20-40 sessile flowers. Root system is very strong and it gives out new fresh shoots even after cutting repeatedly. Infusion of the whole plant is used to cure bronchitis and the powdered root in milk was given to children for stomach-ache and as a vermifuge. Lantana oil is used in the treatment of skin, itches, as an anticeptic for wound healing. In leprosy and scabies decoctions were applied externally.[4]

The essential oil (EO) of aerial parts of *Lantana Camara* L. Verbenaceae, were obtained by hydro distillation and analyzed by GC-FID and GC-MS techniques. In total, 68 compounds were identified. The most representative compounds of the oil were mono and sesquiterpenes. The main compounds found in the oil were β-caryophyllene (10.5%), sabinene (7.98%) limonene (7.68%), spathulenol (11.64%). The oil from stems of *L. Camara* was characterized by a largest amount of sesquiterpenoids, with spatulenol (15.9%) and caryophyllene oxide (17.1%), as main components. β-Gurjunene (32.7%) was the most prominent compound in the stems` oils, which was absent or at very low relative abundance in leaves. *L. Camara* essential oils from leaves were cytotoxic to V79 mammalian cells and also to *Artemia salina*, showing 50% lethal concentration (LC50) values from 0.23µg/mL. The in vitro data obtained in this study suggested that EO may also be effective treating yeast infection in patients infected with fluconazole and terbinafine resistant isolates, but its toxicity must be monitored carefully.[5]

Role of Triterpenes in hepatocellular carcinoma treatment

Triterpenes can obtain naturally in many plants and it will serve as a defense mechanism system for anti-cancer properties, anti-inflammatory, anti-viral, anti-bacterial and anti-fungal, anti-oxidative and for many more. These substances can be isolated from plants, animals or fungi. Nowadays, when neoplasms become a main cause for death then triterpenes can become an alternative method for treating cancer because of their cytotoxic properties and chemo preventive activities.[6]

Basic structures of triterpenes are alkenes which are available naturally from animal, vegetable and also fungal origin. It is classified among an extensive and structurally diverse group of natural substances, referred to as triterpenoids. Their structure includes 30 elements of carbon atom and they are constituted by isoprene units. Taking into consideration the structure, triterpenes may be divided into linear ones—mainly derivatives of squalene, tetracyclic and pentacyclic, containing respectively four and five cycles, as well as two- and tricyclic ones. Triterpenes families show anti-cancer properties as well as anti-inflammatory, anti-oxidative, anti-viral, anti-bacterial and anti-fungal ones. The betulinic acid and its derivatives which have been investigated for their strong cytotoxic properties could be a good example. The other representatives could be originated from squalene, oleane (e.g., oleanolic acid), lupane (e.g., lupeol, betulin, betulinic acid), dammarane, lanostane, ursane (e.g., ursolic acid, uvaol, alpha-amyrin) or triterpenoid sapogenins, for example cycloartane, friedelane, filicane and cucurbitane triterpenoids.

A crude extract of *Lantana Camara* leaves had a cytotoxic effect on HeLa cells at 36hours to 72 hour by employing the 2-5 diphenyltetrazolium bromide (MTT) cell viability assays. The result showed that an increase in the concentration or duration of the extract treatment was effective in killing cancer cell.[7]

MATERIALS AND METHODS

Sample Collection

The herb was randomly collected in the Ghandhi Krushi Vignana Kendra (GKVK) Bangalore. The samples were allowed to dry at room temperature under a shade. The dry samples were then crushed in fine powder and stored in tightly sealed polyethylene bags.

Extraction procedure

Plant leaves were washed thoroughly with distilled Water. The leaves were dried at room temperature. The dried leaves of *L. Camara* were finely grinded using electrical grinder and stored in air tight containers for further use. A total of 250 g of the pulverized plant material was extracted for 4 d in Methanol, ethanol and sterile Water. Then it is filtered through Whatman's No.1 filter paper then the Methanol, Ethanol and Sterile water filtrate were separately condensed to dryness using rotary evaporator. The thick extracted mass was then dried at room temperature. Dried extract was collect.[8]

1. Phytochemical analysis

Phytochemical analysis of *Lantana Camara* linn seed extracts were done using the protocols described by ; Akumu Edwin, Anthoney Swamy, Mutuku Chrispus Ngule, Jackie K. Obey and Miyogo Edwin[9] for the following.

Test for Sterols - Liebermann Burchard reaction
Tests for Alkaloids - Mayer's and Wagner's test
Tests for Tannins - Ferric chloride reagent test
Tests for Saponins - Foam test.
Tests for Phenols - Ferric chloride reagent test
Test for cardiac glycosides- Salkowiski test.
Tests for Flavonoids
Test for Terpenoids

2. Nitric Oxide Radical scavenging assay

Principle

Sodium nitroprussidein aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be measured using Griess reagent at 546 nm spectro phtometrically.

Procedure

Nitric oxide scavenging assay is carried out as per the method of Sreejayan and Rao *et al*. In brief, 200 µl of 10mM 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 incubated for 10 mints. at room temperature. Measure the absorbance at 546 nm spectrophotometrically. Test substances are replaced by buffer solution for a control.

3. HPLC analysis of Quercetin

Plant Extraction

10gms plant powder was extracted with 50ml Methanol at 50^{o}C for 4 hours. The Methanol extracts were filtered through Whatmann No.1 filter paper and filtrate was evaporated to dryness. Methanol extract (10mg/ml) was used for HPLC analysis.

Quercetin Standard: 100ug/ml prepared in Methanol.

HPLC Condition

Instrument: Shimadzhu LC- Prominence 20AT
Column: C18 column 250 mm x 4.6 mm, 5u particle
Mobile Phase: Linear
A: HPLC grade Acetonitrile (60%)
B: HPLC grade Water (40%)
Flow Rate: 1.0 ml/min
Injection volume: 10ul
Quantification of Quercetin in plant extracts
Concentration of Standard injected: 100µg/ml
Sample concentration: 10mg/ml

Formula used for quantification of quercetin in plant extract

Quercetin (Microgram/gram) = Sample area / Standard area x Standard concentration injected x Standard concentration injected.

4. Cytotoxicity studies using HepG2 cell line by MTT assay

HepG2 cell line was obtained from American Type Culture Collection (ATCC) (Rockville, MD USA) (ATCC Number-HB 8065). The steps and procedure for cell culture, Thawing, Revival and Propagation of Cells were followed as described by D. F. Basri et al.[10]

Procedure

The collected cells should be at reach about 70-80% confluences.
Check the viability of the cells and centrifuge it.
Take about 50,000 cells / well and seed it in 96 well plates and incubate for 24 hrs at 37^{0} C, 5% CO_2 incubator.
Add plant samples which are to be tested from 0-320µg/ml (2 fold variation) concentration in RPMI without FBS & are incubated for 24 hr.
Add 100µl/well of the MTT (5 mg/10ml of MTT in 1X PBS) to incubated plant samples to the respective wells and incubated for 3to 4 hours.
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.

RESULTS AND DISCUSSION

1. Phytochemical analysis

Table.1: Phytochemical Analysis *Lantana Camara* linn. leaves extracts.

S.No	Tests	Observation	Inference
1	Froth formation test	Formation of stable froth was not observed in Acetone, Methanol and Water extract.	Saponins was absent in Acetone, Methanol and Water extract.
2	Mayer's and Wagner's test	Precipitate was formed in Acetone and Methanol extract but not in Water extract.	Alkaloid was present in Acetone and Methanol extract and absent in Water extract.
3	Ferric Chloride Test	Brownish green color was formed in Methanol extract but not in Acetone and Water extract.	Tannins was present in Methanol extract and Absent in Acetone and Water extract.
4	Salkowiski test	Red brown color was not formed in Acetone, Methanol and Water extract.	Glycosides was absent in Acetone, Methanol and Water extract.
5	Liebermann Burchard reaction	Formation of blue green ring in Methanol extract and not in Acetone and Water extract.	Steroid was present in Methanol extract and absent in Acetone and Water extract.
6	Test for flavonoids	Appearance of yellow color was observed in Methanol and Water extract and not in Acetone extract.	Flavonoid was present in Methanol and Water extracts and Absent in Acetone extract.
7	Test for terpenoids	Grayish color was not formed in Acetone, Methanol and Water extract.	Terpenoids was absent in Acetone, Methanol and Water extract.
8	Test for phenols	Bluish green color was not formed in Acetone, Methanol and Water extract.	Phenols was absent in Acetone, Methanol and Water extract.

From Table1 the quantitative analysis of *Lantana Camara* leaves was found to contain several compounds. The aqua extracts were found to contain only flavonoids. Acetone extracts were found to contain only alkaloids and Methanol extract extracts were found to contain tannins, flavonoids, alkaloids and steroids. Presence of these phytochemical compounds shows a great traditional importance in pharmacological field.

2. Nitric oxide scavenging assay

Table 2: Nitric oxide scavenging assay.

Plants Name	Concentration (µg/ml)	Absorbance 546nm	% Inhibition	IC_{50}
Control	0.0	0.5403	0.0	
Standard (Curcumin)	6	0.4992	7.61	
	12	0.4172	22.78	
	25	0.3128	42.11	
	50	0.1680	68.91	25.52
	100	0.1196	77.98	
	200	0.0380	92.97	
Lantana Camara	5	0.5150	4.68	
	10	0.4822	10.75	
	20	0.3740	30.78	
	40	0.2830	47.62	
	80	0.2389	55.78	22.13
	160		67.39	

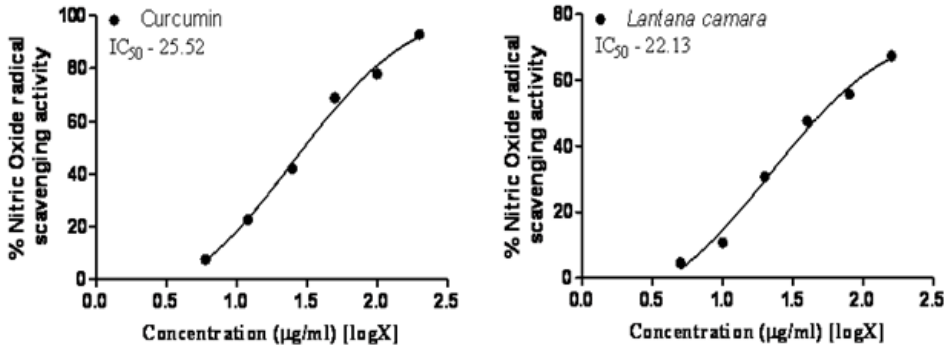

Figure 1: Nitric oxide scavenging assay of Curcumin and *Lantana Camara*.

From Table – 2 and Figure – 1, the results of NO scavenging activity of the selected plant extracts was shown as percentage of Nitric oxide or reactive nitrogen species, formed during their reaction with oxygen or with

superoxides, such as $N0_2$, N_2O_4, N_3O_4, NO^3- and NO^2 found to be very reactive. These ionic compounds are responsible for altering the structural and functional behavior of many cellular components. The Sodium nitroprusside solution is incubated in phosphate buffer saline at 250 C for 2 hours resulted in linear time-dependent nitrite production, which was reduced by the tested Methanol extracts of *Lantana Camara leaves*, This may be due to the antioxidant principles in the extract, which compete with oxygen to react with nitric oxide thereby inhibiting the generation of nitrite. *Lantana Camara* shows approximately equal when compare with standard curcumin. The maximum NO inhibition percentage and IC50 valve of standard curcumin and *Lantana Camara* was found to be 92.97%and 67.39%, 25.52 and 22.13 respectively.

3. HPLC analysis of Quercetin and *Lantana Camara.*

Table 3: HPLC analysis of Standard Quercetin.

S. No.	Retention. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	1.933	373.177	22.881	20.5	14.0	0.20
2	3.107	92.433	3.352	5.1	2.0	0.49
3	3.487	1296.195	133.916	71.3	81.6	0.14
4	4.207	55.054	3.869	3.0	2.4	0.22
	Total	1816.859	164.018	100.0	100.0	

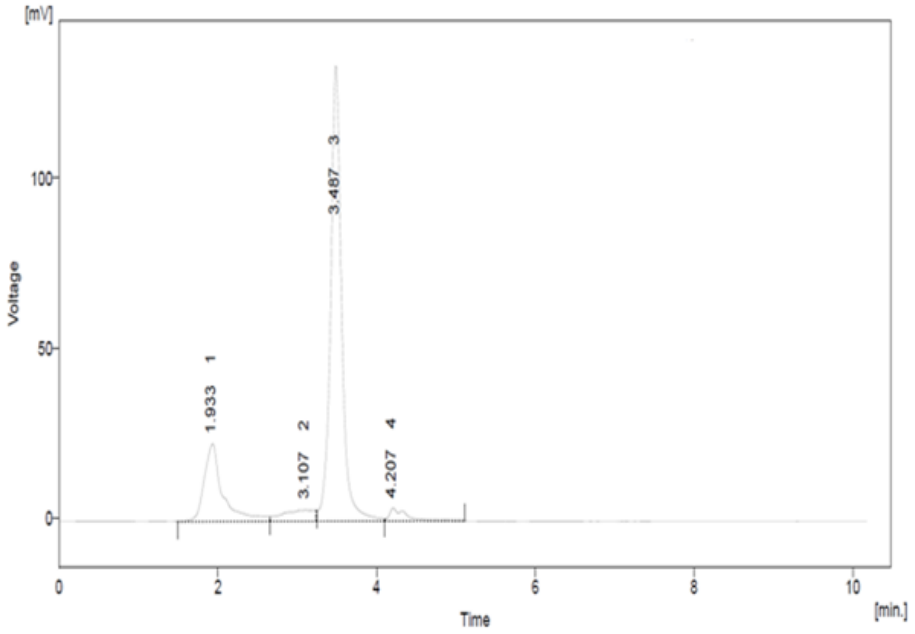

Figure 2: HPLC analysis of standard Quercetin.

From Table – 3 and Figure – 2, the flavonoids were quantified at 254nm using peak area by comparison to a calibration curve derived from the quercetin.

Table 4: HPLC analysis of Quercetin content in *Lantana Camara*.

S. No.	Retention. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	1.708	110.137	12.653	19.6	27.7	0.11
2	2.217	235.556	12.394	41.9	27.2	0.27
3	2.907	64.437	4.695	11.5	10.3	0.31
4	3.017	35.155	4.724	6.3	10.4	0.13
5	3.197	45.665	5.175	8.1	11.3	0.16
6	3.313	62.289	5.405	11.1	11.8	0.17
7	3.880	8.937	0.575	1.6	1.3	0.31
	Total	562.176	45.620	100.0	100.0	

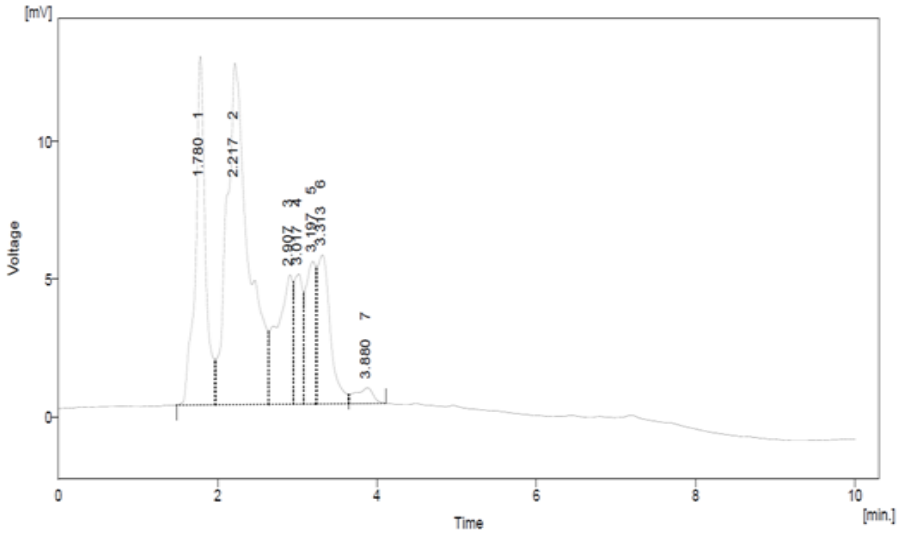

Figure.3: HPLC analysis of Quercetin content in *Lantana Camara.*

From Table – 4 and Figure – 3, the HPLC chromatograms from leaves and flowers of *L.Camara*, the main difference was in peak eluted at 3.4min. External flavonoid aglycones were already analysed using HPLC method in various plant extracts.

The peaks in this study shown marked decreased in peak area in case of *L.Camara* leaves when compared with standard quercetin.

From the calibration curve results, the amount of Quercetin, in the sample injected was calculated. *L. Camara* leaves contain quercetin less than 10%. Other peaks (#1) in both the HPLC chromatogram *L.Camara* leaves extracts indicated the presence of other chemical constituents (euginol, etc.). The present method was applicable for determining quercetin in any aerial part of plant material using HPLC technique.

4. Cytotoxicity studies using HepG2 cell line by MTT assay

Table 5: cytotoxic study of *Lantana Camara.*

Plants name	Conc. µg/ml	OD at 590 nm	% Inhibition	IC_{50}
Control		0.8167	0.00	
Lantana Camara	10	0.7479	8.42	42.35
	20	0.6512	20.26	
	40	0.5089	37.69	
	80	0.3377	58.65	
	160	0.2798	65.74	
	320	0.1588	80.56	

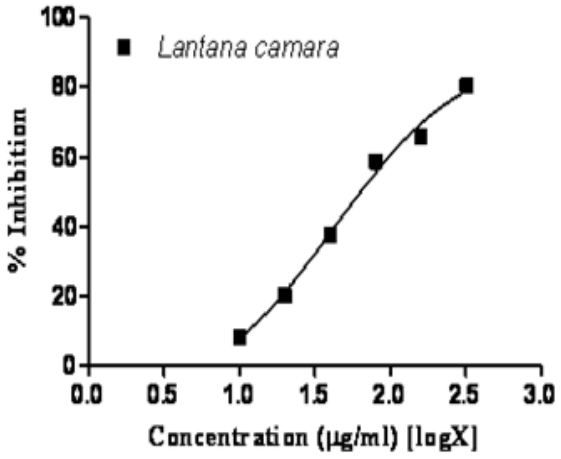

Figure 4: Cytotoxic study of *Lantana Camara.*

From Table – 5 and Figure – 4, *Lantana Camara* treatment extract showed significant dose-dependent inhibition of growth of HepG2 cells at IC_{50} values of 115.2µg/ml. It was found that there were cytotoxic effects with increasing concentration on HepG2 cell line from 10µg to 320µg concentration. Compared to the untreated Hep G2 cells, only 20% growth and cell proliferation is maintained even in the treated cells.

CONCLUSION

Our study in accordance with the above findings showed that *Lantana Camara* leaves has free radical scavenging activity. The maximum Nitric oxide (NO) inhibition percentage and IC50 valve of standard curcumin and *Lantana Camara* is 92.97%and 67.39%, 25.52 and 22.13 respectively. The MTT assay shows that there were cytotoxic effects with increasing concentration on HepG2 cell line from 10µg to 320µg concentration. Compared to the untreated HepG2 cells, only 20%

growth and cell proliferation is maintained even in the treated cells. The reported IC value is 42.35.

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