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Comparative analysis of the cytotoxicity of two weeds, *Ricinus communis* L. and *Lantana camara* L. on HeLa cell line

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Abstract

Cancer is a major health challenge with all treatment modalities face low success rate in bringing down the mortality of humans. Natural plant-based products have received much attention in the search for potential anticancer drugs. Though weeds are unwanted plants, many of them have been used traditionally as medicines. Hence, the current study aims to compare the anticancer activities of two weeds, *Lantana camara* L. and *Ricinus communis* L. leaf extracts against HeLa and HepG2 cancer cell lines. The methanol extracts of the leaves were tested for cytotoxicity on HeLa and HepG2 cells by MTT assay. The crude extracts were purified by Thin Layer Chromatography and the bands separated were also checked for their cytotoxicity to select the best fraction. The results revealed the promising anticancer traits of these weeds, and among the 2 weeds, *L. camara* exhibited higher cytotoxicity and lower IC_{50} value than *R. communis*, on the HeLa cell line. The findings highlight the significance of weeds towards therapeutic exploitations.

Key words : Anticancer, MTT, weeds, LDH activity, *Lantana camara.*

Cancer is a major public health burden in both developed and developing countries, with a high mortality rate despite the availability of modern treatment techniques. Natural products, especially plants, have been used for the treatment of various diseases for thousands of years. Medicinal plants have long served as important sources for anticancer drug discovery due to their cost-effectiveness, minimal side effects, and high therapeutic efficiency. In this context, weeds have also demonstrated significant ethnomedicinal and pharmacological value[5].

For example, the common weed *Achyranthes aspera* (Amaranthaceae), found in India, has therapeutic applications in bronchitis, rheumatism, rabies, skin diseases, and malaria[4]. The mimosine alkaloid extracted from *Mimosa pudica* (Mimosaceae) has been shown to exhibit apoptosis-inducing and anti-proliferative activities. In Ayurveda and Unani

systems of medicine, *Mimosa pudica* is used for treating leprosy, jaundice, smallpox, and ulcers[9].

Similarly, *Lantana camara* L., considered both a weed and a popular ornamental plant, is widely used in the traditional medicine of several Middle Eastern and Asian countries to treat skin allergies, wounds, and ulcers. Previous studies have reported its antifungal, anti-proliferative, and antimicrobial activities[13]. Another example is *Ricinus communis* L., a weed whose seeds yield castor oil, traditionally used as a medicine. In India, the plant's leaves are used as food for Eri silk worms, while its stalks are used as fuel[6].

Given the urgent need for novel compounds to treat liver and cervical cancers, the present study aims to investigate two common weeds, *L. camara* L. and *R. communis* L., and conduct a comparative analysis of their cytotoxic activities in vitro on HepG2 and HeLa cancer cell lines.

Collection of the plant material and preparation of the extract :

The weeds *R. communis* and *L. camara* were collected from various locations in and around Bengaluru, India. The leaves were dried under shade conditions, ground into a fine powder, and 50 grams of the powder was packed in Whatman filter paper. The extraction was carried out using dichloromethane as the solvent in a Soxhlet apparatus. The extract was then concentrated using a rotary evaporator, dried, and stored at 4°C until further use. A stock solution of 1 mg/mL was prepared using dimethyl sulfoxide (DMSO) and subsequently diluted to the desired concentrations with phosphate-buffered saline (PBS).

Cell lines :

HeLa and HepG2 cell lines were procured from National Centre for Cell Science (NCCS), Pune. The cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) and Minimum Essential Medium (MEM), respectively, supplemented with 10% fetal bovine serum (FBS).

Cytotoxicity Assay :

MTT [3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay :

MTT assay was performed to assess the cytotoxicity of the weed extracts. HeLa and HepG2 cells were cultured onto a 96 well microtiter plates for 24h using a multichannel pipette at an initial cell concentration of 1×10^4 cells/ml. Cells were treated with varying concentrations of the samples and incubated in the CO_2 incubator for 24, 48 and 72h. At the end of the treatment, to each well 20µl of MTT was added. The absorbance was read at 540nm using the ELISA reader[11].

Partial purification of crude extract by Thin Layer Chromatography (TLC) :

Thin layer chromatography (TLC) is a chromatographic technique used to separate compounds present in the mixture. TLC was carried out using pre-coated TLC plates Silica gel 60 F254 (Merck)[19]. The partially purified fractions obtained from preparative TLC were again checked for cytotoxicity by MTT assay.

Phytochemical screening of plant extracts and Active fractions :

A qualitative phytochemical analysis was carried out for the determination of the chemical groups of the active fraction using standard methodologies[8]. The assays were carried out with 0.5-1 mL of extract solutions. For testing carbohydrates, Fehling's and Benedict's tests were performed. Wagner's and Mayer's tests were for alkaloids, Salkowsky test was done for sterols, tests for the detection of phenolic compounds (test with neutral $FeCl_3$) and tannins, Biuret test for proteins, and also tests for the detection of saponins and flavonoids, were performed. Fehling's test: Equal volumes of Fehling's reagent A and B were added to 50µl of each samples and gently boiled it. It was observed for brick red precipitate to confirm the presence of reducing sugars.

Lactate Dehydrogenase (LDH) Activity :

Lactate dehydrogenase (LDH) is a soluble cytosolic enzyme that is released into the culture medium following loss of membrane integrity from either apoptosis or necrosis. HeLa and HepG2 cells were treated with L. camara and R. communis bioactive fractions for 48h, and the LDH assay was performed as per instructions provided in the kit (G Biosciences: kit #786-210).

LDH activity was calculated as

$$\frac{\text{Test } OD_{490} - \text{Blank } OD_{490}}{\text{Control } OD_{490}} \times 100$$

DNA Fragmentation Analysis :

DNA fragmentation analysis was performed using the mammalian genomic DNA mini preparation kit (Bangalore Genei), as per the instructions provided in their manual[3]. HeLa and HepG2 cells (2×10^4 cells /ml) were cultured in $25cm^2$ tissue culture flasks for 24h. Different concentrations of weed extracts were added and incubated again for 24 h. A control was maintained with only cells. Cells were washed with PBS, trypsinized, centrifuged and the cell pellet was lysed in buffer containing 10mM Tris HCl, 10mM EDTA, 0.5%Triton X 100. To avoid RNA and Protein contamination, 200µg/ml of RNAse and 200µg/ml proteinase K were added. DNA was precipitated with ice cold ethanol and suspended in Tris-EDTA solution. Samples were resolved using 0.8% agarose gel and visualized.

Statistical analysis :

All experiments were carried out in triplicates. The results were calculated as mean ± standard error (SE) Values. Statistical significance was calculated using one way analysis of variance (ANOVA) and Dunnett's multiple comparison test. The values < 0.05 were taken as significant.

Viability of Cancer Cells as per MTT Assay:

When the methanol extract of *R. communis* and *L. camara* were treated to Hela cells at different concentrations (1, 10, 50 and 100 µg/ml) for 48 hours, the percentage viability of treated cells decreased at all the tested concentrations (Figure 1A). The highest inhibition was observed (33%) for 10µg/ml concentration for the crude extract of *Ricinus communis* and the highest inhibition (30%) was observed for 100µg/ml concentration for *Lantana camara* on HeLa cell line.

When the methanol extract of *Ricinus communis* and *Lantana camara* were treated to HepG2 cells at different concentrations (1, 10, 50 and 100 µg/ml) for 48 hours, the percentage viability of treated cells decreased at all the tested concentrations (Figure 1B). The highest inhibition of 21% was observed for 100µg/ml concentration after 48h for the crude extract of *Ricinus communis* and the highest inhibition of 32% was observed for 100µg/ml concentration for the crude extract of *Lantana camara* on HepG2 cell line.

Partial purification of Crude extracts by Thin Layer Chromatography :

The extract from *Ricinus communis* and *Lantana camara* were subjected to partial purification by thin layer chromatography (TLC). Fractionation of the crude extract of *R. communis* was performed using toluene: ethyl acetate: formic acid: chloroform as the solvent which revealed the presence of 6 major fractions (Figure 1C). The fractionation of crude extract of *L. camara* was carried out using ethyl acetate as the solvent which revealed the presence of 6 major fractions (Figure 1D). The Rf values of all the fractions from both the extracts were calculated and presented in the (Table-1).

Effects of Ricinus and Lantana TLC purified fractions on HeLa *and* HepG2 *cells:*

Effect of *Ricinus* and *Lantana* TLC purified fractions on HeLa and HepG2 cell lines were checked for a treatment period of 48h at 1µg/ml, 10µg/ml and 25µg/ ml concentrations. The effect of all fractions of *R. communis* were checked on both HeLa and HepG2 cell lines. The 3rd fraction exhibited the highest inhibition (50%) among all the fractions, for the Hela cell line (Figure 2A), which was significant ($p< 0.001$). The IC_{50} value for the fraction 3 was found to be 4.88µg/ml on HeLa cell line.

The results on HeLa cell lines were good compared to HepG2. In HepG2, the percentage viability was more than 100% at all the tested concentrations (Figure 2B). The two-way ANOVA for TLC purified fraction 3 on HepG2 cell line was found to be not significant for 48h and hence further studies were carried out on the HeLa cell line.

The effect of all the fractions of *L. camara* were checked on both HeLa and HepG2 cell lines. The 6th fraction showed the highest inhibition among all the bands, on the Hela cell line, which was significant ($p< 0.001$). The IC_{50} value for the fraction 6 was found to be 3.49µg/ml on HeLa cell line.

The results on HeLa cell lines were good compared to HepG2. In HepG2, all the values were more than 100%. The two-way ANOVA for TLC purified fraction 6 on HepG2 cell line was found to be non-significant for 24h ,48h and 72h. The IC_{50} value for the fraction 6 was found to be 12.95µg/ml on HepG2 cell line. So further studies were carried out on HeLa cell line.

Phytochemical screening :

The phytochemical screening of the *R. communis* crude extract and fraction 3 (R3) indicated the presence of alkaloids, glycoside, flavonoids and steroids and the *Lantana* crude extract and the fraction 6 (L6) indicated the

presence of alkaloids, glycoside, flavonoids, steroids, terpenoids (Table-2). These fractions represent partial purification with several phytochemicals being present and they require further purification to isolate a pure compound.

Cytotoxicity Assay for Partially Purified Bioactive Fractions :
Effect of Fraction 3 from R. communis :

When treated with fraction 3 of *Ricinus* for 24 hours, the percentage viability of HeLa cells decreased from 85.13 at 1µg/ml to 83.76 at 25µg/ml (Figure 3A). The viability of the cells further decreased when the treatment period was increased to 48 hours. It was 77.06, 50.45, and 53.71% at concentrations of 1.0, 10.0, 25.0 µg/ml respectively, when treated for 48 hours. Higher effects were observed at 10µg/ml rather than at 25µg/ml treatment concentration. Here we could see a progressive time and dose dependent anti-proliferative effects of the fraction on HeLa cells. The IC_{50} value from the dose-response curve was found to be 4.8µg/ml for this fraction, which is within the range specified by FDA for plant based active compounds for cancer treatment to be taken up for purification, characterization and clinical studies.

Effects of Fraction 6 from L. camara :

The treatment decreased the percentage viability of HeLa cells from 103.72 at 1µg/ml to 85.57 at 25µg/ml when treated with fraction 6 of *Lantana* for 24 hours (Figure 3B). The viability of the cells further decreased when the treatment period was increased to 48 hours. It was 64.74, 52.82, and 54.31% at concentrations of 1.0, 10.0, 25.0 µg/ml respectively, when treated for 48 hours. Highest inhibitory effect was seen at 10µg/ml rather than at 25µg/ml treatment concentration. Here also we could see a progressive time and dose dependent anti-proliferative effects of the fraction on HeLa cells. The IC_{50} value from the dose-response curve was found to be 3.9µg/ml for this fraction, which is well within the range specified by FDA for plant based active compounds for cancer treatment to be taken up for purification, characterization and clinical studies.

Table-1. Rf values of the TLC separated fractions of *R. communis* & *L. camara*

FRACTIONS	Rf values	
	R. communis	*L. camara*
Band 1	0.35	0.38
Band 2	0.43	0.52
Band 3	0.53	0.67
Band 4	0.76	0.84
Band 5	0.78	0.87
Band 6	0.81	0.96

LDH Activity :

Release of LDH in to the cytoplasm is the consequence of damage that occurs due to the apoptotic cell death or due to the cytotoxicity of the test compound. In cancer drug development and research, the activity of a test compound can be determined by LDH release assay in the treated cancer cells, as it is regarded as a simple, reliable and quick technique to estimate the cell death in cancer cells. In the current study, LDH assay was performed to measure the Lactate dehydrogenase released into the media from damaged cells, which is a biomarker for cellular cytotoxicity. LDH activity was 74.2% in *Lantana* fraction treated cells and it was 70.9% in *Ricinus*

Table-2. Phytochemical screening of the plant extract and active fractions

TESTS	*R. communis*		*L. camara*	
	CRUDE EXTRACT	FRACTION 3	CRUDE EXTRACT	FRACTION 6
Alkaloids	+	+	+	+
Saponins	+	-	-	-
Glycosides	+	+	+	+
Flavonoids	+	+	+	+
Carbohydrates	-	-	-	-
Steroids	+	+	+	+
Terpenoids	+	-	+	+

Table-3. Cytotoxicity (%) of *R. communis* and *L. camara* fractions through LDH assay

Sample	Percentage cytotoxicity (%)
Blank	0
Control	100
Fraction 3 (*R. communis*)	70.9
Fraction 6 (*L. camara*)	74.9

fraction treated cells as compared to the control cells (Table-3). The activity of LDH present in the supernatant of the treated cells were clearly higher than the control sample, demonstrating the cytotoxic effect of the samples on HeLa cells.

Observation of cell morphology under the Inverted Microscope :

Several morphological changes that occur in cells due to natural or induced apoptosis can be generally detected by routine observation under the inverted microscope. Hence, in the current study, we attempted to observe the morphological changes in the cells treated with the bioactive fractions. It was observed that the control HeLa cells were firmly attached to the flask, were fibroblastic in appearance and were found in higher concentrations than the treated cells (Figure 4A). That HeLa cells, after treatment with fraction 3 of *Ricinus* and fraction 6 from *Lantana* for 24 hours, were found to shrink and detached from the surface, became rounded and formed several apoptotic bodies (Figures 4 B & C).

DNA fragmentation analysis :

DNA fragmentation is considered one of the hallmarks of apoptosis, occurring due to the significant biochemical changes. DNA previously isolated from cells treated with 10µg/ml of bioactive fraction for 24h were loaded in the wells of a 0.8% agarose gel, electrophoresed, and visualized under UV transilluminator. The gel showed a smear of DNA in *Lantana* fraction 6 treatment compared to the intact DNA of the control untreated cells (Figure 4D). Appearance of fragmented DNA of the extract treated cells on agarose gel as compared to the distinct band of the control

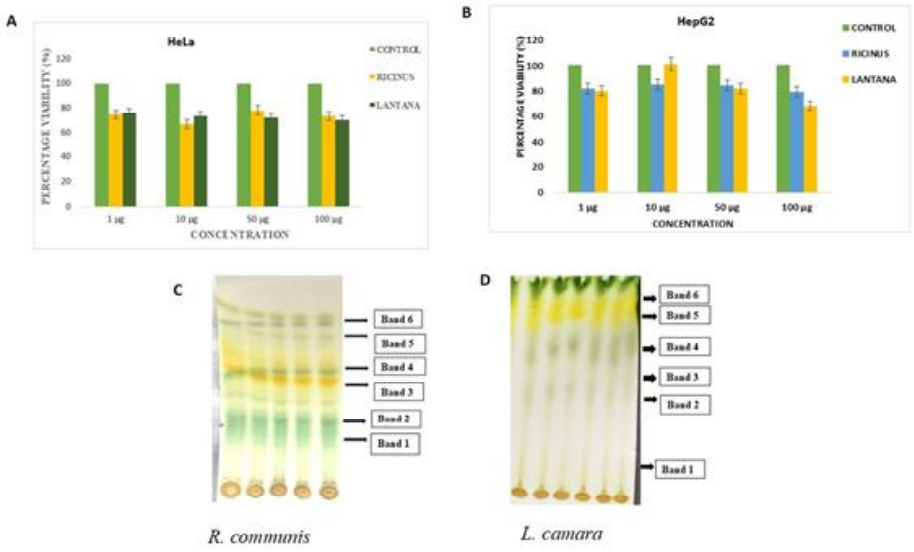

Figure 1: A) Effect of methanol extract of *R. communis* and *L. camara* on HeLa cells; **B)** Effect of methanol extract of *R. communis* and *L. camara* on HepG2 cells; **C)** Thin layer chromatography fractionation (TLC) of *R. communis;* **D)** TLC of *L. camara* showing the different bands separated under the visible light.

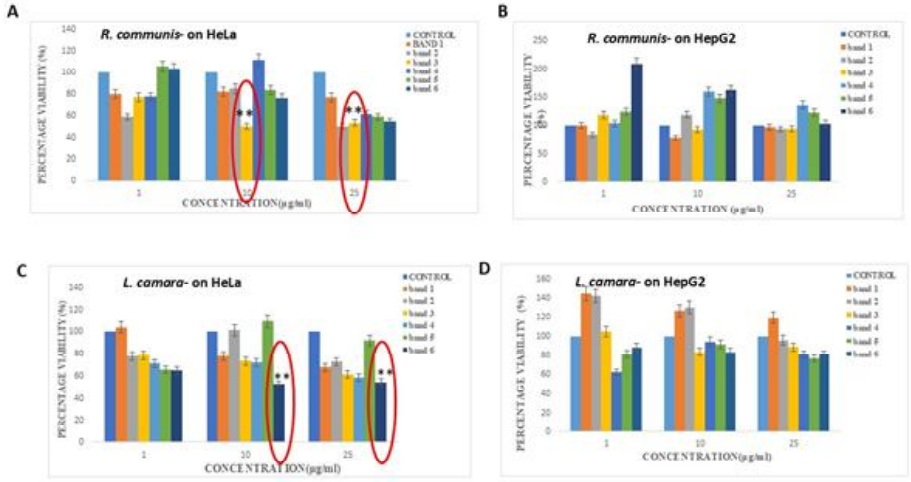

Figure 2: A) Percentage viability of HeLa cell line treated with TLC purified fractions from *R. communis* for 48 hours. B) Percentage viability of HepG2 cell line treated with TLC purified fractions from *R. communis* for 48 hours. C) Percentage viability of HeLa cell line treated with TLC purified fractions from *L. camara* for 48 hours. D) Percentage viability of HepG2 cell line treated with TLC purified fractions from *L. camara* for 48 hours. **denotes 0.001 level of significance. The red circle indicates fraction 3 from *R. communis* and fraction 6 from *L. camara* having highest cytotoxic effects to HeLa and HepG2 cells.

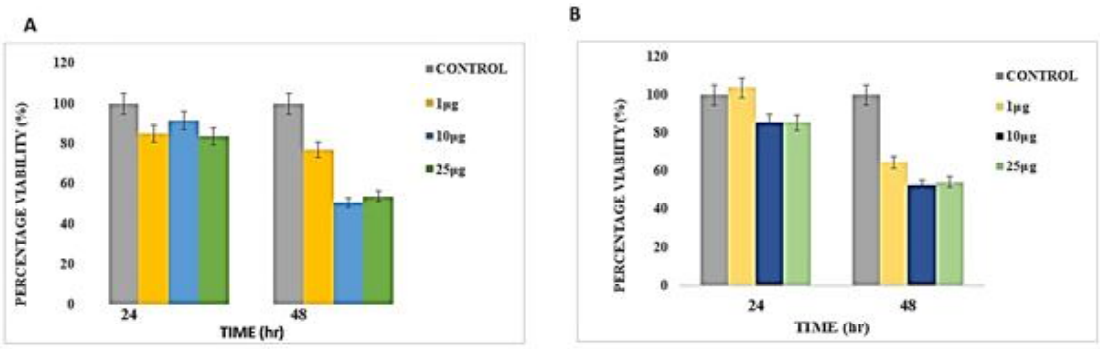

Figure 3: A) Percentage viability of HeLa cell line treated with TLC purified **fraction 3** from *R. communis* for 24 and 48h. B) Percentage viability of HeLa cell line treated with TLC purified **fraction 6** from *L. camara* 24 and 48h.

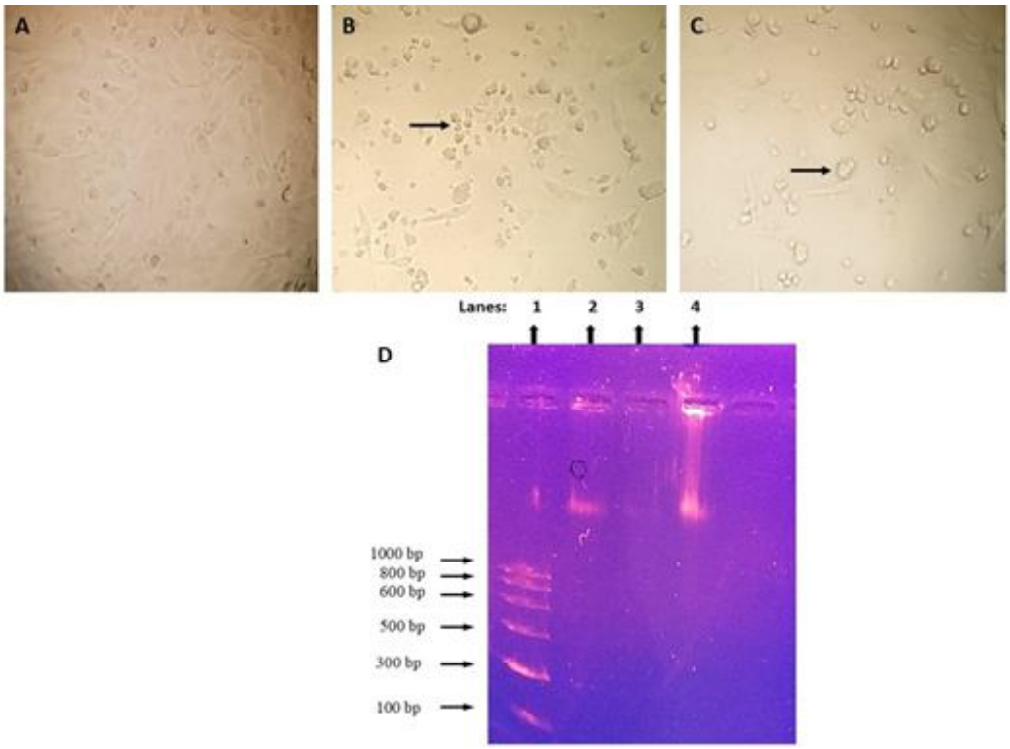

Figure 4: Upper Panel: Morphology of HeLa cells under the inverted Microscope & Lower panel: DNA fragmentation analysis. **A)** Control HeLa; **B)** HeLa cells treated with fraction 3 of *R. communis*; **C)** HeLa cells treated with fraction 6 of *L. camara*. Arrow indicates cells undergoing apoptosis. **D)** Analysis of DNA on 0.8% Agarose gel. Lanes: 1) Ladder DNA, 2) Control HeLa cell DNA 3) DNA from *R. communis* treated HeLa, 4) DNA from *L. camara* treated HeLa.

cell is clear evidence for the induction of apoptosis. The treatment with *Ricinus* fraction 3 did not show any clear indication of DNA fragmentation.

Cancer remains as the one of the leading causes of death of millions of people all over the world despite the advancement in modern techniques for diagnosis and treatment. Utilization of medicinal plants as sources for drug discovery leads a prime position in the fields of cancer and other infectious diseases. Among the FDA approved drugs for cancer and infectious diseases 60% and 75% respectively are from natural sources[12]. Among plants, weeds are important because they are either harmful (nuisance) or beneficial as many of them have pharmacodynamic importance[17]. There are many weeds having ethnomedicinal and pharmacological value. A number of weeds, such as the dandelion (*Taraxacum officinale*) are edible, and their leaves and roots may be used for food or herbal medicine. *Centella asiatica* (L) Urban (Apiaceae), locally known as "ondelaga" (in Kannada). Plant juice is considered as refrigerant to the body, when given orally. *Rubia cordifolia* L. (Rubiaceae), locally known as "Sappli Koth". Decoction of stem is orally administered as a restorative tonic. Root juice is given orally to cure jaundice[5]. *Argemone mexicana L. (Papavaraceae),* has antimicrobial, antidiabetic, atiarthritic and wound healing[16]. The badaga community in Nilgiris uses the flower decoction externally to treat eye infections[2,18].

Leucas aspera (Willd.) Link. (Lamiaceae), is commonly known as common leucas and locally known as thumbaigidu. It has antifungal, antioxidant, antimicrobial, antinociceptive and cytotoxic activities[15]. In the current study, we wanted to do a comparative analysis of the cytotoxicities of two weeds *Ricinus communis* and *Lantana camara* very commonly found in Bangalore.

We collected *R. communis* and *L. camara* from vacant sites in Bangalore, dried their leaves and extracted the active components using the solvent methanol. When we screened our extracts for cytotoxicity on HeLa cells and HepG2 cells, we found both the plant materials exhibited anticancer potentials. Both the extract has shown significant cytotoxic and apoptotic potentials on cultured HeLa cells than the HepG2 cells. For the *Ricinus* the highest inhibition was observed at 100µg/ml concentration for 48h and for the *Lantana sp.* as the concentration of the extract increased from 1 to 10 to 50 and to 100µg/ml the extract inhibited the proliferation in a dose dependent manner (from 76.21% to 73.63% to 72.12% and 70.29%) for the HeLa cell line.

For HepG2 cell line the highest inhibition was observed at 100µg/ml concentration of *Lantana* extract, when the concentration of the extract increased from 1 to 50 and 100µg/ml the extract inhibited the proliferation in a dose dependent manner *i.e.*, from 80.08 to 81.61 and to 68.64% respectively.

When the crude extracts were fractionated by TLC, 6 fractions were separated from both the plant extracts. Among these, the 6th fraction from *Lantana* and 3rd fraction from *Ricinus* were found to have highest inhibitory activity against HeLa cell line, where as their effects were not that significant on HepG2 cell line. The HeLa cell line was found to be more

sensitive to the treatment as compared to the liver carcinoma cell line HepG2. Hence, further invitro assays were performed on HeLa cell line.

Fraction 6 from *L. camara* exhibited antiproliferative effects on HeLa cell line where higher inhibitions were observed at increasing concentrations and after 48h of treatment than 24h. The highest inhibition (56.82%viability) was seen after 48h of treatment with 10µg/ml of fraction 6. the IC_{50} being 3.50µg/ml. Fraction 3 from *Ricinus* also had similar effects on HeLa cell line with highest inhibition at 10µg/ml concentration (50%viability) after 48h. The $IC_{50\ value}$ of fraction 3 of *Ricinus* was 4.9µg/ml Among these two, *Lantana* fraction was having higher toxicity to the cervical cancer cell line HeLa with a lower IC_{50} value. There are reports about the use of magnetic nanoparticles (MNPs) to isolate positively charged alkaloids from the methanolic extract of *L. camara* leaves, where the crude alkaloid was fractionated using HPLC to separate the highest peak of the alkaloid fraction (HPAF)[1] (Al-Hakeim *et al*., 2021). The alkaloid fraction exhibited a profound anticancer effect against MCF-7 and HeLa cell lines (with IC_{50} = 0.027 µg / mL and 5.90 µg/mL, respectively, which displayed mild cytotoxicity against the HCT-116 cell line (IC_{50} = 8.38 µg / mL). The CA also demonstrated a significant anticancer effect against MCF-7 and HeLa and a weak cytotoxic effect against colon cancer HCT-116 cells.

LDH cytotoxicity assay also were supportive of the above observations where higher cytotoxicity was exhibited by *Lantana* fraction 6 (74.2%) than *Ricinus* fraction 3 (70.9%) on HeLa cell line. Even the DNA fragmentation analysis indicated that *Lantana* fraction was more effective in inducing apoptosis in the treated HeLa cell line as compared to the *Ricinus* fraction, where only in *Lantana* treatment, we could observe a DNA smear in the agarose gel.

Our results are suggesting that some plants which are considered as a waste and of no use are also having pharmacological significance as it was reported in certain reviews[5].

As per literature, there are few reports about the anticancer activity of the crude extracts of *L. camara* and *R. communis*[1,7,10,14]. They did not attempt to purify and characterize the active components. The value of IC_{50} reported for Vero cell line after treatment with *L. camara* leaf extract and Triton X 100 were 361.44 ± 10.68 µg/ml for 24h and 319.37 ± 99.80 µg/ml for 72h (Pour *et.al.,* 2011). When compared to these studies, the results from our plant extracts of *R. communis* and *L. camara,* were promising with low IC_{50} values on the cervical cancer cell line HeLa (4.9 µg/ml for *Ricinus* and 3.5 µg/ml for *Lantana*). Hence, these can be explored further for characterization studies.

It can be concluded that the two common weeds *Lantana* and *Ricinus* have demonstrated therapeutic potentials towards the disease cancer. Among the two *L. camara* was more potent than *R. communis* in suppressing the proliferation of HeLa cells.

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