"ISOLATION ESTIMATION AND STUDY OF IN-VITRO ANTICANCER ACTIVITY OF D-LIMONENE FROM *MURRAYA KOENIGII*"

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Abstract:

The main aim of the study was to screen the D-limonene isolated from extract of *Murraya koenigii* leaves and their for their *in vitro* anticancer activity against HEP G2 and COLO205 human colon cancer cell lines. D-limonene is plant extract with widespread application, and it has been reported to have ant proliferative effect on cancer cells . However the mechanism by which D-limonene achieves these effects, especially in liver cancer ,are clear. therefore ,the goals of this study was to examine the effect of D-limonene on liver cancer and explore its mechanism of action.

Key words: D-limonene, extraction, separation, confirmation, in-vitro anticancer activity

1. INTRODUCTION:

Murraya koenigii, usually known as *curry* leaves or *karipatta*in India from Family "Rutaceae" which characterize 150 genus and 1600 type. *Murraya koenigii* Spreng- is called as "Surabhinimba" in Sanskrit and Curry leaves is call by various names in the different cultural, In Tamil we called as Karivempu, in Bengali as 'Barsung' in Hindi known as Kurrypatte. Among 14 worldwide class belong to the species of *Murraya*, only *Murraya koenigii (L)* Spreng and *Murraya paniculata* (Linn) is found in India· *Murraya koenigii* is a important plant for its quality fragrance and therapeutic value. It is an important sell abroad product from India as it fetches good overseas income. A amount of chemical constituents from each fraction of the plant have been extracted. The most essential chemical constituents dependable for its strong characteristic odor are P- gurjunene, P-caryophyllene, P-element as well as O-phellandrene. The plant is poor source for carbazoles alkaloids, bioactive coumarone, acridine alkaloids and carbazoles alkaloids. The Murraya genus also used in conventional medication in eastern Asia. Previous studies on the Murraya species contain information of coumarins, terpenoids and many study on carbazoles alkaloids.**(1)**

The curry leaves are used as constituent in Ayurvedic medicine. Their properties contain much importance as an anti-diabetic and hepatoprotectant. Study on carbazoles extracted from *curry* leaves have antioxidant and antimicrobial activity**.** **(2)**

1.1.Review of the plant:

Table no 1 Review of plant-

BOTANICAL NAME.	*Murraya koenigii* (L.) Spreng
FAMILY-	Rutaceae
Vernacular names:	
Karnataka-	karibevu
English-	Curry leaf
Tamilnadu	Karivempu
Hindi-	curry patta
Sanskrit	Mahanimb
Gujarat	Kadhilimbdo

Fig.1. *Murraya koenigii* leaves

1.1.1 Morphology:

Flowers are white, funnel-shaped, strong fragrant, total, bracteate, usual, actinomorphic, hypogynous, the usual length of a fully opened flower is 1.12 cm; inflorescence, a fatal cyme, each contains 60 to 90 flowers; calyx, 5-lobed, persistent, lesser, green; white, polypetalous, lower, with 5 petals,; length, 5 mm; androecium, polyandrous, lesser, with 10 stamens, dorsifixed, set into circles of five each; lesser stamens, 4 mm. long while the longer ones, 5 to 6 mm; gynoecium, 5 to 6 mm long; stigma, bright, sticky; style, short; ovary, superior. Fruits, around to rectangle, 1.4 to 1.6 cm long, 1 to 1.2 cm in width; weight, 880 mg; volume, 895 microlitres; fully ripe fruits, black with a very shining surface; pulp, the number of fruits per group changeable from 32 to 80. Seed, one in each fruit, 11 mm long, 8 mm in diameter, colour spinach green 0960/3; weight, 445 mg; capacity, 460 microlitres.

1.1.2 Phytoconstituents:

1.1.3 Aroma Constituents.

The aroma components consist of β-caryophyllene, β-gurjunene, β -trans-ocimene, β-thujene a-sabinene, β-bisabolene, furthermore D- limonene, β-element, β -phellandrene and β -cadinene.

1.1.4 Essential Oil Components.

The essential constituents of curry leaf are: monoterpene including β-phellandrene, a-pinene, β-pinene d-limonene. In some species sesquiterpenes is then main constituents.

1.1.5 Medicinal Uses.

The flavor is supposed to have a lot of medicinal properties. It is used in the conventional medicinal system for improving the digestive system, skin situation and treatment for diabetes.

1.2 D-Limonene:

It is terpenes in nature. It is a main ingredient in some citrus oils. D-limonene is scheduled in the Code of Federal Regulations as generally recognized as safe (GRAS) for a flavoring agent and can be found in general food items like fruit juices, soft drinks, baked goods, ice cream. D-limonene is considered to have quite less toxicity. It is tested for carcinogenicity in mice and rats. Although primary results showed d-limonene increased the occurrence of renal tubular tumor in male rats, female rats and mice in both genders showed no substantiation of any tumor. consequent studies have determined how these tumors happen and recognized that d-limonene does not create a mutagenic, carcinogenic, or nephrotoxic hazard to humans. In humans, d-limonene has established little toxicity after single and repetitive dosing for up to one year. Being an outstanding solvent of cholesterol, d-limonene is used clinically to melt cholesterol-containing gallstones. Because of its gastric acid neutralizing effect and its hold up of normal peristalsis, it has also been used for

release of heartburn. D-limonene has well-established chemo defensive action against many types of cancers.. [3]

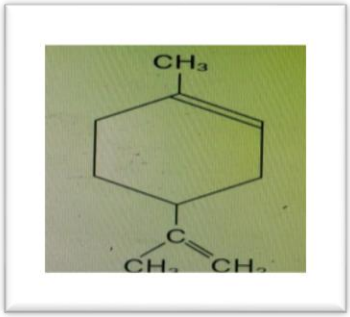

Fig.2. Structure of D-limonene

Molecular Formula: $C_{10}H_{16}$

Molecular Weight:136 g mol^{-1}

Chemical Name :(1-methyl-4-(1-methylethenyl) cyclohexane)

Boiling Point:176ºC

Solubility: N-hexane,

Therapeutic Category: Monoterpene

D-limonene (1-methyl-4-(1-methylethenyl) cyclohexane) is a monocyclic monoterpene with a lemon-like odor and is a main ingredient in several citrus oils. Because of its citrus smell, it is widely used as a taste and smell preservative in perfumes, soaps, foods, chewing gum, and beverages. It is programmed in the Code of Federal Regulation as generally recognized as safe (GRAS) for a flavoring agent. The usual concentration of d-limonene in orange juice, ice cream, candy, and chewing gum is 100 ppm, 68 ppm, 49 ppm, and 2,300 ppm, correspondingly. nutritional ingestion of d-limonene varies depending on the types of foods consumed.

1.3. CANCER:

Cancer can be defined as a disease in which a group of abnormal cells grow uncontrollably by disregarding the normal rules of cell division. Normal cells are constantly subject to signals that dictate whether the cell should divide, differentiate into another cell or die. Cancer cells develop a degree of autonomy from these signals, resulting in uncontrolled growth and proliferation. If this proliferation is allowed to continue and spread, it can be fatal. In fact, almost 90% of cancer-related deaths are duetotumor spreading – a process called metastasis.

The foundation of modern cancer biology rests on a simple principle – virtually all mammalian cells share similar molecular networks that control cell proliferation, differentiation and cell death. The prevailing theory, which underpins research into the genesis and treatment of cancer, is that normal cells are transformed into cancers as result of changes in these networks at the molecular, biochemical and cellular level, and each cell there is a finite number of ways this disruption can occur. Phenomenal advances in cancer research in the past 50 years have given us an insight into how cancer cells develop this autonomy. We now define cancer as a disease that involves changes or mutations in the cell genome. These changes (DNA mutations) produce proteins that disrupt the delicate cellular balance between cell division and quiescence, resulting in cells that keep dividing to form cancers. [4,5]

Cancer is a growing public problem whose estimated worldwide new incidences about 6 million cases per year. It is the second major cause of deaths after cardiovascular diseases. It is a disease characterized by unregulated proliferation of cells. The search for natural products as potential anticancer agents dates back, at least, to the *Ebers papyrus* in 1550 BC, but the scientific period of this search is much

more recent, beginning with the investigations by Hartwell and co-workers in late 1960s onapplication of podophyllotoxin and its derivatives as anticancer agents.

Plant, marine, and microbial have been tested as leads, and many compounds have survived the potential leads. Over the years, a number of approaches developed for clinical use and a number of anticancer drugs have come out of these as a result. The main problem with these agents is the toxicity associated with them due to their lack of specificity, as these agents also kill healthy cells. Nowadays, combination therapy is used to combat this problem, which seems to be a temporary one. But this approach threatens the possibility of the progress of drug resistance. Still a good number of anticancer agents developed from plants or their resulting agents, progress of a safe, economic and site-specific anticancer drug are still a challenge. [6]

1.3.1 LIVER CANCER:

Liver cancer is cancer that grown in the cells of liver. live is football-sized organ which is in the upper right portion of abdomen, beneath you diaphragm and above stomach. various types of cancer can form in liver .the most common type of liver cancer is hepatocellular carcinoma, which occur in the main type of liver cell (hepatocyte).other types of liver cancer, such as intrahepatic cholangiocarcinoma an hepatoblastoma, are less common. Cancer that spreads to the liver is more common than cancer that occurs in the liver cell. It is begins in another area of the body-such as the colon, lung or breast-and spreads to the liver is called metastatic cancer somewhat than liver cancer. This type of cancer is named after the organ in which it begins –such as metastatic colon cancer to explain cancer that begin in the colon and spread to the liver.

1.3.2 Types –

Hepatocellular carcinoma

1.3.3 Symptoms –

Losing weight without tryng, loss of appetite, upper abdominal pain, nausea and vomiting, abdominal swelling, white, chalky stools

1.3.4 Causes:

It happens when liver cells develop changes in their DNA. Cell's DNA is the fabric that provides directions for every chemical process in your body. Once result is that cells may start to grow out of control and finally form a tumor-a mass of cancerous cell**.** [6]

MATERIAL AND METHODS:

2.1. Collection and authentication of plant: The leaves of plant *Murraya koenigii* (L) Spreng were collected from the local area of miraj. The plant was authenticated by Dr. M. D. Wadmare, Head Department of Botany, Kasturibai Walchand college, Sangli.

2.2. Extraction:

1. **Soxhlet extraction:** The material to be extracted in powdered form was placed in a thimble and then inserted into the extractor.20g of powder of *Murraya koenigii* (L) Spreng was placed in a thimble. Then 250 ml of n hexane was added to it. The extraction was carried out for 48 hrs.

2. **Microwave assisted extraction:** The application of microwave heating for the isolation of essential oils from plant material has generated interest. An advantage of this technique is the reduction of extraction time and reduced use of organic solvent. For MAE, the dried leaves was crushed and screened through 24 mesh sieve. 20 gm of *Murraya koenigii* (L) Spreng was transferred to a 500 ml conical flask. 100 ml of n hexane was added. [7]

- The mixture was shaken well and kept for some time so that the drug absorbs the solvent.
- In this way the bumping of solvent was avoided and extraction was better when the flask kept in microwave oven and treated for microwave process.

- Extraction temperature was set at 20 min and irradiation power set at 480 W.
- The samples were extracted in triplicates.
- The final extract was weighed and the percentage of yield content was calculated using formula:

2.2.1 Phytochemical investigation:[8]

Various phytochemical tests such as alkaloids, carbohydrates, glycosides, saponins, phytosterols, phenols, tannins, flavonoids, diterpenes, proteins and amino acids was performed using leaf extract of *Murraya koenigii*. The extract was subjected to preliminary qualitative phytochemical screening for the determination of various primary and secondary metabolites

Table no.2 Phytochemical tests for *Murraya koenigii* leaves extracts

Test	Procedure	Inference
1. Tests for Alkaloids:		
a) Mayer's test	Test solution + Mayer's reagent (Potassium mercuric iodide solution).	Cream colour precipitate
b) Dragendroffstest	Test solution + Dragendroffs test (Potassium bismuth iodide solution).	Reddish brown precipitate
c) Wagner's test	Test solution + Wagner's test (Iodine potassium iodide solution)	Reddish brown precipitate
d) Hager's test	Test solution + Hager's test (Saturated solution of picric acid),	Yellow precipitate.
2. Tests for Glycosides:		
a) Borntrager'sTest	Test solution + 1 ml of sulphuric acid, boil for 5 mins. Filter while hot. Cool the filtrate and shake with equal volume of chloroform. Separate the lower layer of chloroform and shake with half of its volume of dilute ammonia.	Red colour appears.
b) Baljet's test	Test solution + picric acid.	Orange colour is formed.
3.Test for Flavonoids:		
a) Shinoda Test	Test solution + Magnesium turnings + conc. Hydrochloric acid. Pink, scarlet,	Crimson red colour appears.
b) Zinc – HCL reduction Test	Test solution + zinc dust + conc. Hydrochloric acid.	Red colour appears after few minutes.
4. Test for Steroids:		
a) Liebermann-Burchard Test	Test solution + Acetic anhydride, boil and cool. + Conc. Sulphuric acid, from the side of the test tube.	Brown ring forms at junction. If upper layer turns green it shows presence of steroids.
b) Salkowaski Reaction	Test solution + conc. Sulphuric acid.	Red colour at lower layer indicates presence of steroids.
5. Test for Saponins:		
a) Foam test:	Test solution + water, shake well.	Stable foam is formed
6.Test for Phenolic Compounds and tannins		
a) Ferric chloride test.	Test solution + ferric chloride solution.	Blue or green colour
b) Gelatin test	Test solution + 1% gelatin solution	Precipitate is formed

	containing 10% sodium chloride.	
c) Lead acetate test	Test solution + Lead acetate solution	White precipitate indicates presence of tannins and phenols
d) Bromine water	Test solution + Bromine water	Decolouration of bromine water
e)Dilute iodine solution	Test solution + Dil.iodine solution	Transient red colour
f)Acetic acid solution	Test solution +Acetic acid	Red colour.
g)Dilute HNO_3	Test solution +Dil.HNO_3	Reddish to yellow colour
7. Test for Proteins		
a) Millon's test	Test solution + Millon's reagent, heat.	Reddish brown color
8. Test for Terpenoids	Test solution +2ml of chloroform +5ml of Concsulphuric acid was carefully added to form A layer and observed.	Reddish brown color

2.2.2 Isolation of d-limonene from the extract:

Column chromatography:

In column chromatography, a vertical glass column was used in which the mobile phase, n-hexane: ethyl acetate (8:2) was added. The stationary phase, a solid adsorbent i.e. crystalline silica gel 100-200, was added in the column with continuous tapping on the column, so as to prevent air gaps in it. The silica gel was added such that some solvent was remaining on it i.e. below the 1 cm from the solvent meniscus. Then solvent system was added till the neck of the column, and was packed properly and kept for saturation.

Fig 3. - Isolation of D-limonene form extract of *Murraya koenigii* by column chromatography

Then the solvent in the column was eluted up to level of silica gel and to the top of it concentrated extract was added, the solvent was eluted so that extract was adsorbed properly on silica. The solvent system was added with the help of glass rod so that the column was not disturbed. The solvent was eluted drop wise, such that 4-6 drops per minute so that better resolution was obtained. 1st two bands obtained was eluted. The eluted bands where concentrated to dryness in a rotary evaporator at 50°C under pressure to remove the solvent and were stored at 4°C for further analysis.[(9)]

2.2.3. Confirmation of d-limonene

1. Thin layer chromatography

   Commercially available standard TLC plate was used with standard particle size range to improve reproducibility. The absorbent silica gel coated on an TLC plate of 7.3 cm length, 2.5 cm breadth and 0.3 cm thick plate. Small spot of the solution containing the sample was applied on the plate 1.0 cm from the bottom marked.

Spotting and development, Visualization and Detection

The sample spotted on the plate was allowed to dry before the plate was placed into the chromatographic tank which is completely saturated with mobile phase. The reaction was then monitored as the solvent moved up the plate (elutes the sample) using mobile phase solvent ratio (8:2), n-hexane and ethyl acetate, respectively. When the solvent reaches the top of the plate, it is removed, marked and dried. Following separation of the solvent, the plate was removed and dried; the spots detected using various techniques and reagents. This includes visualization in daylight; viewing under UV at 254 and 366 nm

2. **UV-Visible spectrophotometer**

Determination of λ_{max}:

For ultraviolet spectroscopic analysis of standard of d-limonene in n-hexane of concentration 10 mg in 100 ml .this solution was stand at wavelength 400 - 200 nm on jasco double beem UV-Visible spectrophotometer against blank (n-hexane) and maximum absorbance.

Preparation of standard solution of D-limonene:

10 mg of d-limonene was diluted in n-hexane up to 10 ml in volumetric flask.

Stock solution was prepared by diluting 1 ml of this solution with methanol up to 100 ml in volumetric flask to give 10ug/ml.

Preparation of sample solution:

10 mg of sample diluted up to 10 ml of n-hexane (stock solution) was prepared by diluting 1 ml of this solution with n-hexane up to 10 ml in volumetric flask to give 10 ug/ml.[10]

3. **HPTLC method:** Photo documentation study of D-limonene:

2.3. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC): [13]

1 Experimental:

Preparation of standard solution:

A stock solution of standard D-limonene was prepared in 5 ml of volumetric flask by dissolving 1.7 mg of accurate weighed d-limonene std in about 3ml of solvent methanol : water (60:40) followed by sonication for 5 min and finally making the volume up to mark with solvent .

Stock solution (for STD and sample)

(Further dilution)

2, 4, 6, 8, 10 µg/ml

(Up to 10 ml)

Preparation of sample solution:

Stock solution of sample was prepared by transferring 2.8 mg of d-limonene in 10 ml of solvent methanol: water (60:40) in volumetric flask and then sonicated for 15 min at room temperature. Then filtered through whatmenn filter paper no 41 .the filtrate was collected and use for further analysis

Chromatographic conditions:

Injection volume: 20 µl.

Flow rate : 0.5ml/min.

Mobile phase : Methanol: water (60:40).

Detection wavelength: 265 nm

Mode: Isocratic

Analyses were performed using a PEAK-7000 HPLC system. The HPLC detector was a PEAK UV detector with 265 nm. A hypersilbds c18 column (250 mm×4.6 mm, 5 µm) column was combined with a guard column of the same stationary phase (10 mm×4.6 mm×5 µm). Column temperature was maintained at room temperature. The mobile phase consisting of Methanol and water as mobile phase in the ration of 60%:40% at a flow rate of 0.5 ml/min. The injection volume was 20 µl.

2.4. In-vitro cytotoxicity screening:

2.4.1.Brine shrimp lethality assay

Procedure for assay:

Preparation of sea water: Artificial sea water for hatching of brine shrimp was prepared according to the hatching instruction given by Aquatic remedies; Chennai, India. Crude 25 gm/Lsea salt (without iodine) and dried yeast 6mg/L was weighed, dissolved in one litter of distilled water and filtered off to get clear solution.

1. Hatching of Brine Shrimp: *Artemiasalina*leach (brine shrimp eggs) collected from Aquatic remedies; Chennai, India was used as the test organism. The 3.5 L of artificial sea waterways added to the egg hatching chamber and 1 capsule of brine shrimp eggs was added to dark side of the chamber then this side was covered. Two days were allowed to hatch the shrimp and to be matured as nauplii. Constant oxygen supply was provided throughout the hatching time. The hatched shrimps areto the light (photo taxis) and so nauplii free from egg shell was collected from the illuminated part of the tank. The nauplii was taken from the fish tank by a pipette and diluted in fresh clear sea water to increase visibility and 10 nauplii were taken carefully by micropipette

2.Preparation of standard and test solutions:

Samples of the test and standard D-limonene was prepared by dissolving 10mg of each test and standard solution in 20ul ofDMSO and volume make up to 10 ml with distilled water to get 1000µg/mlstock solution. From this stock, 100µl, 250µl, 500ul, 1000µl, and 2500µl were took and volume was made up to 5 ml with distilled water to get the final drug concentration20µg/ml, 50µg/ml,100µg/ml,250µg/ml and 500µg/ml. Three replicates were prepared for each dose level. Control vials were prepared by adding equal volumes of artificial sea water.

Bioassay:

The bioassay experiment was performed according to the procedures described by Meyer et. Al.1982. Nauplii were collected in a glass pipette along with water, and 10ml of such shrimps were transferred to each

drug conc. Test tube containing 4.5 ml brine solution (specific volume and yeast suspension). In each experiment, 0.5 ml of test and standard d-limonene was added to 4.5 ml of brine solution at various concentrations 20-500µg/ml respectively. In control test tube added 4.5 mlof artificial sea water and 0.5ml of distilled water. The test tube was maintained under illumination. After 24 hr. survival of nauplii were counted, by 3Xmagnifying glass against dark background, and the percentage lethality andLC50 values were calculated by dose-response data were transformed into straight line by means of a tread line fit linear regression analysis (MS-Excel version-10) and the LC50 was derived from the best fit line obtained.[(11)]

2.4.2.Trypan blue dye cell exclusion assay

Experimental procedure:

Trypan blue: 100mg trypan blue dye was dissolved in 100ml PBS and stored at4ºC.

preparation of standard drug:

Samples of standard prepared by dissolved 10mg of 5-FU in in 20µl of DMSO and volume make up to 10 ml with phosphate buffer saline (PBS-7.4 pH), to get 1000µg/ml stock solution. From this stock 20 µl/mlwas took and volume was made up to 10 ml (in 10 ml vial capacity) with PBSsolution to get the final drug conc. 20µg/ml.

Preparation of sample solution:

Sample of standard and isolated D-limonene was prepared by dissolving 10 mg of each in 20µl of DMSO and volume made up to 10 ml with phosphate buffer saline (PBS),to get 1000 µl/ml stock solution. From this stock 10 µl,20 µl,40 µl,100 µl were taken and volume was made up to 10 ml with PBS solution. Three replicates were prepared for each dose level. Control vials were prepared by adding equal volume of 0.2% DMSO with PBS..

Procedure for cytotoxicity assay:

In the stock cell suspension, cell count determined and cells were found EACcells 2.7×105/0.1ml. From this stock, cell suspension was taken in micro wellsofmicro titer well plates. In the 1st well added only 0.1ml DMSO (0.1 % V/V with PBS-7.4 pH) and considered as control groupIn the next 6 wells, 0.1 ml standard and isolated sample of concentration ranging from 10µg/ml, 100 µg/ml and 500µg/ml were added in respective micro wells considered as test groups. In the next 6 wells, concentration ranging from 10 µg/ml, 100 µg/ml and500µg/ml of 5-FU were added in respective 3 micro wells of micro titer well plate. Considered as standard groups. Further, micro titer well plate was incubated at temperature 37ºC and 7% CO2incubator for period of 3 hours. After the incubation, in each micro well of micro titer well plate individually. 0.1ml of trypan blue was added and mixed well.

Cell counting:

The total numbers of dead and living cells in all the four corner squares of the Neuber's chambers were counted by using haemocytometerandthe total no. of viable cells was calculated by using following formula [(12)]

Total number of viable cells = A×B×10^4

A= Mean no. of unstained cells (Viable cells).

B= Dilution factor of Trypan blue dye (1:5).

C= Mean number of dead cells.

104=Conversion of 0.1 mm3 to ml.

Percentage of cell viability for cytotoxicity was calculated using following

FORMULA: **% Viability = Viable cell count / Total cell count × 100**

2.4.3.MTT Assay:

Principle-

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) is a yellow colored water soluble dye. Mitochondrial lactate dehydrogenase produced by metabolically active cells reduce MTT to water insoluble formazone crystals. When dissolved in appropriate solvent (DMSO) these formazone crystals exhibit purple colour. The intensity of purple colour is directly proportional to number of viable cells and be measured spectrophotometrically at 540 nm.

- **Procedure-**

1. 100ul of cell suspension in a 96-well micro titer plate at the required cell density, with or without the cell growth modifying agent.
2. Incubate the plate at 37oC in a 5% CO2 atmosphere for the required period of time. After the incubation period, remove the plates from the incubator and add MTT reagent to a final concentration of 10% of total volume. This volume should be same as the volume used while determining the optimum cell density.
3. Wrap the plate with aluminum foil to avoid exposure to light. Return the plates to the incubator and incubate for 2 to 4 hours. Remove the plate from incubator after incubation. After incubation period, add 100ul of solubilisation solution to each well. Gentle stirring on gyratory shaker will enhance dissolution. Occasionally, pipetting up and down may be required to completely dissolve the MTT formazone crystals especially in dense culture.
4. Read the absorbance on a spectrophotometer or an ELISA reader at 570nm with a reference wavelength of higher than 650nm. Subtract the average 570nm absorbance values of the control wells from the average 570nm absorbance values of corresponding experimental wells.
5. Measure the absorbance of all the assay wells again at a wavelength higher than 650nm. Subtract these values from the values obtained at 570nm. This reading will help you eliminate non-specific readings from your assay result.
6. Plot the absorbance values on the Y-axis and your experimental parameters on the X-axis.
7. The % inhibition or % cytotoxicity was calculated by using following formula

$$\% \text{ Cytotoxicity} = \frac{(\text{control-Blank})-(\text{Test-Blank})}{(\text{Control-Blank})} \times 100$$

RESULTS AND DISCUSSION:

Table No 3 %Yield of extracts

Soxhlet extract	Microwave extract
8.5%	11%

Percentage yield of extracts were as follows, Soxhlet extract(8.5%) and Microwave assisted extract(11%)

3.1.Phytochemical screening:

Table No 4. Phytochemical constituents from *Murraya koenigii* leaves extracts

Sr. no.	Test	Observation	
		Soxhlet extract	**Microwave extract**
1.	**Tests for Glycosides:** a) Libermann-Burchard's test b) Legal's test c) Modified Borntrager's test	 + + +	 + + +
2.	**Tests for Flavonoids:** a) Ferric chloride Test b) Shinoda Test c) Zinc – HCL reduction Test	 + + +	 + + +
3.	**Test for Steroids** a) Salkowski Reaction b) Liebermann's Reaction	 + +	 + +
4.	**Test for Saponins:** Foam test	 +	 +
5.	**Test for the phenolic compounds and tannins:** a) Ferric chloride test b) Lead acetate test	 -	 -
6.	**Test for Proteins** a) Millon's test b) Biuret test	 + +	 + +
7.	**Alkaloids** **a**) Mayers test b) Wagners test c) Dragendroffs test d)Hager's test	 + + + +	 + + + +
8.	**Test for Terpenoids**	+	+

The extracts of *Murraya koenigii* leaves revealed the presence of Alkaloids, flavonoids, Glycosides, Steroids, Proteins, Tannins and terpenoids, saponins etc.

3.2. Isolation of D-limonene by column chromatography:

Fig 4. Isolated D-limonene by column chromatography

In column chromatography, a vertical glass column was used in which the mobile phase, n-hexane: ethyl acetate (8:2) was added. The stationary phase, a solid adsorbent i.e. crystalline silica gel 100-200, was added in the column. The solvent was eluted drop wise, such that 4-6 drops per minute so that better resolution was obtained. 2nd bands obtained was eluted.

3.3. CONFIRMATION OF D-LIMONENE:

1. Thin layer chromatography

2. UV-Visible spectrophotometer

3. HPTLC

3.3..1 CONFIRMATION OF D-LIMONENE BY TLC:

Fig 5. Confirmation of D-limonene by TLC

Table no 5. R_f value of samples compared with standard D-limonene

Sr. no.	Compounds	R_f value
1	Standard D-limonene	0.89
2	D-limoneneisolated from Sohxlet extract	0.91
3	D-limonene isolated from Microwave assisted extract	0.92

The isolated compound from column chromatography was used for the preparation of the sample solutions. The first spot is of standard D-limomene and second spot is of D-limonene isolated from Soxhlet extraction and third spot which is isolated from microwave assisted extraction method. Different solvent systems as mobile phase were tried to obtain the proper resolution. Better resolution of D-limonene was obtained in the solvent system of N-hexane: Ethyl Acetate:(8:2). From, this D-limonene was identified and confirmed by comparing R_f value obtained by using Thin Layer Chromatography. TLC: R_f value of standard D-limonene was found 0.89. This was compared with compound isolated from Soxhlet and Microwave extraction method.

3.3.2.HPTLC METHOD:

Photo documentation study of D-limonene:

Fig 6.Photo documentation of isolated D-limonene from Soxhlet and Microwave extract

3.3.3.UV-VISIBLE SPECTROPHOTOMETER:

Determination of λ_{max}::

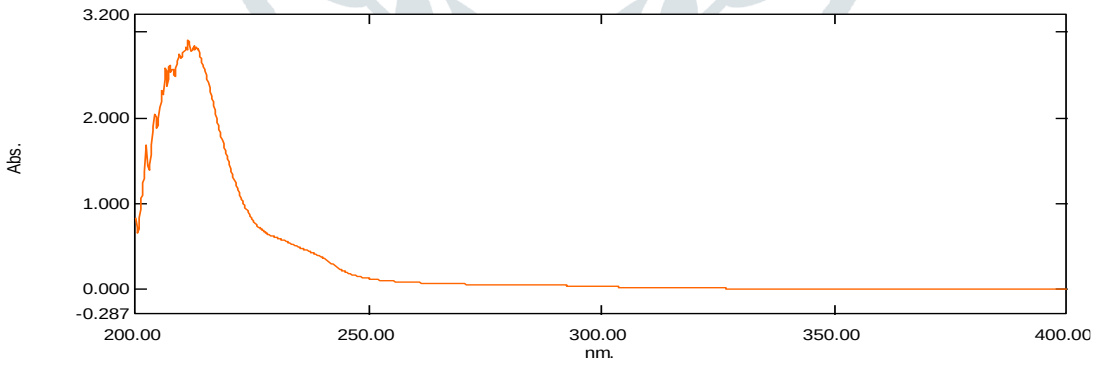

Fig 7.λ max of standard D-limonene

Standard solution- Maximum wavelength-λ max of standard D-limonene was found to be 212 nm

Solvent-n- hexane

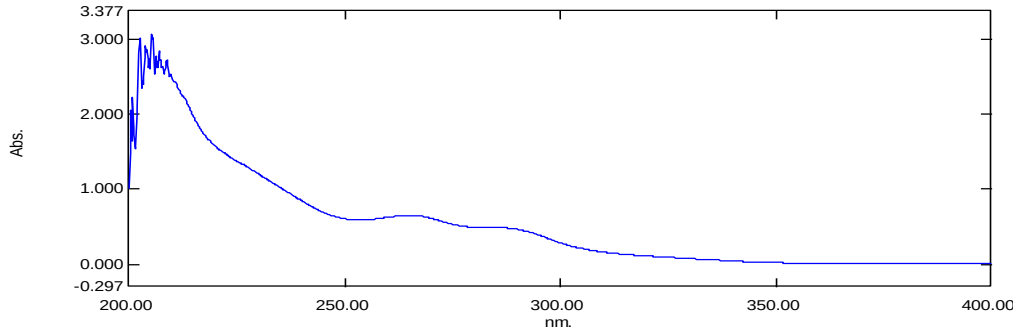

Fig 8- λ max of isolated D-limonene from Soxhlet extract

Sample 1

Maximum wavelength- λ max of isolated D-limonene from Soxhlet extraction was found to be 208nm

Solvent-n- hexane

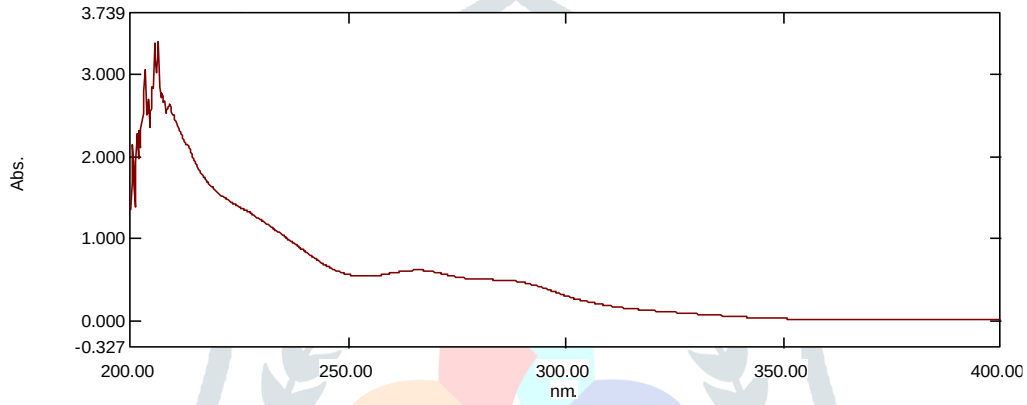

Fig 9 λ max of isolated D-limonene from microwave assisted extract

Sample 2

Maximum wavelength-λ max of isolated D-limonene from microwave assisted extraction was found to be 209 nm

Solvent-n- hexane

3.4.HPLC RESULTS:

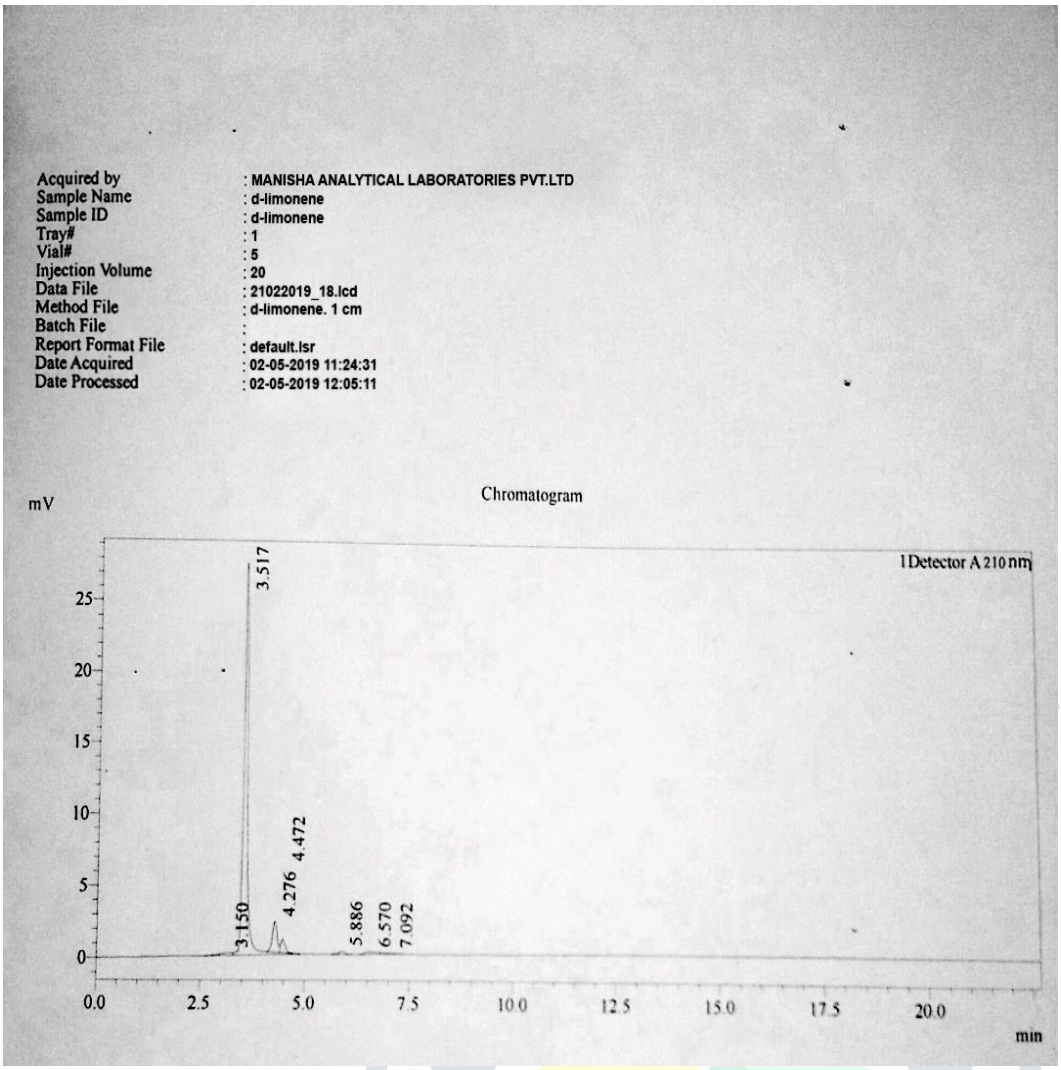

Fig 10: HPLC Result of standard D- limonene

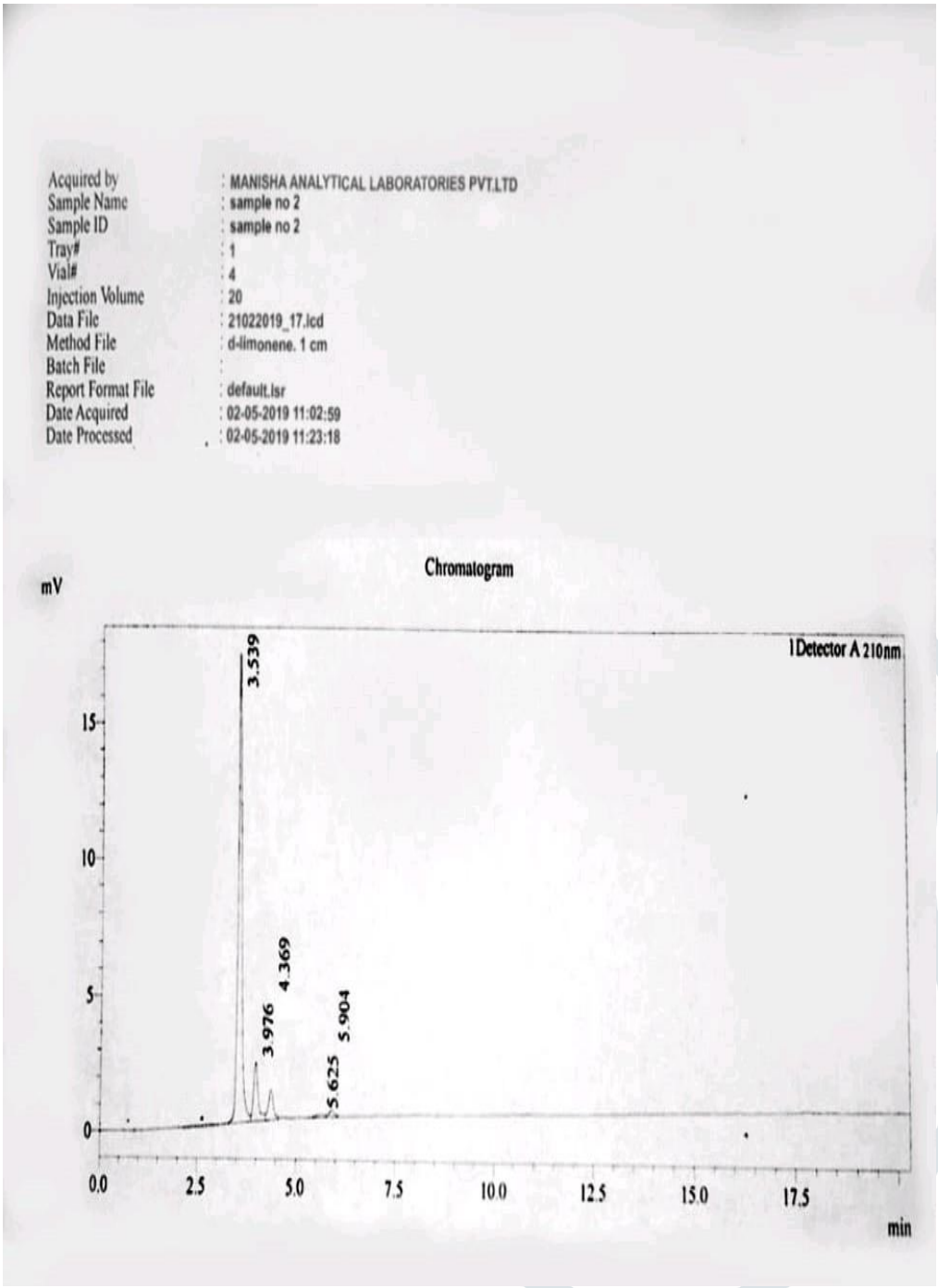

Fig11: HPLC Result of D- limonene isolated from Soxhlet extract

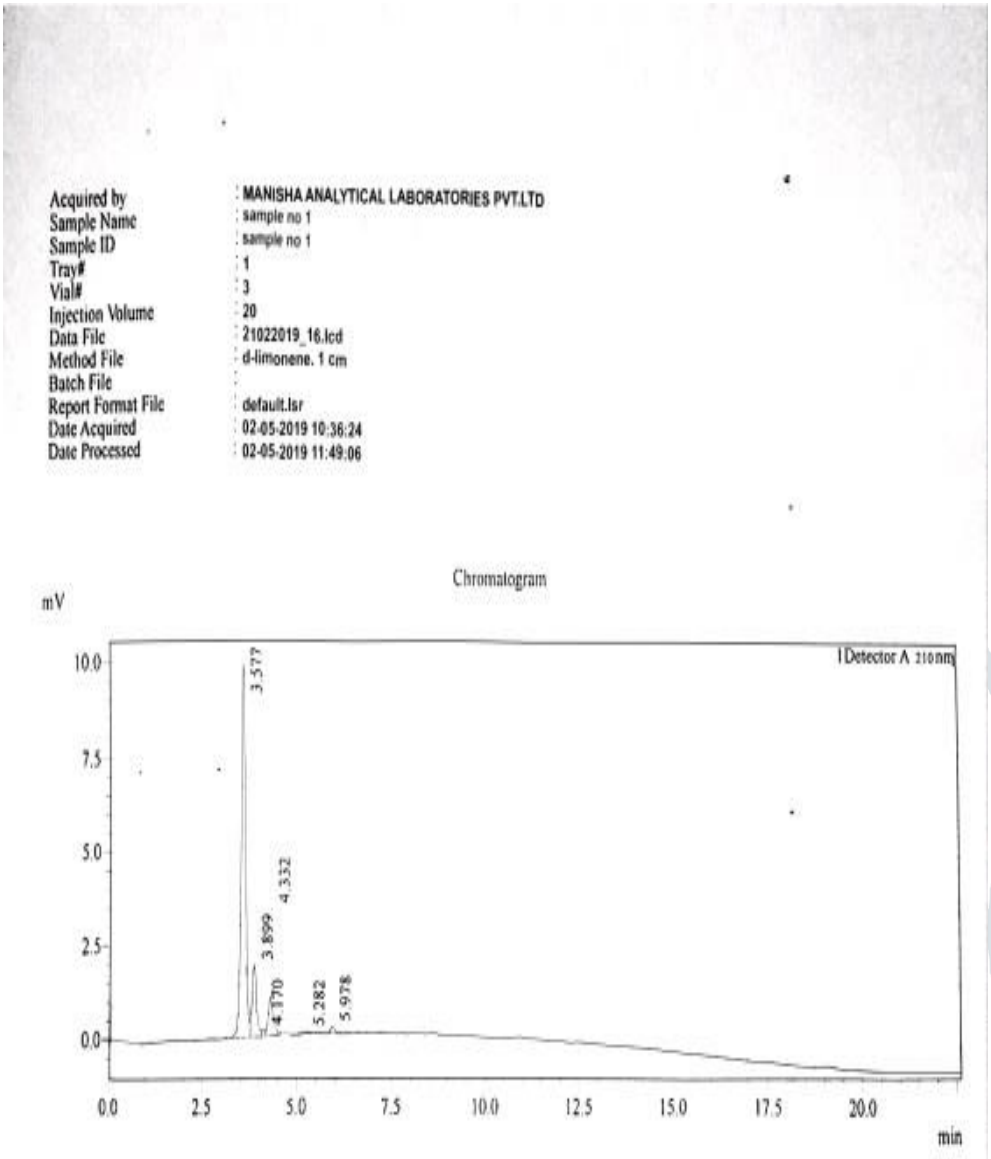

Fig 12: HPLC Result of D-limonene isolated from Microwave extract

FORMULA:

Concentration= Area under curve of test ×100

Area under curve of standard

Concentration of D-limonene isolated from microwave extract

Concentration= Area under curve of test ×100

Area under curve of standard

=89899×100

189991

=46.57%

Concentration of D-limonene isolated from Soxhlet extract:

Concentration= Area under curve of test ×100

Area under curve of standard

=70039×100

189991

=36.86%

Table no. 6 concentration of D-limonene by different method

Sr no	Sample	Area under curve	Concentration
1	D-limonene	189991	83.59%
2	Sample1(Microwave)	89899	46.57%
3	Sample 2(Soxhlet)	700396	36.86%

The Microwave extract shows higher concentration than that of Soxhlet extract.

3.5.IN-VITRO CYTOTOXICITY STUDIES:

3.5.1 Brine Shrimp Lethality bioassay:

Table no: 7 Effect of standard D-limonene on brine shrimp lethality bioassay:

Sr. no.	Concentration (µg/ml)	Total dead nauplii out of 10			Total dead nauplii out of 30	% Mortality	LC 50
		T1	T2	T3			
1.	50	3	1	2	6	20	259µg/ml
2.	100	5	3	3	11	36	
3.	150	5	3	7	15	50.11	
4.	250	5	7	7	17	56	
5.	500	9	5	7	2	70.33	

Table no 8: Effect of isolated D-limonene on brine shrimp lethality bioassay

Sr.no.	Concentration (µg/ml)	Total dead nauplii out of 10			Total dead nauplii out of 30	% Mortality	LC50
		T1	T2	T3			
1.	50	2	0	2	4	13	396.05µg/ml
2.	100	2	1	2	5	16	
3.	150	4	2	3	9	30.11	
4.	250	5	3	3	11	36	
5.	500	6	5	7	18	60	

Bioassay was performed in triplicate manner; each concentration was performed for The 3 replicates. Results of brine shrimp lethality assay on standard D-limonene and D-limonene isolated from microwave extract were done. from above result the percentage of mortality increased with an increase in concentration. these are described as the percentage mortality of standard D-limonene ranging from 20% to 70.33% followed by percentage mortality of isolated D-limonene ranging from 13% to 60%.

Table no 9: Effect of 5-FU (Std.) on brine shrimp lethality bioassay:

Sr. no	Concentration (µg/ml)	Total dead nauplii out of 10			Total dead nauplii out of 30.	% Mortality	LC50
		T1	T2	T3			
1.	20	3	4	2	9	30	123.80µg
2.	50	4	4	6	14	46.66	
3.	100	7	7	8	22	73.33	

4.	250	9	7	8	24	80	/ml
5.	500	9	9	10	28	93.33	

The % lethality concentration of standard D-limoneneby brine shrimp lethality bioassay found that 259µg/ml while the isolated D-limonene were showed significantly increased in lethality concentration (396.05µg/ml) when compared with standard drug 5-flurouracil (123.80µg/ml).

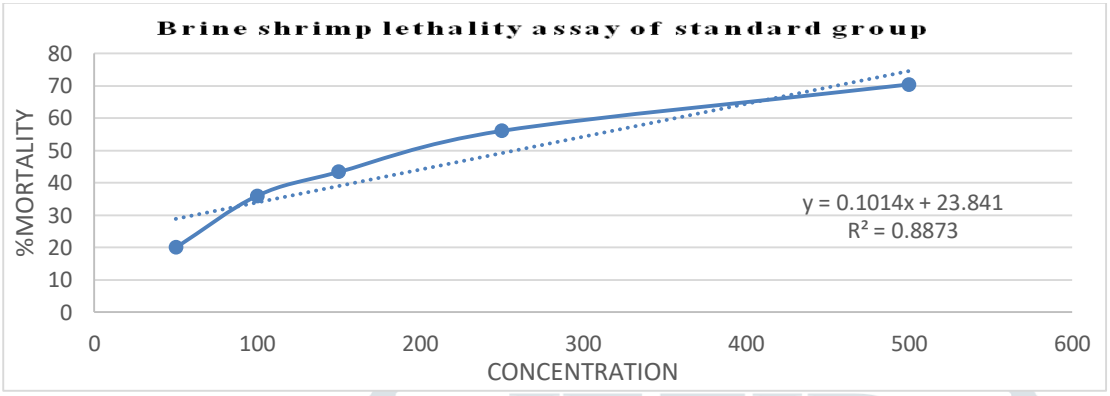

Fig 13. Determination of LC_{50} by linear regression analysis of standard D-limonene

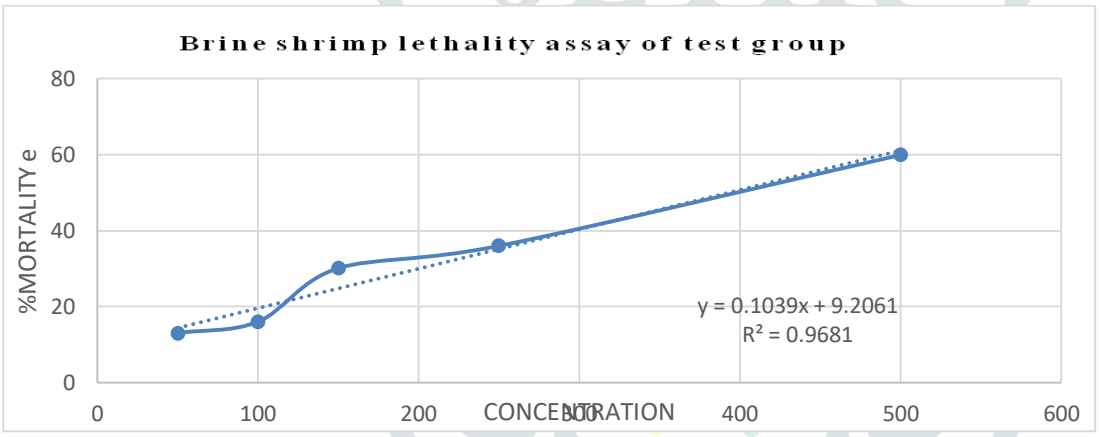

Fig 14. Determination of LC_{50} by linear regression analysis of isolated D-limonene

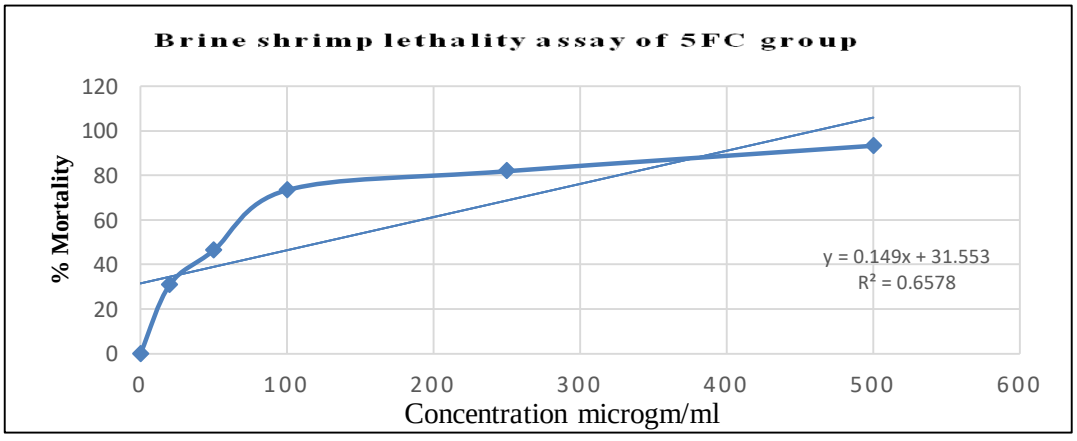

Fig15. Determination of LC_{50} by linear regression analysis of 5-FC.

3.5.2 Tryphan blue dye cell exclusion assay:

Table No 10 .In-vitro Cytotoxic effect of standard D-limoneneon HEP G2 (human liver cancer) cell line by Tryphan blue dye cell exclusion assay.

Groups	Viable cell count (Mean ± SEM) on COLO205 Cell Line	% viability
Control	87.67± 0.6667	79.33± 0.3333
Standard (10µg/ml)	33.67 ± 0.5774****	23.24± 0.2767
Standard (20µg/ml)	26.67± 0.3333****	20.97± 0.2467
Standard (40µg/ml)	22.33 ± 0.3333****	14.96± 0.03667
Standard (100µg/ml)	12.33 ± 0.3333****	16.88± 0.2200
Test I (10µg/ml)	38.00± 0.333****	25.53± 0.3400
Test II (20µg/ml)	26.67 ± 0.3333****	24.63± 0.1667
Test III (40µg/ml)	18.67 ± 0.3333****	23.49± 0.4267
Test IV (100µg/ml)	13.33 ± 0.3333****	19.28± 0.2168

All the values are expressed Mean ± SEM and n=6,****P<0.0001 using one way ANOVA coupled with "Dennett's test", criterion for significance. ****P<0.0001 is considered as significant when standard group and test group compared with control group.

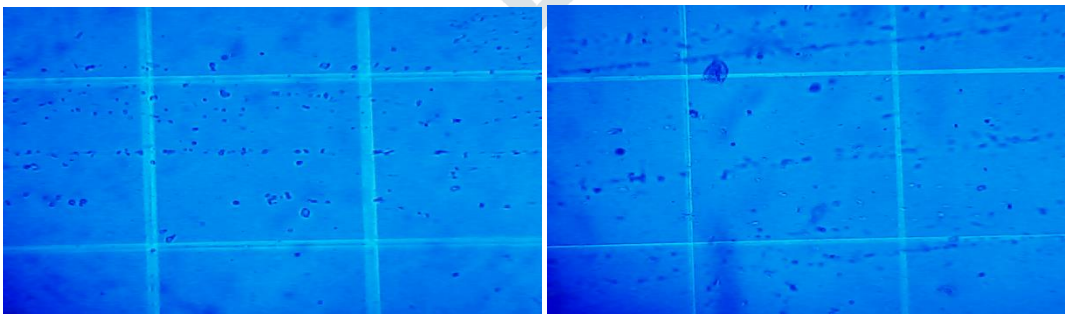

Fig 16 Identification of viable and dead cell

Identification of viable and dead cell in Tryphan blue dye cell exclusion assay: Unstained cells observed in figure indicated viable cells, while completely blue stained cells counted as dead cells**.** On **HEP G2 (human liver cancer) cell line** showed more percentage of cytotoxicity activity at dose 500µg/ml.

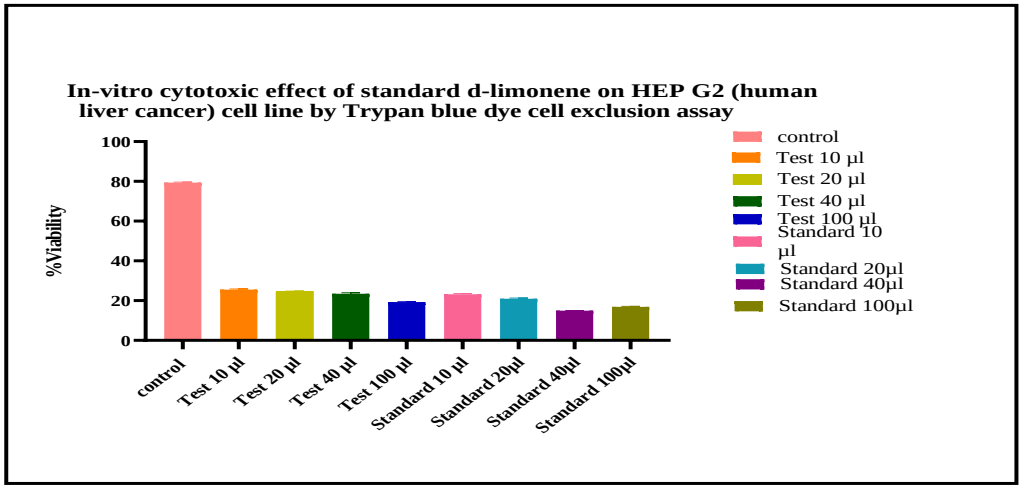

Fig 17. Effect of D-limonene and n-hexane extract of*Murraya koenigii* leaves of on human liver cell line "HEP G2" By tryphan blue dye exclusion assay

3.5.3. MTT assay:

In-vitro cytotoxic effect of standard D-limonene on HEP G2 cell line (human liver cancer) by MTT assay method:

Table no 11. In-vitro cytotoxic effect of standard D-limonene by MTT assay method

Sr.no.	Concentration µg/ml	% Inhibition	IC50 µg/ml
1.	10	21.15	239.58µg/ml
2.	20	22.57	
3.	40	23.45	
4.	100	25.05	

Table no 12 In-vitro cytotoxic effect of extract of *Murraya koenigii* leaves by MTT assay method:

Sr.no.	Concentration µg/ml	% Inhibition	IC50 µg/ml
1.	10	18.48	259.89µg/ml
2.	20	20.67	
3.	40	21.60	
4.	100	23.22	

Table no13 In-vitro cytotoxic effect of isolated D-limonene by MTT assay method:

Sr.no.	Concentration µg/ml	% Inhibition	IC50 µg/ml
1.	10	19.62	265.11µg/ml
2.	20	20.67	
3.	40	21.60	
4.	100	23.22	

3.5.4.In-vitro Cytotoxic effect of 5-Flurouracil on HEP G2 cell line (human liver cancer) by MTT assay method :

Table no 14 In-vitro Cytotoxic effect of 5-Flurouracilby MTT assay method:

Sr.no.	Concentration µg/ml	% Inhibition	IC50 µg/ml
1.	10	33.88	150.14 µg/ml
2.	20	34.24	
3.	40	34.24	
4.	100	35.04	

The IC_{50}value of standard D-limonene were calculated by linear regression analysis at various concentration by MTT assay on HEP G2 human liver cancer cell line was found 239.58 µg/ml; while isolated D-limonene was found that 265.11µg/ml µg/ml and IC_{50} value of n-Hexane extract of *Murraya koenigii* leaves were showed significantly increased (259.89µg/ml) when compared with standard drug 5-Flurouracil (150.14 µg/ml).

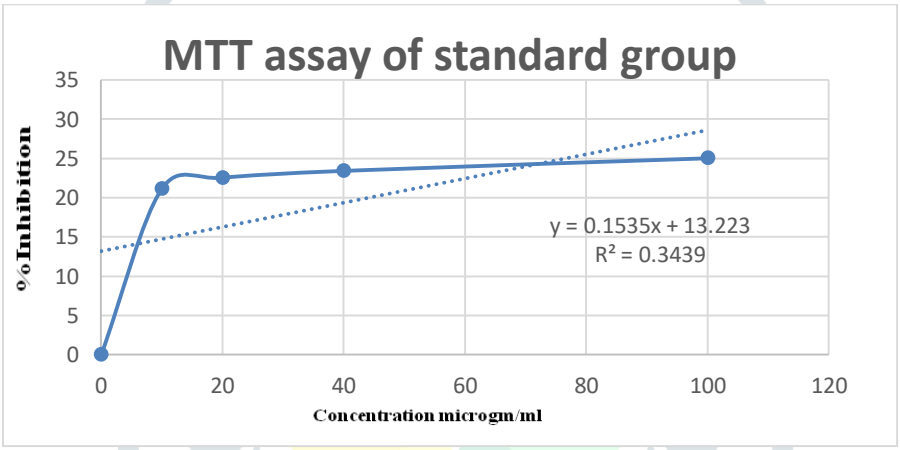

Fig 18.Calculation of IC_{50} by linear regression analysis of standard D-limonene at different concentration by using MTT assay

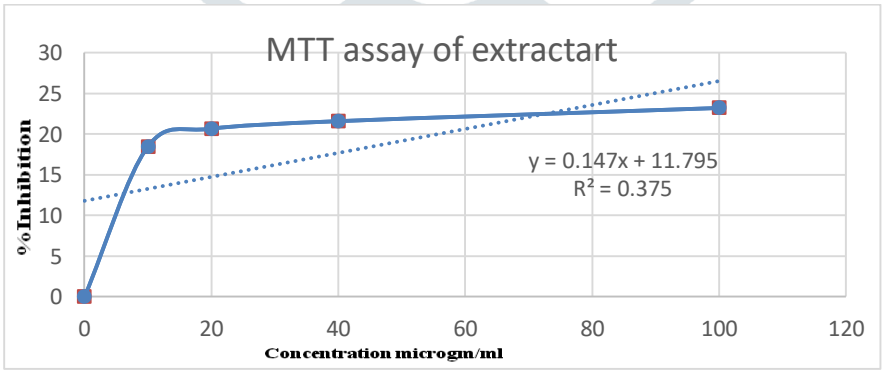

Fig 19.Calculation of IC_{50} by linear regression analysis of extract of *Murraya koenigii* leaves at different concentration by using MTT assay

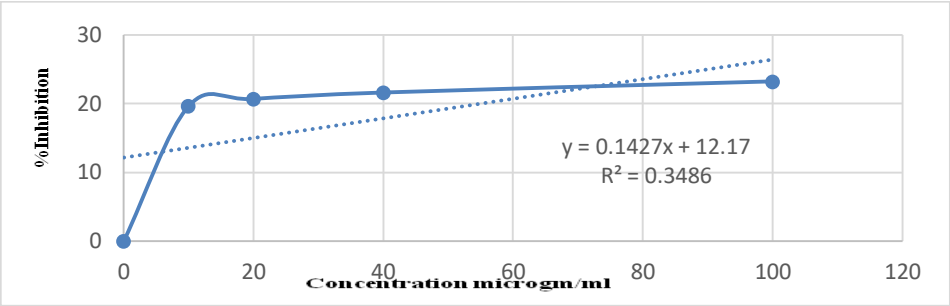

Fig 20.Calculation of IC_{50} by linear regression analysis of isolated D-limonene at different concentration by using MTT assay.

3.5.5.In-vitro cytotoxic effect of D-limonene onCOLO 205 cell line (Human Colon cancer by MTT assay method :

Table no15 In-vitro cytotoxic effect of standard D-limonene by MTT assay method :

Sr.no.	Concentration µg/ml	% Inhibition	IC50 µg/ml
1.	10	34.49	141.06µg/ml
2.	20	35.89	
3.	40	36.03	
4.	100	36.05	

Table no16 In-vitro Cytotoxic effect of extract of *Murraya koenigii* leaves by MTT assay method:

Sr.no.	Dilutions µg/ml	% Inhibition	IC50 µg/ml
1.	10	34.43	153.09µg/ml
2.	20	34.45	
3.	40	34.49	
4.	100	34.65	

Table no17: In-vitro Cytotoxic effect of isolated D-limonene by MTT assay method

Sr.no.	Dilutions µg/ml	% Inhibition	IC50 µg/ml
1.	10	33.98	143.48µg/ml
2.	20	34.97	
3.	40	35.28	
4.	100	35.89	

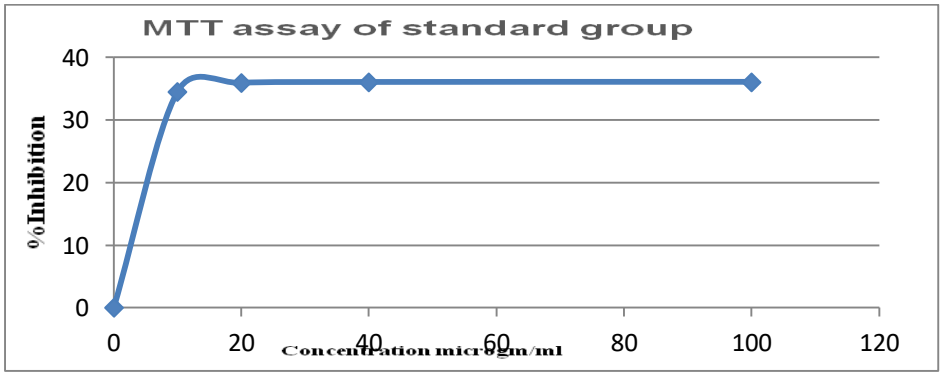

Fig 21.Calculation of IC_{50} by linear regression analysis of standard d-limomeme at different concentration by using MTT assay

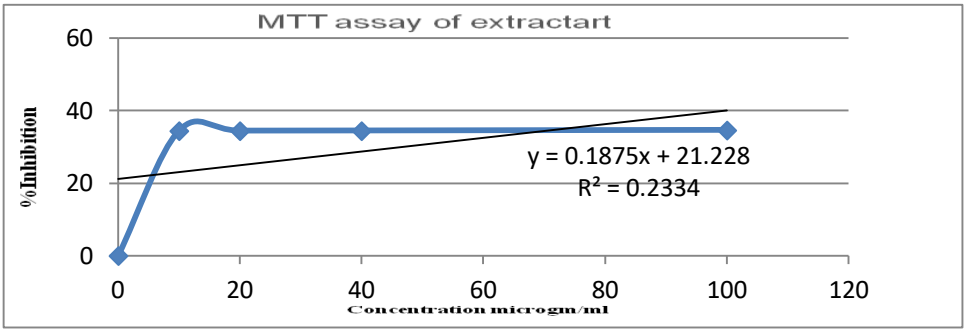

Fig 22 Calculation of IC_{50} by linear regression analysis of extract of *Murraya koenigii*leaves at different concentration by using MTT assay

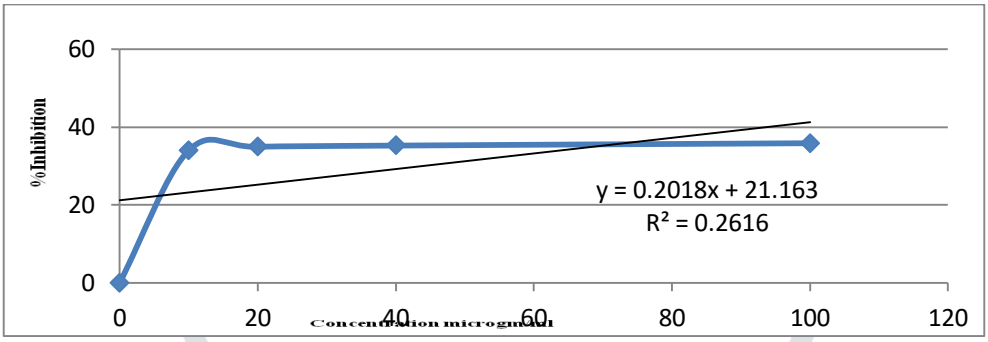

Fig 23.Calculation of IC_{50} by linear regression analysis of isolated D-limonene at different concentration by using MTT assay.

The IC_{50}value of standard D-limonene were calculated by linear regression analysis at various concentration by MTT assayon COLO 205 colon cancer cell line was found (141.06µg/ml)while isolated D-limonene was found that (143.48µg/ml)and IC_{50} valueof n-Hexane extract of *Murraya koenigii* leaves were showed significantly increased (153.09µg/ml)when compared with standard drug 5-Flurouracil (150.14 µg/ml).

CONCLUSION:

In the present study the leaves of *Murraya koenigii* belonging to the family Rutaceae were selected for isolation of phytoconstituents. The plant was authenticated by Dr. M. D. Wadmare, prof and head department of botany, kasturibai walchand college Sangli. The authenticated leaves were subjected to shade drying completely and leaves were subjected to Soxhlet extraction and microwave assisted extraction with solvent n-hexane. The concentrated extracts were used for preliminary phytochemical screening which showed the presence of Alkaloids, Glycosides, Steroids, Proteins, Flavonoids and Terpenoids as major chemical constituents. The D-limonene was identified and isolated from both extract by column chromatography technique. The separated D-limonene was confirmed by its boiling point, TLC, UV visible spectrophotometer and GCMS. The isolated D-limonene was estimated by HPLC instrument.

The proposed HPLC method for the determination of D-limonene in extracts of *Murraya koenigii* leaves. Samples have been shown to be a simple, rapid, precise, and accurate method. The D-limonene from *Murraya koenigii* leaves where extracted by two methods Soxhlet and Microwave assisted extraction. The percentage of D-limonene was higher in microwave extract than that of Soxhlet extract.

The cytotoxicity of D-limonene is evaluated by brine shrimp lethality assay as well as its cytotoxicity effect on human liver cancer cell line (HEP G2) evaluated with Trypan blue and MTT assay method. Also effect on colon cancer cell line (COLO205) evaluated with MTT assay.

The D-limonene was screened for cytotoxic activity by using In-vitro method; Brine shrimp lethality bioassay. The % lethality concentration of standard D-limonene by brine shrimp lethality bioassay found that (259µg/ml) while the isolated D-limonene were showed significantly increased in lethality concentration (396.05µg/ml) when compared with standard drug 5-flurouracil (123.80µg/ml).

In summary we evaluated the anticancer effect of D-limonene on liver cancer by using in-vitro method. The cell viability of the HEP G2 cells was assessed by using trypan blue method. In present study the cytotoxic activity of D-limonene could suppress liver cancer cell growth. There was a concentration dependent cytotoxic activity to HEP G2 cell line. There wasa dose dependent decrease in viable cell count of the treated cells. In different test dilutions, 100µg/ml have shown minimum number of viable cells.

In this study, the cytotoxic activity of D-limonene were also determined by using MTT assay in HEP G2 and COLO205 cell line which were exposed various dilutions viz. 10, 20, 40, 100 µg/ml. There was a dose dependent increase in percentage inhibition and it was found that at the dose level 100µg/ml maximum % inhibition were observed The IC50 value of standard D-limonene were calculated by linear regression analysis at various concentration by MTT assay on HEP G2 human liver cancer cell line was found 239.58 µg/ml; while isolated D-limonene was found that 265.11µg/ml µg/ml and IC50 value of n- Hexane extract of *Murraya koenigii* leaves were showed significantly increased (259.89µg/ml) when compared with standard drug 5-Flurouracil (150.14 µg/ml). while The IC50 value of standard D-limonene were calculated by linear regression analysis at various concentration by MTT assay on COLO 205 colon cancer cell line was found (141.06µg/ml) while isolated D-limonene was found that (143.48µg/ml) and IC50 value of n- Hexane extract of *Murraya koenigii* leaves were showed significantly increased (153.09µg/ml) when compared with standard drug 5-Flurouracil (150.14 µg/ml).

REFERENCES:

1. Azwanida NN "A review on extraction methods use in medicinal plants, principle, strength and limitation". Medicinal and aromatic plants 2015;4:(3)1-6.
2. Chandsainisatish and Dr. Gopubala show reddy "A review on curry leaves {Murraya koenigii}: Versatile multi-potential medicinal plant. American journal of phytomedicinal and clinical therapeutics, 2015;363-368.
3. Jidong sun, review on d-limonene: Safety and clinical application. Alternative medicine review,2017;12:(3) 259-264.
4. Hejmadi M. Introduction to cancer biology, 2nd ed, ebooks, bookboon.com: 1-11.
5. Narang A. S., Desai D. S., Anticancer drug development. In. Pharmaceutical perspectives of cancer therapeutics, 2009; 49-92.
6. Desai D. S., Anticancer drug development. In. Pharmaceutical perspectives of therapeutics, 2009: 49-92.
7. Rao, L. J. M.; Ramalakshmi, K.; Borse, B. B.; Raghavan, B. *Food Chemistry* 2007, 100- 142.
8. Pande M, Ingale S, Gupta S. The Pharmacognostic and phytochemical studies on the leaves of *Murraya koenigii* (L) Spreng. Ind J SciTechnol 2009;2:(3)53-54.
9. Sethi P D: High performance thin layer chromatography for quantitative analysis of pharmaceutical formulation, C.B.S Publisher New Delhi, First edition 1996.
10. Column chromatography, CU Boulder Organic Chemistry undergraduate Course, Lab Techniques
11. Khandalwal KR, Sethi VK. Practical Pharmacognosy Techniques and Experiments. NiraliPrakashan. 1999;25:(1)25.9.
12. Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DJ, McLaughlin JL.Brine shrimp: a convenient general bioassay for active plant constituents. Plantamedica. 1982; 45 (05): 31-4.
13. Skoog, A.; Holler, F.; Nieman, T. *Principles of Instrumental Analysis*. 5th SaundersGoldeSunburst Series; Harcourt Brace College Publishers: Philadelphia, PA, 1998