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TLC, HPTLC Study, and Invitro Anticancer Activity of Cinnamomum Malabatrum Leaves Extract

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Abstract

Extracts of Cinnamomum malabatrum leaves were subjected to primary phytochemical investigations, and extracts showed positive results for alkaloids, saponins, flavonoids, and phenolic compounds. These extracts were subjected to Thin layer chromatography; in petroleum ether extract, yellow dots on the top represent phenolic compounds, and the orange dots represent flavonoid compounds. The Rf values are 0.13, 0.412. The yellow spot at the top represents flavonoids in methanol extract, and the orange tailing represents Phenolics. The calculated Rf values are 0.23, 0.34. The yellow spots at the top of the water extract are flavonoids, and the orange ones with fluorescent tails are phenolic compounds. The Rf value was calculated as 0.32 and 0.37. Ethyl acetate fraction of methanol extract, when subjected to HPTLC fingerprint analysis, gave 6 peaks, among 6 peaks, four the peak Rf value and 6th peak Rf value very close to the Gallic acid and Quercetin respectively. HPTLC fingerprint analysis of ethyl acetate fraction of methanol extract gave 8 peaks; among 8 peaks, 4, 6, and 7th peak Rf values were compared with reference standard in 7th Rf value was very close to the Phenolic compound Thymol. These confirmations indicate that methanol extract's Ethyl Acetate fraction verified flavonoids and Phenolic compounds. The pharmacological activity was carried out using the MTT Assay Method; based on the viability of the cells, anticancer activity was evaluated.

Key words: Cinnamomum malabatrum, TL, HPTLC Fingerprint analysis, MTT Assay Method, Trypan Blue Dye Exclusion Method

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Introduction

Cancer is a disease that impacts around 200 different types of cells. The primary attribute is the absence of regulation over the growth, specialization, and demise of cells, encompassing the invasion of organs and tissues. The treatment is fraught with numerous challenges, with medication resistance, toxicity, and limited specificity being the most prevalent. [1] The use of natural chemicals as cancer treatments has a long history, reaching back to folk medicine and

eventually making its way into allopathic and orthodox medicine. The majority of the chemotherapy drugs used today either originated in plants or were adapted from existing ones. Vinblastine, vincristine, and other vinca alkaloids derived from Catharanthus roseus are among the substances contained. Two partially synthetic derivatives of epipodophyllotoxin derived from Podophyllum species are etoposide and teniposide. Taxanes originate from taxanes, which are themselves derived from taxus species. Camptothecin, irinotecan, and topotecan are semi-synthetic derivatives of camptothecin obtained from Camptotheca acuminata. There are also several other compounds included. [2] More than half of the anti-cancer drugs under clinical trials, according to Cragg and Newman, are either naturally occurring or have some link to such substances. [3]

Cinnamomum malabatrum belongs to the Lauraceae family. The Cinnamomum genus consists of over 250 species, with 20 found in India. This species is a closely related to Cinnamomum verum C. malabatrum is a tree of intermediate size. Wild cinnamons are commonly employed to dilute the cultivated cinnamon and serve as a significant primary resource for the Agarbathy industry [4]. The plant is recognized for its diverse pharmacological effects, including analgesic and anti-inflammatory properties [5], hepatoprotective benefits [6], antioxidant activity [7,] anticancer potential [8], and more.

The present work aims to investigate the phytochemical analysis of leaf extract by using TLC and HPTLC methods and also investigate the in vitro cytotoxic potential of various solvent leaf extracts of Cinnamomum malabatrum using MTT (3-(4,5-Dimethylthiaxol-2yl)-2,5-Diphenyltetrazolium bromide measures metabolic activity of living cells.
Itis suited to measure cell viability or cytotoxicity and cell proliferation.

Materials and Methods

Extraction procedure

The desiccated leaves of Cinnamomum malabatrum were fragmented, pulverised, and subsequently subjected to extraction using several solvents, including hydrocarbon ether, methanol, and aqueous extract.

Phytochemical screening

Different extracts of Cinnamomum malabatrum were subjected to other qualitative tests for analysis of alkaloids, flavonoids, glycosides, tannins, triterpenoids, steroids, carbohydrates, and proteins.[9, 10, 11]

CHROMATOGRAPHIC STUDIES

Thin-layer chromatography

Among the different solvent systems tested, a mixture of toluene and chloroform in a ratio of 70:30 exhibited the highest level of separation and produced clear spots for the methanolic action of Cinnamomum malabatrum. The detection reagent employed was sulfuric acid, which was subsequently subjected to varying temperatures in different solvents. The resulting spot was then examined using UV light.[12]

High-performance thin-layer chromatography

The HPTLC plates (E. Merck No. 5548) constructed of silica gel 60 G F254 + 366, which were pre-coated and pre-activated, were resized using aluminium sheets measuring 20 × 20 cm and having a thickness of 0.1 mm. [13]

Application of sample

A bandwidth of 9 mm, spacing of 9 mm, and flow rate of 15 µL/sec were maintained when the various solvent extract samples were applied using the CAMAG LINOMAT V, an automated equipment for the sample application. Between 5 and 10 microliters of the sample was used.[14]

Test sample preparation

The test samples were produced using ethyl acetate stock solutions containing 100 mg/ml of the methanolic extract of Cinnamomum malabatrum. The initial stock solution had been diluted in

DMSO stepwise, Practical solutions were obtained, featuring concentrations ranging between 0.01µg/ml, 0.1µg/ml, 1µg/ml, 10µg/ml, and 100 µg/ml. A 0.1% DMSO solution was used as a reference during the entire culture.

HPTLC development

The mobile phase used in the experiment was chosen through empirical selection as a mixture of Benzene, Toluene, and Acetic acid in a ratio of 3:6:1. The plates were soaked in a tank containing the mobile phase for 2 hours. The leaves were desiccated using solvent evaporation at room temperature or by employing an air dryer emitting heated air.

EVALUATION OF ANTICANCER ACTIVITY

MTT assay method:

MTT, also known as 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide, is a substance used to assess the metabolic activity of live cells. This non-radioactive assay can be conducted in microtiter plates (MTP). This assay is specifically developed to quantify the rate at which cells divide, determine the overall health and survival, or assess the potential toxicity of substances on cells. The interaction between MTT and "mitochondrial dehydrogenase" forms a formazan salt not soluble in water.

The process entails placing cells on a microtiter plate with 96 wells and subjecting them to incubation in an MTT-containing solution for two hours. Living cells undergo a process of converting MTT into the water-insoluble formazan dye while in culture. The formazen dye in MTP is dissolved and measured using a micro plate reader. Absorbance is directly proportional to the amount. This pertains to the adhering cells in the multi-well tissue culture plate (MTP). The HBL-100 cell line, which is derived from breast cancer cells, was utilized. If required, adherent cells were detached using trypsin and then suspended in a fresh medium following centrifugation. Thoroughly homogenize the cell suspension by pipetting it multiple times to achieve a uniform mixture. The extract was diluted in the culture medium to create various concentrations. The DMSO concentration in the well is below one percent. Using an aseptic technique, carefully transfer 100 µl of the cell suspension into each well of a 96-well plate. Then, add 100 µl of 1% medium to each well. Position the plate within a carbon dioxide incubator set at 300°C for 72 hours. Following a 72-hour incubation period, introduce 20 µl of MTT to each well and cover the plate with aluminum foil to inhibit dye oxidation. Re incubate for a further 2 hours. Dispense 80 microliters of lysis buffer into each well and set the plate on the shaker for the night. Measurements of absorbance were taken within the wavelength range of 570 to 600 nm. Determine the percentage of inhibition by comparing the absorbance of the test sample with that of the blank control.[15, 16]

Results and Discussion

Phytochemical investigation for various extracts

The three extracts were subjected to a Primary Phytochemical test for active compounds.

Table 1: The preliminary phytochemical study of Cinnamomum malabatrum various extracts

TEST	PE	ME	AE
Alkaloids	+	+	+
a)Dragendorff's test	-	-	-
b)Mayer's test	-	+	+
c) Wagner's test	+	+	+
d) Hager's test			
Carbohydrates	+	+	+
Glycosides	-	-	-

Steroidal	+	-	+
Saponins	+	+	++
Flavonoids	+ +	+ ++	+ +
Phenol	+	+ +	+ +
Tannins	+	+	+

Keywords: '-'absent. '+' presence, '++' more clarity, '+++' significant

ME: Methanolic extract; PE: Petroleum ether; AE: Aqueous extract

Thin layer chromatography

TLC profile of Petroleum ether extract

Spots appeared on a thin layer of the chromatogram of a petroleum ether extract after acid application and heating to a temperature of 700˚C. At 254 nm, however, only one spot was visible under UV light before acid application utilizing benzene as the mobile phase. In Table 2, the hue of the dots and their Rf values are documented. The petroleum ether extract was eluted in Benzene solvent. The extract shows that the yellow dots on the top represent phenolic compounds (figure 1), and the orange dots represent flavonoid compounds. The Rf values are 0.13, 0.412.

Table 2: TLC of petroleum ether leaf extract of Cinnamomum malabatrum

Extract	Adsorbent	Solvent system	Observation / R_f values	
			Under UV light 254 nm	After acid spray and heated at 70°C
Petroleum ether	Silica Gel 60GF 254 Pre coated plate	Benzene	1 spot: 0.13 (yellow color)	spots: 0.412 (brown), (orange),

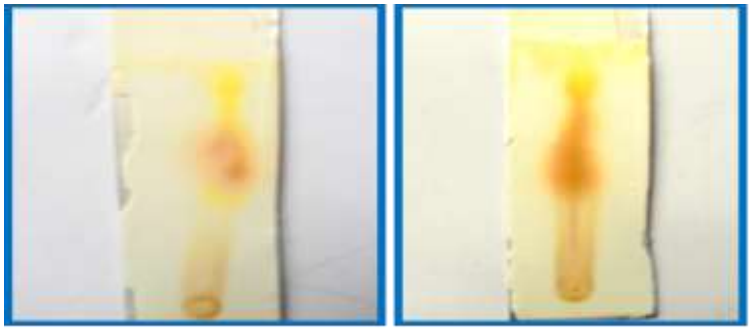


Figure 1: TLC plate of petroleum ether extract of Cinnamomum malabatrum

TLC profile of Methanolic extract

The TLC profile of methanol extract shows three spots when acid is sprayed and heated up to 94^{0}C under UV light at 254nm. A single spot was seen before acid spray using toluene: chloroform (70:30) as the mobile phase. The color and Rf value of the stones are shown in Table 3. Menthol extract was eluted in 70% toluene in 30% chloroform (70:30). The extract showed the presence of compounds. The yellow color spots at the top represent flavonoids, and the orange color tailing represents phenolic compounds (figure 2), the R_f value is calculated as 0.23, 0.34

Table 3: TLC of methanol leaf extract of Cinnamomum malabatrum

Extract	Adsorbent	Solvent system	Observation / R_f values	
			Under UV light 254 nm	After acid spray and heated at 95°C

Methanolic extract	Silica Gel 60GF$_{254}$ Precoated plate	Toluene: Chloroform (70:30)	spot 1 : 0.27 (deep brown)	2 spots : 0.23, 0.34,

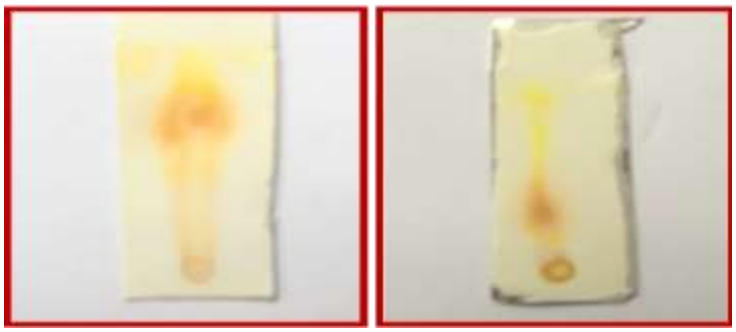


Figure 2: TLC plate of methanol extract of Cinnamomum malabatrum

TLC profiles of aqueous extract

The TLC profile of the aqueous extract showed two spots under UV light at 254 nm and two florescent spots at 360 nm using Benzene: Toluene (3:6) as the mobile phase. The R_f values are recorded in Table 4. The aqueous extract was eluted in 4% Benzene in 6% Toluene (40: 60). The section showed the yellow spots at the top indicate flavonoids and the orange ones with fluorescent tails are phenolic compounds (figure 3). Color spots with fluorescent tails are phenolic compounds.

Table 4: TLC of methanol leaf extract of Cinnamomum malabatrum

Extract	Adsorbent	Solvent system	Observation / R_f values	
			Under UV light 254 nm	Under UV light 360 nm
Aqueous extract	Silica Gel 60GF 254 Precoated Plate	Benzene: Toluene (4:6)	2 spots : 0.19, 0.22	2 florescent spots: 0.32,0.37

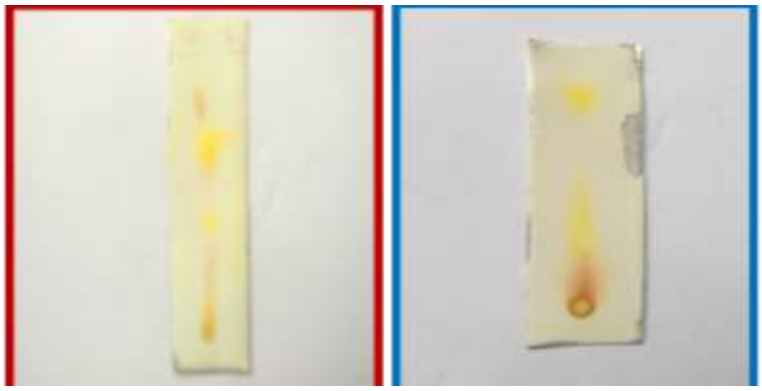


Figure 3: TLC plate of aqueous extract of Cinnamomum malabatrum

HPTLC Fingerprint analysis

The chemical constituents of the methanol extract were characterized by the HPTLC fingerprinting profile using a solvent system consisting of benzene, toluene, and acetic acid in a ratio of 3:6:1. The developed plates were photographed in the UV chamber in normal light at 254nm and 365 nm (figure 4). Profile of the HPTLC fingerprint of extract from methanol in solvent system Benzene: Toluene and glacial acetic acid (3:6:1) Chromatogram a, b, and c by HPTLC are illustrated in Figure 5.

(a) (b) (c)

Figure 4: HPTLC plate of methanolic extract (a) Normal light (b) 254 nm Ultraviolet light, (c) 365nm Ultraviolet light

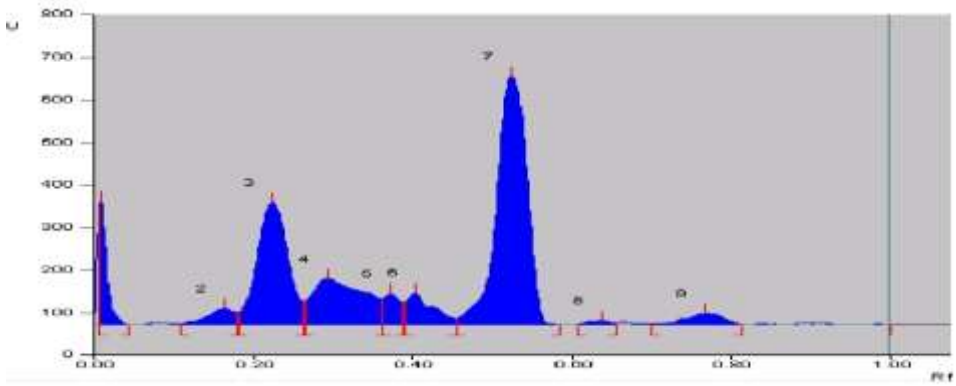


Figure5: HPTLC chromatogram area and peaks of methanolic extract

Table 5: HPTCL analysis of methanolic extract

Peak	Start Rf	Max. Rf	Max. Height
1	0.01	0.01	292.4
2	0.11	0.16	39.4
3(Gallic acid)	0.18	0.22	287.2
4	0.26	0.29	109.1
5	0.36	0.37	73.1
6	0.39	0.4	74.9
7(Quercetin)	0.46	0.52	584.5
8	0.61	0.64	10.3
9	0.7	0.77	26.4

HTLC screening profiles of the Ethyl Acetate fraction of the methanol extract for flavonoids TLC profile of the Ethyl acetate fraction of methanol extract for flavonoids showed 11 spots formed after the sulphuric acid spray was applied and heated to 100 °C. Before spraying, a mobile phase consisting of n-butanol, acetic acid, and water (4:1:5) produced sixteen marks when observed under ultraviolet (UV) light at a wavelength of 365 nm (figure 6). HPTLC fingerprint analysis of ethyl acetate fraction of methanol extract gave 6 peaks, among 6 peaks 4 the peak Rf value and 6th peak Rf value very close to the Gallic acid and Quercetin, respectively, as shown in figure 7 and table 5. As per the above evidence, we have concluded flavonoids are present in this fraction but need further analytical procedures to confirm the flavonoids.

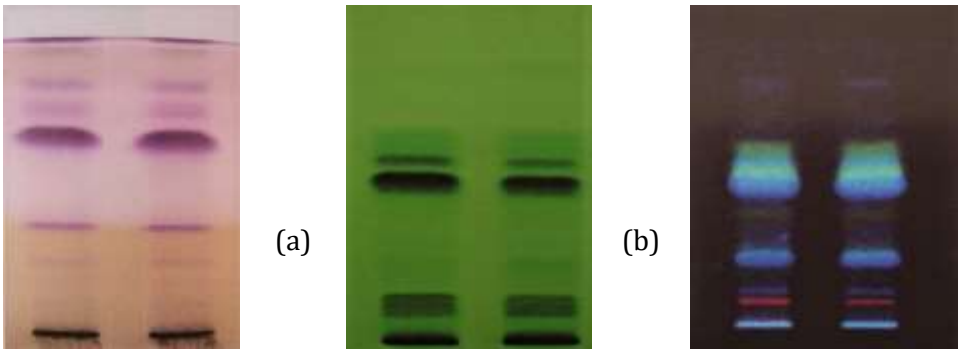


Figure 6: HPTLC plate of Ethyl Acetate fraction of the methanolic extract flavonoids (a) Normal light, (b) 254 nm Ultraviolet light, (c) 365nm Ultraviolet light

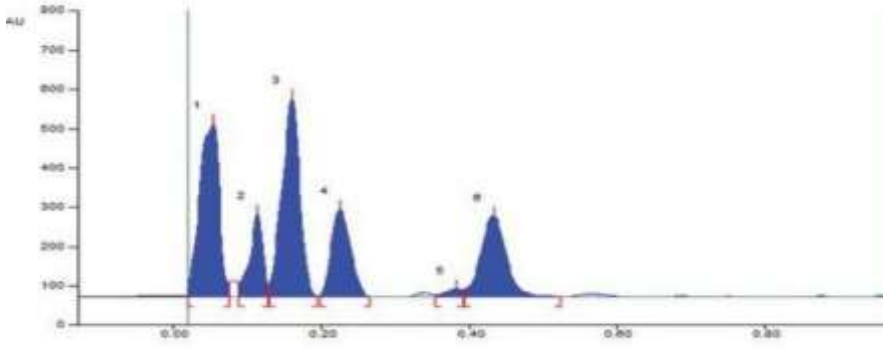
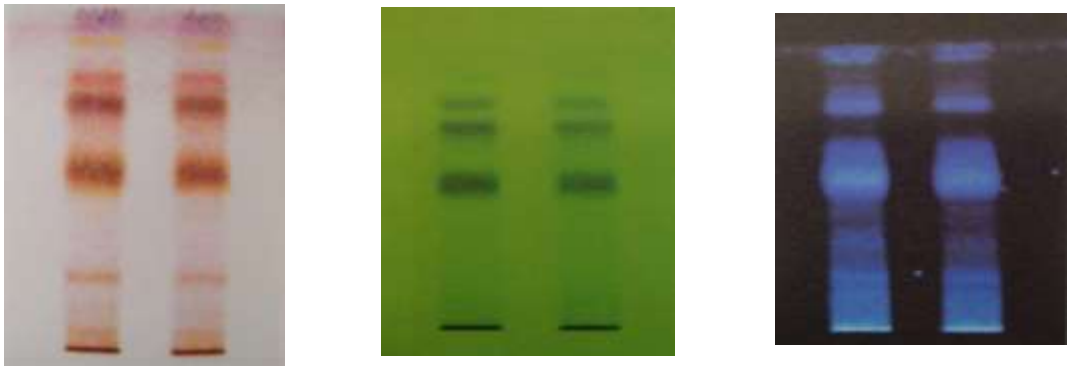


Figure7: HPTLC chromatogram area and peaks of of flavonoids

Table 6: Rf values of flavonoids

Peak	Rf value	Area %
1(Unknown)	0.05	28.74
2 (Unknown)	0.11	9.06
3(Unknown)	0.16	29.59
4(Gallic acid)	0.23	14.12
5(Unknown)	0.38	1.13
6(Quercetin)	0.46	17.37

HTLC screening profile of Ethyl acetate fraction of methanol extract for Phenolics showed 18 spots after $FeCl_3$ Solution spray using Chloroform: Ethyl acetate: Formic acid (5:4:1). HPTLC fingerprint analysis of Ethyl Acetate fraction of methanol extract gave 8 peaks, among 8 peaks 4, 6, 7^{th} peak Rf values were compared with reference standard in 7 th Rf value was very close to the Phenolic compound Thymol, as per the above evidence we are concluded Phenolic compounds are present in this fraction, as shown in the figure 9 and table 7.



(a) (b) (c)

Figure 8: HPTLC plate of Ethyl Acetate fraction of the methanolic extract of phenolic compounds (a) Normal light, (b) 254 nm Ultraviolet light, (c) 365nm Ultraviolet light

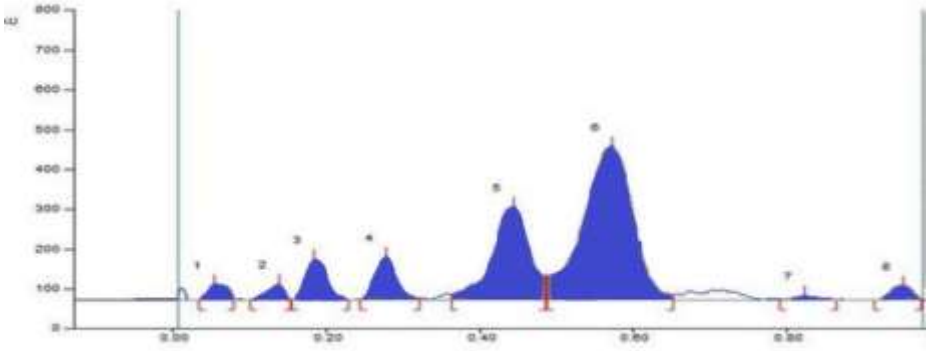


Figure 9: HPTLC chromatogram area and peaks of phenolic compounds

Table 7: Rf values of phenolic compounds

Peak	Rf value	Heights	Area %
1(Unknown)	0.05	110	2.32
2 (Unknown)	0.14	105	2.09
3(Unknown)	0.19	185	6.41
4(Gallic acid)	0.28	186	6.84
5(Unknown)	0.45	218	25.08
6(Quercetin)	0.57	486	53.12
7(Thymol)	0.84	46	0.89
8(Unknown)	0.96	58	2.36

MTT Assay Method

The Ethyl acetate fraction of the methanol extract of Cinnamomum malabatrum was cultured in 96-well plates with HBL-100 cell lines at various doses (0.125, 0.25, 0.5, 1, and 2.5 mg/ml) for 72 hours. Through metabolism, active (viable) cells reduce MTT, leading to the formation of formazan crystals. As shown in Figure 10, the Ethyl acetate fraction of the methanol extract of Cinnamomum malabatrum resulted in a percentage decrease in proliferation. After creating the graph in Excel, we determined the cell viability %. According to Table 8, the ethyl acetate portion of the methanolic Cinnamomum malabatrum extract reduced cell viability by 8% at a concentration of 2.5 mg/ml after 72 hours. The percentage of viable cells during growth and proliferation decreased significantly as the dose and duration increased compared to control cells that were not treated.

TABLE 8: In vitro cytotoxicity of methanol extract of Cinnamomum malabatrum on HBL-100 cell lines measured by the MTT assay

S.No	Concentration (mg/ml)	Average reading at 570 to 600 nm	% cell viability
1	0.125	2.24132	96
2	0.25	1.68	72
3	0.5	1.161	51
4	1	1.163	30
5	2.5	0.66480	8

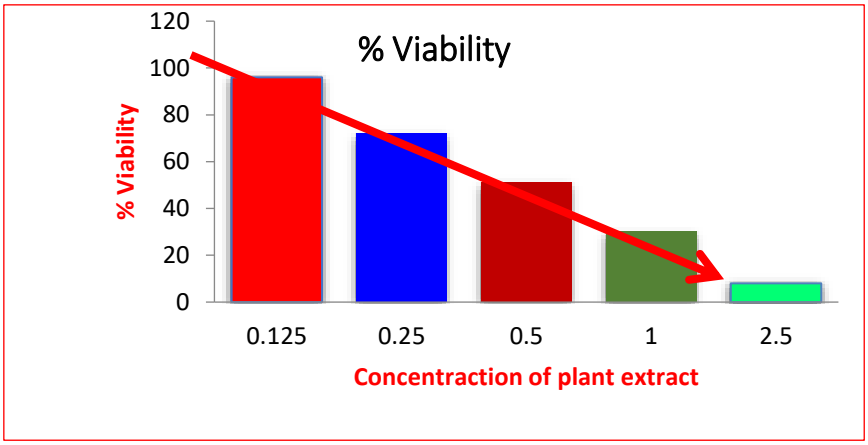


Figure 10: Average concentrations vs. %Viability

DISCUSSION

Successfully searching for anti-cancer medications from organic materials to treat human tumours has been made possible by isolating active components. Ethnopharmacological knowledge helps direct the hunt for cytotoxic plants.[17] This research aimed to determine whether the traditional plant Cinnamomum malabatrum leaves had any cytotoxic effects on cancer cells or cancer-related diseases. Identifying biologically active chemicals from plants has often used ethnopharmacological data, which is information derived from the historical medical use of plants.[18] The wealth of ethnopharmacological literature can provide some justification for the purported use in terms of its biological potential.[19] The anti-cancer activity of the Cinnamomum malabatrum plant was chosen for the investigation based on its known traditional usage. Cinnamomum malabatrum leaves, thought to contain primarily semi-polar chemicals, including flavonoids and phenolic compounds, were utilized in this study as a cancer treatment in an ethanolic extract. According to traditional healers, water decoction was given to patients when asked about the extract preparation process. This provides more evidence that the anti-cancer benefits were caused by the polar chemicals. The MTT test was used to study the anti-cancer efficacy of a methanolic extract of Cinnamomum malabatrum leaves on HBL-100 breast cancer cell lines. Living cells contain an enzyme called succinate-dehydrogenase, which breaks the tetrazolium ring and turns MTT into a purple formazan that cannot be dissolved. Hence, the formazan generated is proportionate to the number of live cells. Mosmann [15] demonstrated that, at a concentration of 2.5 mg/ml, the presence of chemicals in the methanol extract considerably arrested the proliferation of cancer cells.

Conclusion

The extraction of Cinnamomum malabatrum leaves is done by using different solvents. Cinnamomum malabatrum has other phytochemical compounds like alkaloids, flavonoids, phenols, saponins, and tannins. TLC HPTLC was analyzed further extract. The thin-layer chromatography indicates that methanolic extract had more active constituents. HPTLC study indicates that Ethyl Acetate fraction of Methanol extract showed confirmation of flavonoids and Phenolic compounds. The MTT assay method has given clear evidence of the anti-cancer activity

of the ethyl acetate fraction of methanol extract; in this method, extracts of Cinnamomum malabatrum leaves show anticancer activity. Based on the above evidence, Cinnamomum malabatrum leaf extracts were used to treat Cancer.

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