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Characterization and in-vitro cytotoxicity of lupeol isolated from leaf extract of ficus mysorensis

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***Abstract*---**Medicinal plant extracts gain more attention in research of modern medical sciences due to their non-lethal activity. The use of traditional medicine practices from plants has been accepted as a main sources for drug discovery in various health disorder especially cancer. Ficus mysorensis has a long history of usage as traditional medicine. The leaf extract of this plant possess phytochemicals such as alkaloids, flavonoids, terpenoids, steroids, saponins, Phenolics and glycosides. The ethanol leaf extract has been subjected to compound isolation and confirmed by GCMS, HPLC Chromatogram, ^{13}C and ^{1}H nuclear magnetic resonance (NMR), IR spectra. Based on spectral studies the isolated compound confirmed that Lupeol. Lupeol compound was evaluated against MCF-7 cell lines by MTT assay. Lupeol induced an effective change in the cell viability of MCF-7 cells with IC50 concentration (198.75 g/ml). Induction of cell death, change in cell morphology and cancerous cells population was observed in the treated cells, but the normal cells was not affected.

***Keywords*---**Ficus mysorensis, in-vitro cytotoxicity, Lupeol, MCF-7.

1. Introduction

In the Indian traditional medicinal system, thousand of diverse plant species and their components are recognized to have therapeutic properties, and they are utilized as an alternative to prevent the long-term immune adverse effects of contemporary drugs (Mukherjee et al., 2014; Gokhale et al., 2003). The use of traditional medicine practices from plants has been accepted as a main sources for drug discovery and development progress in various health disorder especially cancer (Aruoma et al., 2003). *Ficus (Moraceae)* is a genus with over a thousand species found in subtropical and tropical zones (Wagner et al., 1999), including figs and sacred trees endemic to India. These plant leaf juice has been practiced for the cure of respiratory disorders, cough, diarrhea, hematuria, ear-ache, toothache, migraine, eye problems, gastric problems and scabies. The fruits and stem bark also used for the treatment of asthma, gonorrhoea, bleeding, paralysis, diabetes, diarrhoea, bone fracture, antiseptic, astringent, and antidote and scabies (Lambertini et al., 2005). *F. Religiosa* is said to have anticonvulsant activity (Siddique et al., 2011), acetyl cholinesterase inhibitory activity (Tarapore et al., 2013), and antianxiety activity (Saleem et al., 2004) in Ayurveda. Terpenoids, alkaloids, steroids, lignans, phenolic acids, flavonoids, volatile compounds, megastigmanes, and many pheophorbide have all been found in Ficus (Bafor et al., 2013; Nascimento et al., 1999). Modern drug discovery strategies concentrate on identifying structurally new chemotypes with strong and selective biological action by screening plants and other natural materials. [Kinghorn & Balandrin, 1993; Gullo, 2013; Colegate & Molyneux, 2007; Cragg et al., 1997). Breast cancer are revealed to be the most probable users of alternative herbal medicines accounting for a large share of this group (Morris et al., 2000; Newman et al., 1988). This preference for herbal medicines have less adverse effects than synthetic/artificial chemicals (Dubey et al., 2004).

In-vitro cytotoxicity tests are used to assess the efficacy of a target medicine in inducing apoptosis [Akter et al., 2016). Their primary goal is to detect cell death and growth impairment. The adoption of the appropriate model in cancer research is critical for the study of proliferation and cancer progression (Ferreira et al., 2013). Till date many cancer cell lines are available and consider as a appropriate tools in biological research for the development of new therapeutic medications for cancer treatment. They are a good model for studying cancer-related biological mechanisms (Pandey et al., 2007).

Lupeol, a natural pentacyclic triterpenoid found in many medicinal plants, it is a biologically active phytoconstituent and reported most effective anti-diabetic and anticancer drug in *in-vitro* studies (Lilly, 2011). Many fruits and vegetables such as mango, olive, and fig, contain lupeol, and possess the various biological properties including anti-inflammatory and antioxidant characteristics (Jin et al., 2012). Lupeol inhibits the proliferation of MDA-MB-231 cells in a dose-dependent manner and has the ability to activate estrogen receptor (ER) expression in this ER-negative breast cancer cell line [Kunwar & Bussmann, 2006]. It's also clear that the molecule could be used to fight tumors such human prostate, colorectal, skin, liver, and stomach cancer. [Vyawahare et al.., 2007; Vinutha et al., 2007; Ratnasooriya et al., 1998; Liu et al., 2013; Lee et al., 2011; Wu et al., 2013).

Researches on Ficus has centered on its edible component (fruits) followed by aerial roots and barks, while the leaves are rarely investigated. Surprisingly, no extensive research on the *F. mysorensis* species has been published. In this paper, we will discuss the qualitative analysis of extracts, isolation and confirmation of Lupeol compound and evaluation of cytotoxic potential against MCF-7 cell line.

2. Materials and Methods

2.1 Plant material collection and extraction: Leaves of *Ficus mysorensis* plants were collected from Kuvempu University campus in Shankaraghatta in the month of June 2016, the plants were identified by Dr. V. Krishna, Professor, Department of Biotechnology, Kuvempu University. Sequential Soxhlet extractions with Ethyl acetate, chloroform, ethanol, and aqueous extract were performed on the collected samples. The resulting extracts were concentrated and desiccated until further examination using a rotary evaporator.

2.2 Phytochemical Screening:
Standard techniques were used to conduct the phytochemical analysis, which was done qualitatively (Harborne, 1998; El-Olemy et al., 1994). The leaf extract was used further studies like of phytochemical ingredients, isolation, characterization and their cytotoxicity studies. The extracts were subjected to column chromatography on silica gel (60-120 mesh) with a gradient elution using chloroform: Methanol as the mobile phase. Single spot Single spot was observed on TLC at Rf 0.75 using chloroform: Methanol (8.5:1.5) as mobile phase.

2.3 GC- MS analysis:
A Shimadzu GC – MS – QP 2010 gas chromatograph with a DB1 (methylphenylsiloxane, 30 m 0.25 mm i.d.) capillary column was used for the GC – MS analyses. Column temperature is 70° C, 5 min in 180°C, 180-260°C at 3°C/min, 5 min in 260°C, 260-280°C at 0.2°C/min, and finally 5 min in 280°C; injector temperature 280°C, detector temperature 290°C 1 litre of trimethylsilyl (TMS) ether derivatives in n-hexane (2%) was injected; the split ratio was 3:0. Ionization potential 70 eV; ion source temperature 200°C; quadrupole 100°C, Solvent delay 6.0 min, scan speed 2000 amu/s and scan range 30-600 amu, eV voltage 3000 volts were the MS operating parameters. The GC/MS instrument receives the concentrated extract. Under increasing temperature, the sample was volatilized at the injection port and eluted through a capillary column [Cherkaoui et al., 2010).

2.4 HPLC Chromatogram:
For the separation of bioactive components, a ethanol leaf extract of *F. mysorensis* was subjected to HPLC analysis using a C18 HPLC column (150x4.6mm), 5 m diameter. Plant extract (5 l) was introduced into the apparatus, and an isocratically used mobile phase of methanol: water (90:10) was employed at a flow rate of 0.2 mL/min. Compounds were detected using a UV-detector at 254 nm (SHIMADZU, JAPAN HPLC instrument) (Christophoridou et al., 2005).

2.5 IR spectra:
Infrared spectroscopy is used to determine the presence of various functional groups in compounds. Purified compound from *F.mysorensis* leaf extracts was appropriately dried in an oven, and approximately 5 mg of the chemical was course fine with potassium bromide (KBr) pellets for IR analysis in the 4000-500 cm-1 range (Popova et al., 2009).

2.6 ^{13}C and ^{1}H- NUCLEAR MAGNETIC RESONANCE (NMR):
The purified compound from Ficus sp. leaf extract was dissolved in DMSO and subjected to 125MHz 13C and 1H NMR spectroscopic investigations (Bruker). The 13C-spectra provides information on several carbon functional groups, whereas the 1H-NMR shows the overall number of protons associated with many groups (Kemp, 1991).

2.7 Screening for Cytotoxic Activity:
Trypsinized cells were aspirated into a 15ml centrifuge tube. Centrifugation at 300 x g yielded a cell pellet. Using DMEM media, the cell count was adjusted so that 200l of suspension contained roughly 10,000 cells. 200l of the cell suspension was added to each well of the 96 well microtitre plate, and the plate was incubated for 24 hours at 37°C in a 5% CO2 environment. The used medium was aspirated after 24 hours. 200l of various test drug concentrations (10, 20, 30, 40, and 50 g/ml from B NPs stock) (50, 100, 150, 200, and 250 g/ml from B extract stock) were added to the appropriate wells. The plate was then incubated for 24 hours at 37°C in a 5% CO2 environment. The drug-containing media was withdrawn from the incubator. Thereafter, 100l of media containing 10% MTT reagent was added to each well to achieve a final concentration of 0.5mg/ml, and the plate was incubated for 3 hours at 37◦C in a 5% CO2 environment. The culture media was fully drained without affecting the crystals that had grown. The plate was then gently shaken in a rotary shaker with 100l of solubilisation solution (DMSO) to solubilize the produced formazan. The absorbance was measured using a microplate reader at 570 nm and 630 nm wavelengths. After subtracting the background and blank, the percentage growth inhibition was obtained, and the concentration of test drug required to inhibit cell growth by 50% (IC50) was calculated from the cell line's dose-response curve (Gerlier & Thomasset, 1986; Mosmann, 1983).

3. Results and Discussion
3.1 Qualitative phytochemical analysis:
The extracts so obtained from *F.mysorensis* were subjected to preliminary phytochemical screening and the results are displayed in table 1.

Table 1: Ficus Mysorensis Extracts Were Screened For Phytochemicals

Sl.No.	Test	Procedure	Observation	Ethyl Acetate	Chloroform	Distilled water	Ethanol
1	Flavanoids	Extract + zinc + Hcl reduction test	Magenta color	–	–	+	+
2	Anthroquinone	Extract + Anthrone + H2SO4 +Heat	No Purple color	–	+	+	+

	Glycosides						
3	Phenols	Extract + $FeCl_3$+1ml of sample	Deep violet color or black precipitate with ferric ions was absent.	−	+	−	+
4	Tannins	Extract + lead acetate + water	No White ppt.	−	−	−	−
5	Terpenoids	Extract 2ml + chloroform + con. H2SO4	Lower layer turns yellow	+	−	+	+
6	Alkaloids	Extract (2-3 ml filtrate) + add Dragondroffs reagent	No orange ppt.	−	−	+	+
		Extract (2-3 ml filtrate) + Mayer's reagent	No white ppt.	−	+	+	+
		Extract (2-3 ml filtrate) + Hager's reagent	No yellow ppt.	−	−	+	−
7	Saponins	Extract + 20ml water + Shake well	Formation of stable froth	−	−	-	+

+ = Present, **--** = Absent

3.2 GC-MS analysis:

GC-MS is one of the most effective methods for identifying volatile constituents such as branched chain hydrocarbons, alcohols, acids, and esters. The existence of 25 chemicals (phytochemical ingredients) in F. mysorensis leaves was discovered using GC-MS analysis, which could contribute to the plant's therapeutic qualities. The peak area and retention duration were used to confirm the phytochemical components identity. The active principles with their Retention time (RT), molecular formula, peak area (%), peak height (%) and peak area are depicted in Table 2 and Fig 1. The less retention time (12.487) compound is 5-Methyl-phenyltetrahydro-1, 3-oxazine-2-thione, and longest retention time (47.851) compound is 6-Aza-2-thiothymine was identify.

Table 2: GC-MS Analysis Revealed the Presence of Phytochemical Components in Ethanol Leaf Extract of F. Mysorensis

S. No.	**Apex RT**	**Molecular Formula**	**Peak Area (%)**	**Peak Height (%)**	**Identification**
1	12.487	$C_{11}H_{13}$	0.45	1.40	5-Methyl-phenyltetrahydro-1,3-oxazine-2-thione
2	12.908	C_8H_{10}	0.36	1.37	Cis-Bicyclo[4.2.0]octa-3,7-diene

3	14.222	C14H12N2	0.75	2.66	Pyrazolo[1.5-a]pyridine , 3-methyl-2-phenyl
4	14.661	$C_8H_5N_3O_4$	1.36	4.45	2,3-Dihydroxy-6-nitroquinoxaline
5	15.708	$C_{14}H_{12}N_2O_2S$	0.18	0.61	Benzimidazole , 2-benzylsulfonyl
6	16.428	C10H9NO	0.09	0.40	Trans-1-Cyano-2-phenylcyclopropanol
7	17.076	C7H5N5O3	1.04	1.73	3-Amino-7-nitro-1,2,4-benzotriazine 1-oxide
8	18.73	C12H11NO2	0.33	0.85	Trans-Acetoxy-1-cyano-2-methyl-2-phenylcyclopropane
9	19.038	$C_{15}H_{12}N_4O_2$	6.04	3.87	Pyrazolidinetrione ,phenyl-,4-(phenylhydrazone)-
10	19.216	$C_{11}H_{17}NO$	0.67	2.30	Methylephedrine
11	21.995	$C_{10}H_{12}$	7.41	4.36	Dispiro[2.2.2.2]deca-4,9-diene
12	23.266	C10H12	0.46	1.58	Pentalane
13	23.873	$C_{10}H_9NO_2S$	0.65	0.99	1-Benzenesulfonyl-1H-pyrrolo
14	27.809	C10H12N4	0.40	1.43	2,6-Bis(diazo)adamantane
15	29.969	C3H5NO	23.16	11.01	N-Benzenesulfonylazetidin-3-one
16	34.719	C8H11NO	0.28	1.07	Benzyloxymethylimine
17	35.95	C11H17N	0.23	0.77	2,3-Dimethylamphetamine
18	36.175	C12H17NS	0.23	0.65	(+)-trans-3,4-Dimethyl-2-phenyltetrahydro-1,4-thiazine
19	36.343	C16H24O2	0.91	2.47	2H-Pyran,tetrahydro-2-(2,5-undecadiynyloxy)-
20	39.311	C10H12N2O	0.18	0.58	Phenylacetaldehyde N-methyl-N-formylhydrazone
21	39.683	$C_{12}H_{17}NO$	2.37	0.80	Pentanal O-benzyloxime
22	41.694	$C_7H_{10}O_2S$	0.27	0.83	Acetoacetic acid , 1-thio-, S-allyl ester
23	44.034	C9H16ClNO	0.62	1.69	Benzenemethanol , α-(1-aminoeethyl)- .[R,(R*,R*)]
24	47.116	C9H14S	0.14	0.53	2-Cyclopentene-1-thione , 2,3,4,4-tetramethyl-
25	47.851	C4H5N3OS	0.57	1.79	6-Aza-2-thiothymine

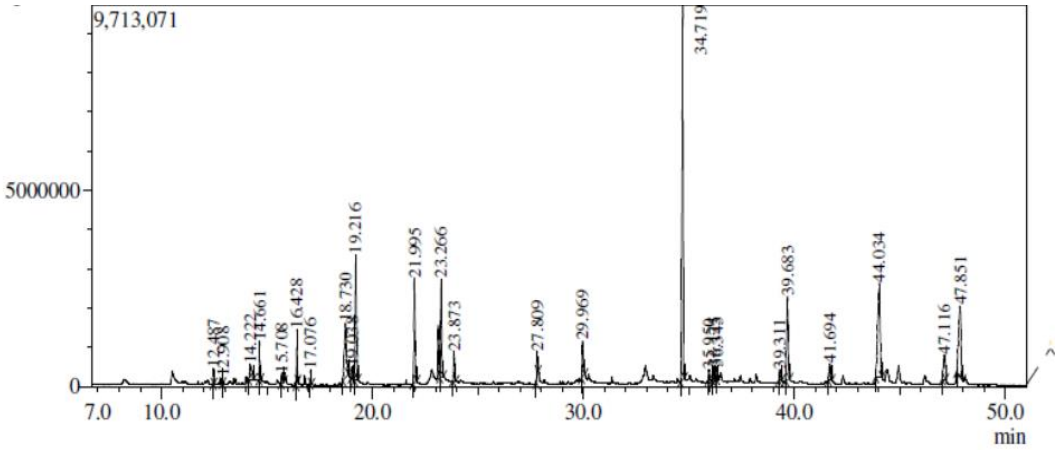
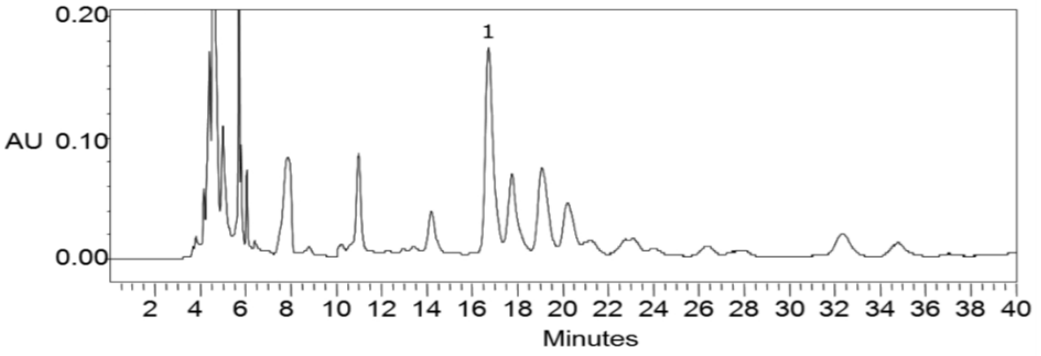


Figure 1: GC-MS chromatogram of ethanol leaf extract of F. mysorensis

3.4 HPLC Chromatogram:

Lupeol was discovered as a white amorphous powder. Its IR spectra revealed a very broad band at 3425 cm^{-1} for the hydroxyl group's O-H bond vibration. The accompanying C=C vibrations were seen as a slightly concentrated band approximately 1658 cm^{-1}. The ion peak at 34.4 m/z, corresponding to the chemical formula C30H50O, was discovered in the mass spectra of compound lupeol. The spectral studies found to be consistent with lupeol characteristics previously published in the literature. It was observed that the presence of six tertiary methyl groups signals at 0.74, 0.76, 0.84, 0.91, 0.99, and 1.01, as well as one isopropenyl moiety at 1.65, indicated a lupane skeleton in the ^{1}H NMR data. The most prevalent ^{1}H NMR signals at 4.58 and 4.69 relate to vinyl hydrogen present at C-29, which is sufficient to support lupeol skeleton. Also evident in the form of hydrogen signals at 3.17 and 2.35 for C-3 and C-19, respectively. The existence of C-29 is confirmed by ^{13}C NMR, which shows a signal at 105. In addition, as indicated in Table 3, the 13C NMR spectra revealed 30 carbon signals, which are typical of lupeol. The large coupling constant (J = 10 Hz) between H-3 and H-2, H-4 are axially oriented, implying that the hydroxyl group at C-3 is equatorially orientated (Lee, C. K., & Chang, 2000). The molecule was recognized as lupeol based on the foresaid evidence, and its spectral data were in good accord with the known literatures [Iman et al., 2007]. Figure 2 shows one distinctive peak with retention time of 16.885 with concentration of 86.658 mg/L.



Peak#	Ret. Time	Area	Height	Conc.
1	16.885	11187839	2255595	86.658
Total		11187839	2255595	

Figure 2: HPLC Chromatogram of ethanol leaf extract of F. mysorensis

3.5 ^{13}C and ^{1}H- Nuclear magnetic resonance (NMR)

The purified leaf extract of *Ficus mysorensis* was dissolved in DMSO and subjected to ^{13}C and ^{1}H- NMR spectroscopic studies 125MHz (Bruker). ^{13}C-spectra gives the information about various carbon functional groups (table 3 & figure 3) while ^{1}H- NMR indicates the total number of protons associated with several groups as follows ^{1}H NMR (DMSO, 400 MHz) δ 0.74 (s, Me-24), 0.76 (s, Me-28), 0.84 (s, Me-25), 0.91 (s, Me-27), 0.99 (s, Me-23), 1.01 (s, Me-26), 1.65 (s, H-30), 3.14 (dd, J= 5.0, 11.1 Hz, H-3), 4.58 (brs, H-29b), 4.69 (brs, H-29a) (figure 4).

Table 3: ^{13}C-NMR spectra of lupeol (DMSO, 100 MHz)

Position	Frequency (MHz)	Position	Frequency (MHz)
1	39.01	16	35.78
2	27.06	17	42.97
3	79.04	18	47.99
4	39.06	19	48.06
5	54.99	20	149.96
6	18.54	21	30.01
7	33.98	22	39.98
8	39.96	23	28.33
9	51.01	24	15.55
10	36.99	25	15.97
11	21.05	26	16.23
12	24,97	27	15.02
13	37.96	28	17.98
14	41.98	29	110.06
15	27.54	30	20.03

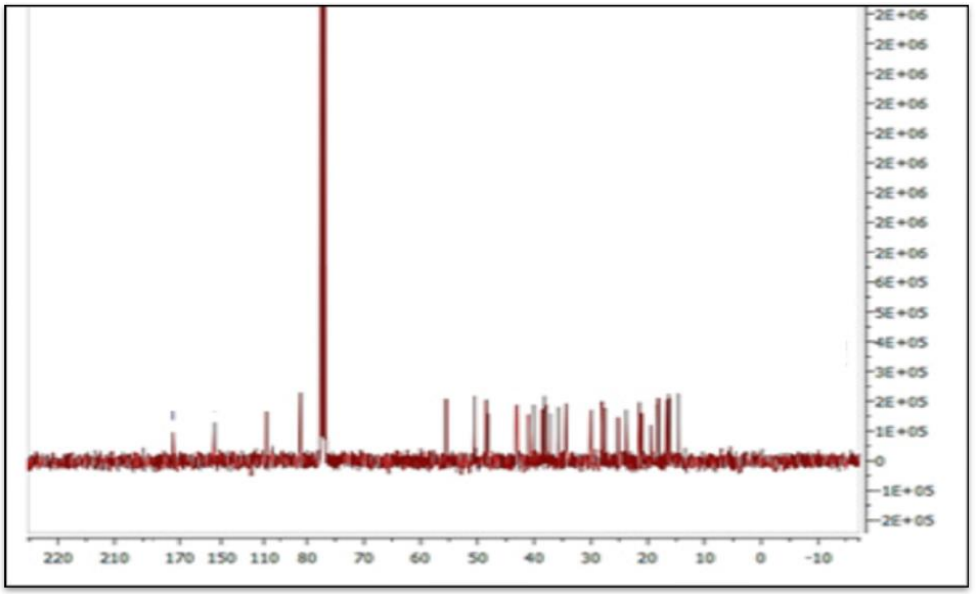


Figure 3: Spectra of ^{13}C-NMR of Lupeol.

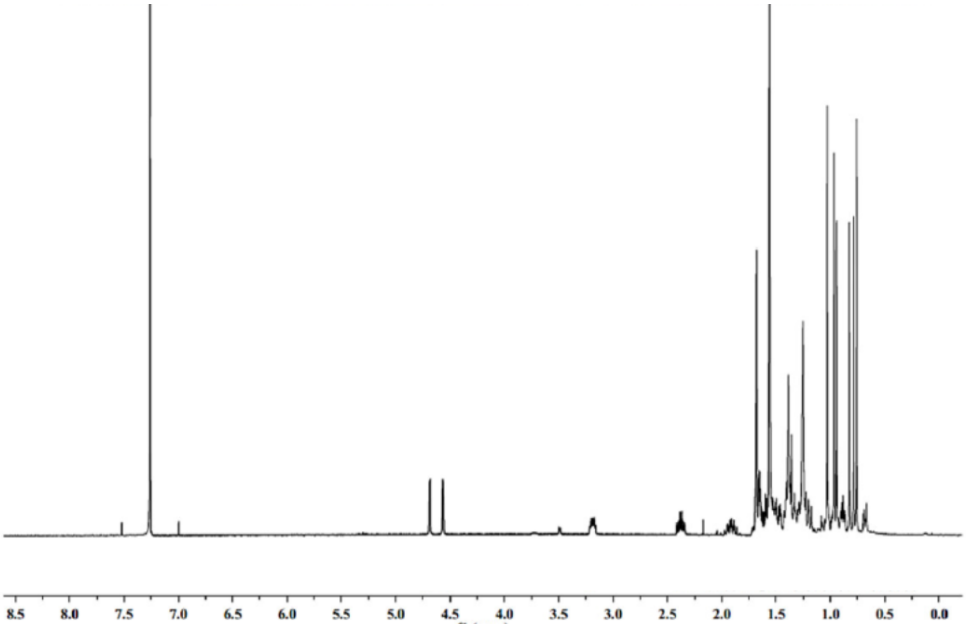


Figure 4: Spectra of ^{1}H-NMR of Lupeol

3.6 IR spectra:

The IR spectrum of Compound, as shown in Figure 5 showed the C-H stretch absorption presence at 2949 cm-1, as well as C-H bond stretching at a wavelength of 1037.6 cm-1. At the IR, a single C=O bond stretching at 1539.2 cm-1 was also seen. The IR spectrum was found to be very similar to previously reported in the literature (Kalegari et al., 2011).

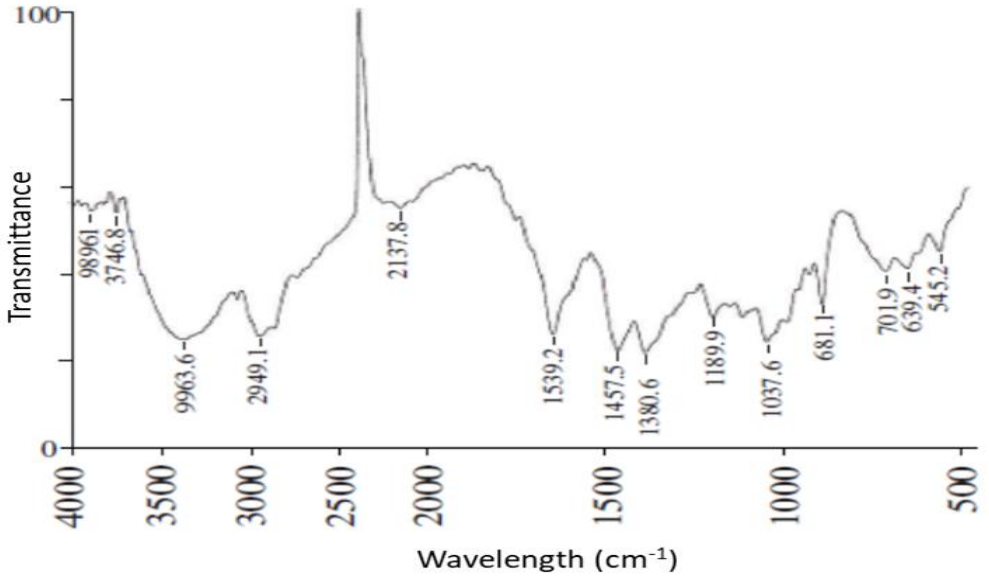


Figure 5: Spectra of IR of Lupeol

3.7 In-Vitro Cytotoxicity:

The cytotoxic effect of Lupeol isolated from *F. mysorensis* leaf ethanol extract was dose dependent and reported as inhibitory concentration 50 (IC_{50}). Lupeol showed IC50 of (198.75g/ml) cell line. The goal of employing anticancer medications is to stop cancer cells from multiplying without harming healthy cells. The viability of cancer cells was influenced by Lupeol compound in the current investigation, but normal cells was not affected. As a result, the current investigation backs up the traditional medical claims that *F. mysorensis* leaves exhibits considerably good *in-vitro* cytotoxic activity against MCF-7 cell-line. Table 4 shows the IC50 values of the test compounds for the MCF-7 cell-line after 24 hours of treatment. cytotoxicity of cell line was observed in figure 6 & figure 7. Lupeol is a natural triterpenoid found in many medicinal plants. A vast number of triterpenoids has been reported to have cytotoxicity against a range of cancer cells while causing no damage in normal cells. They also indicate antitumor effectiveness in preclinical cancer animal models (Setzer, W. N., & Setzer, 2003; Liby, 2007; Laszczyk, 2009).

Table 4: IC50 values of test compounds

Sample name	**MCF-7 cell line IC_{50} (in µg/ml) 24hr**
Lupeol compound	198.75
Cisplatin	10.19

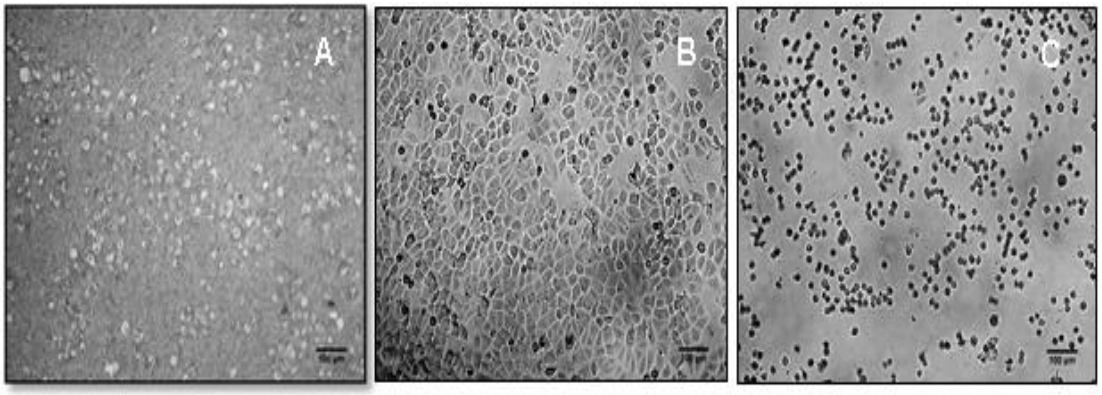


Figure 6: Cytotoxicity activity (A) MCF-7 Lupeol, (B) Standard Cisplatin treated cells (C) Untreated cells

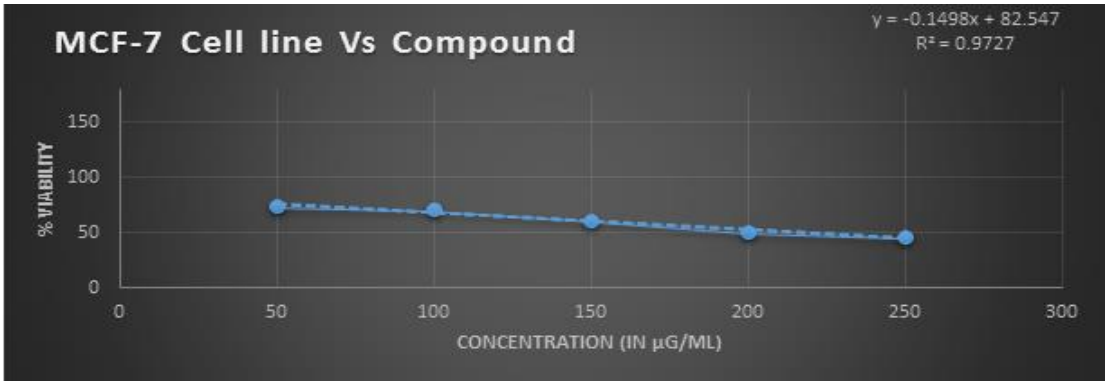


Figure 7: Graph showing MCF-7 of Lupeol

4. Conclusions

The current research culminates with the isolation and identification of lupeol, as well as its efficacy as a triterpenoid isolated. The Lupeol from the leaf extract of *F. mysorensis* inhibited the development of MCF-7 breast cancer cells and significantly reduced their viability. The current study began with the goal of determining the compound's anticancer effect, and additional *in-vivo* studies will be required in the future to identify other targets of the compound and to investigate its effect on other cancer signaling pathways, which could lead to more effective knowledge about the compound's use in breast cancer treatment.

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