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REVIEW ARTICLE

Anticancer activity of *Hybanthus enneaspermus (Linn) F. Muell* extracts on A549-CCL185 (Lung Adenocarcinoma cell)

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ABSTRACT:
Aim: This study aims to elucidate the fundamental apoptotic mechanisms and evaluate the anticancer efficacy of whole plant extracts of *Hybanthus enneaspermus (Linn.) F. Muell* against human lung adenocarcinoma (A549-CCL185) cells. **Methods:** The whole plant was gathered, analyzed, shade-dried, and then extracted in stages with water, ethanol, ethyl acetate, and chloroform using the Soxhlet and cold maceration techniques. We used the MTT assay to find out how toxic the extracts were to A549-CCL185 cells. To verify apoptosis, DNA fragmentation was analyzed through agarose gel electrophoresis, and morphological alterations were assessed using acridine orange/ethidium bromide (AO/EtBr) dual staining. **Results:** The ethanolic extract (EEHE) demonstrated the most significant cytotoxic effect among all tested extracts (IC_{50} = 37.88µg/ml), while the ethyl acetate extract (EAEHE) displayed moderate activity (IC_{50} = 215.39 µg/ml). AO/EtBr staining showed clear signs of apoptosis, such as breaking up of chromatin, condensation of the nucleus, and shrinking of the cell. In cells treated with EEHE, apoptotic cell death was verified by DNA ladder formation. Phenolic and flavonoid compounds that encourage mitochondrial dysfunction brought on by reactive oxygen species (ROS) and cause programmed cell death are thought to be responsible for the cytotoxicity. **Conclusion:** *Hybanthus enneaspermus's* ethanolic extract significantly inhibits the growth of A549-CCL185 lung adenocarcinoma cells and induces apoptosis. These results point to its potential as a natural source of chemotherapeutic agents and validate its use in ethnomedicine. It is necessary to conduct additional research that includes in vivo validation and the isolation of active ingredients.

KEYWORDS: *Hybanthus enneaspermus*, A549-CCL185 cells, apoptosis, MTT assay, cytotoxicity.

INTRODUCTION:
Cancer:
Cancer is a cellular disorder marked by the unregulated proliferation of atypical cells that can infiltrate and disseminate throughout the body. The human body comprises trillions of cells, allowing cancer to potentially arise in nearly any tissue. Normally, cells grow and die through regulated processes of division and apoptosis. However, injury or genetic abnormalities may cause cells to multiply excessively, forming tumors. Tumors may be benign or malignant, and cancer refers to malignant forms capable of spreading and destroying normal tissue.[1-4]

Lung Adenocarcinoma:
Lung adenocarcinoma originates from the epithelial cells of the lungs. Approximately 80% of cases are due to tobacco use, which is a major risk factor not only for lung cancer but also for several other malignancies. Nitrosamines and polycyclic aromatic hydrocarbons are among the nearly fifty known carcinogens found in tobacco smoke. Tobacco use is responsible for one-fifth of cancer deaths globally and one-third of cancer deaths in developed countries.[5-9] mall cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC) are the two main types of lung adenocarcinomas that are distinguished by their cell morphology. Accurate classification is essential for treatment because these subtypes vary in their growth pattern, spread, and therapeutic response.[8,10,11]

Plant Profile – *Hybanthus enneaspermus (L.) F. Muell*
Hybanthus enneaspermus of the Violaceae family is a small perennial herb or shrub (15-30 cm tall), found throughout tropical and subtropical regions. It is commonly known as "spade flower" or "pink lady's slipper".[3,12,13]

Taxonomy:

Hierarchy	Name
Kingdom	Plantae
Phylum	Tracheophyta
Class	Magnoliopsida
Order	Malphighiales
Family	Violaceae
Subfamily	Violoideae
Genus	*Hybanthus*
Species	*Enneaspermus*

Vernacular Names:
Ayurveda-Sthalakamala, Hindi-Ratan Purush, Kannada-Purusharathna, Malayalam-Orithalthamara, Tamil – Orilaithamarai.[13,14]

Parts used:
Whole plant

Ethnomedical uses:
In conventional medicine, it is used to treat cholera, sterility, leucorrhea, diarrhea, urinary tract infections, inflammation, and dysuria. Whole plant: a general tonic for expectant mothers. Root infusion: a diuretic used to treat urinary tract infections and gonorrhea. Leaves: applied externally to wounds for healing purposes. The anti-venom properties of the fruit protect against scorpion and snake stings.[3,13-15]

Figure 1: Entire plant of *Hybanthus enneaspermus* in natural habitat

2. MATERIALS AND METHODS:

Phytochemical Studies:
Phytochemical investigation includes authentication, extraction, and characterization of plant material to identify bioactive constituents and assess potential pharmacological activity.[16-19]

Generation of *Hybanthus enneaspermus* Extract:
Extracts were prepared using the Soxhlet reflux extraction technique, where solvent vapors condense and repeatedly wash the plant material to extract phytoconstituents. The condensed solvent carrying dissolved compounds is collected and concentrated by distillation.[17-19]

Solvents Used:
Chloroform, Ethyl acetate, Ethanol, and Water.

2.1 Plant Collection and Identification:
The whole plant of *Hybanthus enneaspermus* was collected in January from Tindivanam, Villupuram District, Tamil Nadu, India. Authentication was done by Dr. P. Jayaraman, Director, Plant Anatomy Research Centre (PARC), Chennai. A voucher specimen (Reg. No. PARC/2022/4636) was deposited for reference.

2.2 Preparation of Plant Material:
The fresh plant was washed, shade-dried, crushed mechanically, and sieved (No. 40). The powdered material was stored in an airtight container for extraction.

2.3 Successive Extraction Method[17-19]
Defatting: Petroleum ether (60–80°C) was used in a Soxhlet apparatus to defatten coarsely ground plant material until a clear solvent was produced.

Extraction:
The defatted marc was successively extracted with chloroform, ethyl acetate, and ethanol by continuous hot extraction, followed by aqueous extraction through cold maceration. A rotary evaporator was used to concentrate each extract at 40°C under reduced pressure after it had been filtered through Whatman No. 1 filter paper, and stored below 10 °C. Extraction was completed when the siphon solvent became colorless, indicating exhaustion of plant material

Extraction yields were weighed and calculated using the formula as followed

$$\text{Yield (\%)} = \frac{\text{Dry weight of extract}}{\text{Dry weight of plant of extract}} \times 100$$

Each extract's color, consistency, and yield percentage were recorded.

Figure 2: Soxhlet extraction set-up of *Hybanthus enneaspermus*[19,20]

Pharmacological Studies:

Cell Line: A549-CCL185 obtained from NCCS Pune; cultured in F12/K medium (Gibco) with 10% FBS, 100 U/ml penicillin, 100µg/ml streptomycin at 37°C, 5% CO_2. For cryopreservation, medium was supplemented with 5% DMSO (1ml aliquots, ~5 × 10^6 cells).[21,22]

MTT Assay: Cells (1 × 10^4/well) were seeded in a 96-well plate with 10% FBS medium. After 24h, samples of varying concentrations were added and incubated for 24h. Then, 20µl MTT (2mg/ml in PBS) was added and incubated for 4h. After removing supernatant, 100 µl DMSO was added to dissolve formazan crystals. Absorbance was measured at 570nm, and viability (%) was calculated as (Sample Abs/Control Abs) × 100.[21-23,27-29]

Morphology by Dual Staining (AO/EtBr): A549 cells were treated with sample at IC_{50} and below IC_{50}, incubated 24h, fixed with 4% paraformaldehyde (15 min), and stained with 50µM AO/EtBr for 10 min in dark. Cells were washed with PBS and examined under an inverted fluorescence microscope.[24-26]

DNA Fragmentation Assay: Treated A549 cells (10^6) were lysed in Tris-EDTA buffer (pH 8.0), followed by Tris-EDTA-SDS-proteinase K solution (37°C, 3h). DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1), treated with RNase (20µg/ml, 45min, 4°C), and precipitated using sodium acetate and ethanol. DNA (10µg) was run on 2% agarose gel (20V, 1h) with ethidium bromide for fragmentation analysis.[26]

3. RESULTS:

3.1 Preparation of extracts:

Each extract's percentage yield was determined using the dried weight of the plant material. Table No. 1 lists the concentrated extracts' color and consistency.

Table 1: *Hybanthus enneaspermus* extracts' percentage yield

Extract	Extraction Technique	Physical nature	Color	Yield (% w/w)
Chloroform	Soxhlet apparatus for continuous hot percolation method	Semisolid	Dark green	6.55
Ethyl acetate		Semisolid	Dark green	3.46
Ethanol		Semisolid	Dark green	4.2
Aqueous	Cold maceration	Semisolid	Dark brown	3.9

The polarity and solubility specificities of the metabolites in the plant powder have been revealed the usage of extraction values. The share yield of the numerous became as follows: ethyl acetate (3.46%), ethanol (4.2%) and chloroform (6.55%). Among the extracts, the chloroform extract showed the best extraction yield.

3.2 In vitro Cytotoxicity assay- MTT assay (A549-CCL185 cell line)

Ethyl acetate extract of *Hybanthus enneaspermus* (Linn) F. Muell

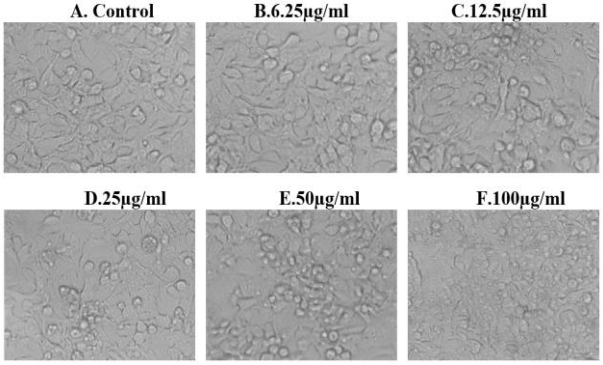

Figure 3: (A-F) Cytotoxic effect of EAEHE on A549-CCL185 Cell Line

Table 2: *Invitro* cytotoxicity assay for EAEHE (MTT Assay)

Sample	Concentration	Singlet OD	Duplicate OD	Triplicate OD	Blank Mean OD	Mean OD-Blank Mean OD	STD DEV	% of Viability	IC50
Blank	0	0.022	0.022	0.021	0.021	-	-	-	-
Control	0	1.254	1.243	1.256	1.251	1.229	0.007	100	-
EAEHE	6.25	1.218	1.225	1.216	1.219	1.198	0.0047	97.451	-
	12.5	1.112	1.119	1.114	1.115	1.093	0.0036	88.937	215.39
	25	0.983	0.976	0.989	0.982	0.961	0.0065	78.172	-
	50	0.852	0.865	0.847	0.854	0.833	0.0092	67.760	-
	100	0.775	0.784	0.782	0.780	0.758	0.0047	61.713	-

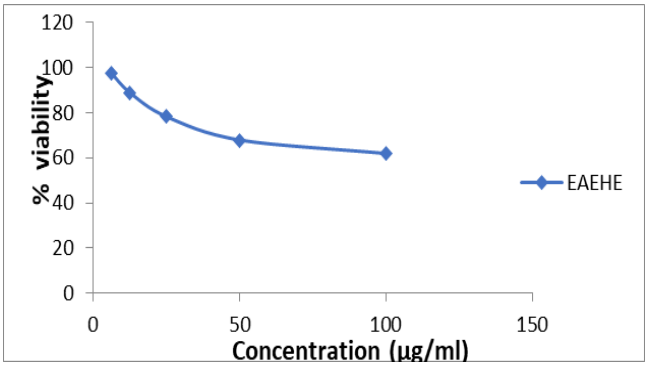

Figure 4: Effect of EAEHE on A549-CCL185 cell death

Ethanolic extract of *Hybanthus enneaspermus* (Linn) F. Muell

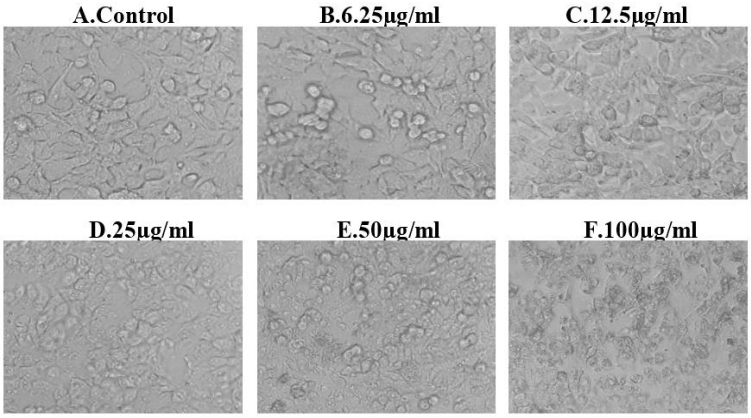

Figure 5: (A-F) Cytotoxic effect of EEHE on A549-CCL185 Cell Line

Table 3: *Invitro* cytotoxicity assay for EEHE (MTT Assay)

Sample	Concentration	Singlet OD	Duplicate OD	Triplicate OD	Blank Mean OD	Mean OD-Blank Mean OD	STDEV	% of Viability	IC50
Blank	0	0.022	0.022	0.021	0.021	-	-	-	-
Control	0	1.254	1.243	1.256	1.251	1.229	0.007	100	-
EEHE	6.25	1.227	1.219	1.232	1.226	1.204	0.0065	97.966	-
	12.5	1.006	1.011	1.004	1.007	0.985	0.0036	80.151	37.88
	25	0.743	0.752	0.768	0.754	0.732	0.0126	59.598	-
	50	0.531	0.547	0.539	0.539	0.517	0.008	42.082	-
	100	0.336	0.329	0.332	0.332	0.310	0.0035	25.271	-

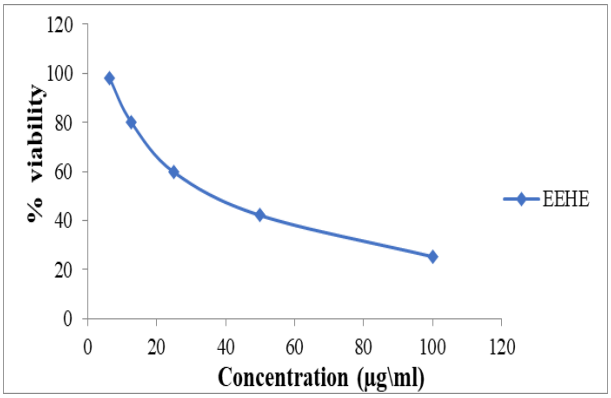

Figure 6*: Effect of EEHE on A549-CCL185 cell death.*

The cytotoxic potential of ethyl acetate (EAEHE) and ethanol (EEHE) extracts of *Hybanthus enneaspermus* was evaluated on A549-CCL185 cells using the MTT assay at concentrations of 12.5, 25, 50, and 100 µg/ml (Fig. 3-6; Table 2 & 3). The IC_{50} values for EAEHE and EEHE were 215.39 µg/ml and 37.88 µg/ml, respectively. Both extracts exhibited antiproliferative activity, with the ethanol extract showing significantly higher cytotoxicity. Based on its lower IC_{50} value, EEHE was selected for further investigations.

3.3 Cell morphological study:

Fluorescence microscopy of A549-CCL185 cells treated with EEHE at IC_{50} and sub-IC_{50} concentrations revealed

distinct morphological alterations (Fig. 7). Cell shrinkage, nuclear condensation, and fragmentation were all visible with AO/EtBr dual staining, suggesting apoptosis. While cells stained orange indicated late apoptotic stages with the formation of apoptotic bodies, green fluorescence indicated viable cells. Increased EEHE concentration confirmed its apoptotic effect by intensifying nuclear fragmentation.

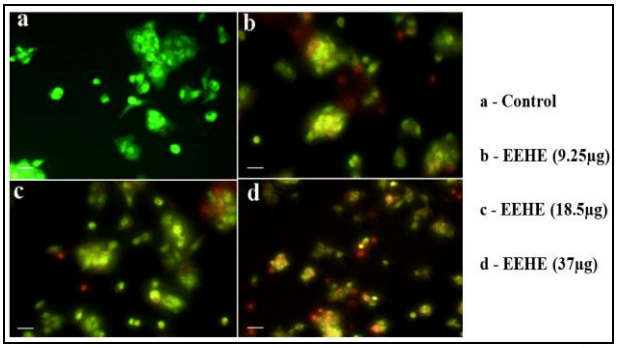

Figure 7: EEHE-treated cells' morphological appearance after being further stained with AO/EtBr

3.4 DNA fragmentation assay:

A genomic DNA fragmentation test was used to evaluate EEHE's capacity to induce cell death in A549-CCL185 cells. While control cells showed intact DNA with little damage, cells treated with IC_{50} and sub-IC_{50} concentrations of EEHE displayed clear DNA ladder formation on a 2% agarose gel, indicating apoptosis. The fragmentation pattern validated the degradation of apoptotic DNA caused by EEHE. A smaller number of G_1-phase cells and a subsequent G_2-phase arrest were linked to this process. Reactive oxygen species (ROS), which functioned as signaling molecules that promoted DNA damage, cell shrinkage, and programmed cell death, most likely caused the induced apoptosis through mitochondrial dysfunction and ROS generation. (Figure 8).

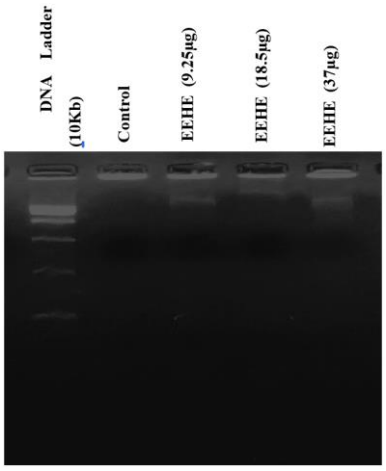

Figure 8: DNA fragmentation patterns in A549-CCL185 cells treated with EEHE

4. DISCUSSION:

The whole plant extracts of *Hybanthus enneaspermus (Linn.) F. Muell* were shown in this study to have anticancer properties against human lung adenocarcinoma (A549-CCL185) cells. Of the extracts that were tested, the ethanolic extract (EEHE) showed strong antiproliferative activity and higher cytotoxicity (IC_{50} = 37.88 µg/ml) than the ethyl acetate extract (EAEHE, IC_{50} = 215.39 µg/ml). The MTT test verified a dose-dependent decrease in cell viability, indicating that EEHE successfully disrupts mitochondrial activity and triggers apoptosis. Apoptotic death was confirmed by fluorescence microscopy (AO/EtBr staining), which showed morphological changes like nuclear fragmentation, chromatin condensation, and cell shrinkage. Additionally, DNA fragmentation analysis revealed distinctive ladder patterns in cells treated with EEHE, which suggested apoptotic DNA cleavage. These outcomes imply that oxidative stress and mitochondrial-mediated pathways are the mechanisms by which EEHE causes programmed cell death. Phenolic and flavonoid compounds like quercetin, rutin, and kaempferol, which are known to modulate intracellular ROS, activate caspases, and arrest cell cycle progression, may be responsible for the observed cytotoxicity. The increased cytotoxic effect of EEHE is correlated with its higher total phenolic and flavonoid contents. Similar phytochemical mechanisms, where ROS generation and mitochondrial dysfunction trigger apoptosis in carcinoma cells, have been documented in other plant-based anticancer studies. In summary, by preventing proliferation and triggering apoptosis, the ethanolic extract of *Hybanthus enneaspermus* demonstrates strong anticancer activity against A549-CCL185 lung adenocarcinoma cells. The results demonstrate its potential as a natural source of chemotherapeutic agents and offer scientific backing for its traditional medicinal use. To prove its therapeutic suitability in the treatment of lung cancer, more research concentrating on the isolation of bioactive compounds, the clarification of molecular pathways, and in vivo validation is necessary.

5. CONFLICT OF INTEREST:

The authors have no conflicts of interest regarding this investigation.

6. ACKNOWLEDGEMENTS:

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7. REFERENCES:

1. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011; 144(5): 646-74.
2. Stewart BW, Wild CP. World Cancer Report 2014. Lyon: IARC Press; 2014.
3. Ramesh B, Vijayalakshmi P. Pharmacological potential of Hybanthus enneaspermus: A review. Res J Pharm Technol. 2017; 10(8): 2769-73.
4. Vignesh R, Arul D, Kumar R. Antioxidant and antimicrobial activities of Hybanthus enneaspermus extracts. Res J Pharm Technol. 2016; 9(9): 1341-5.
5. Thun MJ, Carter BD, Feskanich D, Freedman ND, Prentice R, Lopez AD, et al. 50-year trends in smoking-related mortality in the United States. N Engl J Med. 2013; 368(4): 351-64.
6. Islami F, Torre LA, Jemal A. Global trends of lung cancer mortality and smoking prevalence. Transl Lung Cancer Res. 2015;4(4):327-38.
7. Molina JR, Yang P, Cassivi SD, Schild SE, Adjei AA. Non–small cell lung cancer: epidemiology, risk factors, treatment, and survivorship. Mayo Clin Proc. 2008; 83(5): 584-94.
8. Kumar S, Patel J, Sharma R. Lung cancer biology and therapeutic interventions: An update. Res J Pharm Technol. 2020; 13(7): 3261-8.
9. Gupta A, Banerjee S. Natural products as novel anticancer agents: Mechanistic insights. Res J Pharm Technol. 2021; 14(2): 881-7.
10. Herbst RS, Morgensztern D, Boshoff C. The biology and management of non–small cell lung cancer. Nature. 2018;553(7689):446-54.
11. Kirtikar KR, Basu BD. Indian Medicinal Plants. 2nd ed. Dehradun: International Book Distributors; 1999. p. 442-4.
12. Nadkarni KM. Indian Materia Medica. Vol. 1. Mumbai: Popular Prakashan; 2009. p. 640-2.
13. Kala CP. Ethnomedicinal botany of the Apatani in the Eastern Himalayan region of India. J Ethnobiol Ethnomed. 2005; 1: 11.
14. Nandhakumar L, Dhanalakshmi S, Saravanan R. Antioxidant and free radical scavenging activity of Hybanthus enneaspermus (L.) F. Muell. J Pharm Sci Res. 2011; 3(5): 1190-6.
15. Vignesh R, Arul D, Kumar R. Antioxidant and antimicrobial activities of Hybanthus enneaspermus extracts. Res J Pharm Technol. 2016; 9(9): 1341-5.
16. Harborne JB. Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis. 3rd ed. London: Chapman & Hall; 1998.
17. Kokate CK, Purohit AP, Gokhale SB. Pharmacognosy. 50th ed. Pune: Nirali Prakashan; 2023.
18. Evans WC. Trease and Evans Pharmacognosy. 17th ed. Edinburgh: Saunders; 2002.
19. Priya R, Kumaravel S. Soxhlet extraction and phytochemical analysis of selected medicinal plants. Res J Pharm Technol. 2018; 11(4): 1635-9.
20. Meena M, Ramesh C. Comparative phytochemical profiling of chloroform, ethyl acetate, and ethanolic extracts of medicinal herbs. Res J Pharm Technol. 2019; 12(9): 4358-63.
21. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983; 65(1–2): 55-63.
22. Prasad G, Rani S. Evaluation of cytotoxic activity of herbal extracts by MTT assay. Res J Pharm Technol. 2020; 13(3): 1367-71.
23. Sahu R, Patel A. In vitro anticancer screening of plant extracts using lung carcinoma cells. Res J Pharm Technol. 2019; 12(8): 3689-93.
24. McGahon AJ, Martin SJ, Bissonnette RP, Mahboubi A, Shi Y, Mogil RJ, et al. The end of the (cell) line: methods for the study of apoptosis in vitro. Methods Cell Biol. 1995; 46: 153-85.
25. Elmore S. Apoptosis: a review of programmed cell death. Toxicol Pathol. 2007; 35(4): 495-516.
26. Iqbal J, Verma K. DNA fragmentation and apoptosis studies of plant extracts in cancer cells. Res J Pharm Technol. 2021; 14(9): 4743-7.
27. Rani K, Srivastava V. Antiproliferative potential of polyphenolic compounds: An overview. Res J Pharm Technol. 2018; 11(8): 3611-5.
28. Dinesh P, Arunachalam G. Natural compounds targeting lung cancer: An update. Res J Pharm Technol. 2021; 14(6): 3041-6.
29. Prakash R, Sundar R. Cytotoxic and apoptotic effects of triterpenoids in lung carcinoma cells. Res J Pharm Technol. 2019; 12(12): 6001-6.