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Evaluation of *In-vitro* Anticancer Effect of Hydroalcoholic Extract of Lepidagathis spinosa Wight Ex Nees.

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Abstract

People, for the most part in rustic environments and lately, those unsatisfied with usual medicine, use medicinal plants for their therapeutic effects.This ongoing study deals with the *in vitro* antioxidant and anticancer activity of hydroalcoholic extract of *L.spinosa* wight Ex Nees*.,* (*L.spinosa*) is a herb in the equatorial zone of Asia belonging to the family Acanthaceae. Total phenolic and flavonoid content was dogged**.** Antioxidant activity was valued by total antioxidant capacity, DPPH assay. By using MTT assay cell feasibility of total cell lines such as Rat skeletal muscle cell line (L6), Ehrlich Ascites Carcinoma (EAC), Human Breast cancer cells (MCF 7), Human Cervical cells (He La) and Human Hepatocellular carcinoma cell lines (Hep G2) were dignified against various doses of extracts. Isolate the active compound by using chromatographic performance. Hydroalcoholic extract of *L.spinosa* exposed more powerful activity against EAC & Hep G2 cell lines but average activity against MCF 7 & He La cell lines. The anticancer and antioxidant nature of the hydroalcoholic extract is due to the occurrence of numerous secondary metabolites like phenolic compounds, flavonoids, terpenoids, alkaloids, etc. The results obviously exposed the hydroalcoholic extract of *L.spinosa* as a good antioxidant with a significant anti-cancer effect.

Keywords*: L.spinosa*, Antioxidant, Anticancer, MTT assay, Compound isolation.

Introduction

Cancer is an important worldwide health problem commonly due to the absence of widespread and comprehensive primary detection methods, the related poor prognosis of patients diagnosed in later stages of the disease, and its increasing occurrence on a global scale. Definitely, the struggle to combat cancer is one of the greatest tasks of mankind(1).Almost 70% of deaths from cancer happen in low and middle-income countries. Opportunities to diminish the death rate from cancer through the finding of new drugs are benefiting from the advances in technology and knowledge on neoplastic disease (2).Recently, innovative methods of treating oncological diseases have appeared (nanotherapy, neutron capture, and low-intensity electroresonance therapy, and also, old methods are used – chemotherapy, radiation therapy, and surgery. Though, all of the overhead methods go with a number of side effects, which also negatively affect the patient's health(3). The present research and development personalized towards the detection of new antiproliferative agents from natural products have been buoyed by upgrading the science and technology of anticancer drug discovery. Antitumor drugs have also been linked with change of secondary malignancy(4).

According to the World Health

Organization (WHO), several countries, as well as developing countries, still use plants and natural source–associated products for therapeutic purposes. About 60% of anticancer agents have been initiated from natural sources globally. The nature-derived compounds are freely available, frequently more tolerated, and considered non-toxic to normal human cells (5). Various natural compounds such as terpenoids, alkaloids, lignans, tannins, quinones, phenolic acids, coumarins, and flavonoids have been initiated from plant sources that have major antioxidant activity and play a main part in the therapy of cancer. A number of studies have shown that antioxidant compounds demonstrate anti-inflammatory and ant carcinogenic activity. Natural antioxidant compounds can directly avoid the proliferation of cells and enhance the immune system (6). The development of cancer drugs from plant materials and their use in experimental practice is still a vital task. The occurrence of the above activities certifies the use of the inhibition and treatment of different cancer types such as skin, lung, stomach, liver, breast, prostate, and cervix cancers.

At present, more than 3,000 plants globally have anticancer properties. From the scientific perspective, the search for knowledge of the anticancer herbal raw materials is not new, on the other hand for the correct user, detailed phytochemical and pharmacological studies are forever essential (3). The ultimate aim of the study is to discover the antioxidant, cytotoxic activity of crude extract and the separation of the active constituent of hydroalcoholic extract of *L.spinosa* whole plant.

Materials and Methods

Plant collection and verification: The established and healthy whole plant of *L.spinosa* was collected from Tirunelveli district. The specimen was verified by

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Research Officer-Botany, Central Council for Research in Ayurveda & Siddha, Govt. of India. Tirunelveli, Tamil Nadu. In our college museum, the specimen has been placed.

Preparation of crude extracts: *L.spinosa* whole plant was collected and washed carefully. It was dried out in the air and sliced into small parts. Around 1 kg of dried entire plant *L. spinosa* was extracted in soxhlet consecutively with increasing polarity of solvents [petroleum ether (PE), chloroform (CH), ethyl acetate (EA), hydroalcohol (HA)]. All the extracts were evaporated using rotary vacuum evaporator. The whole thing was weighed and deposited for further use. All the used solvents were of analytical grade.

Preliminary phytochemical analysis of L.spinosa: Extracts were checked for the presence of phytochemicals with the standard procedure as defined in the textbook by Harborne A J (7).

Determination of total flavonoid content: Entire flavonoid content was analyzed via the aluminium chloride colorimetric technique(8).

Determination of total phenolic content: The phenolic content was assessed by Gallic acid equivalents (GAE/g) of dry plant material on the base of a standard curve of Gallic acid (2 - 64µg/mL) (8,9,10).

Antioxidant activity of crude extracts of Lepidagathis spinosa

Total antioxidant assay: The total antioxidant capacity of the test samples (PE,CH, EA, HA) was valued spectrophotometrically by the phosphomolybdenum method (11). Addition of 0.5 mL of extracts with 3 mL of reagent solution, incubated at 95°C for 90 min. After cooling, absorbance was measured at 695 nm by using a UV-Visible spectrophotometer against blank. The total antioxidant capacity is stated as the number of grams equivalent to ascorbic acid. Prepare the calibration curve by mixing ascorbic acid (10 – 80 µg/mL) with ethanol (12).

DPPH antioxidant assay DPPH antioxidant estimation is based on the capacity of antioxidants to decolorize 1, 1-diphenyl-2-picryl-hydrazyl. 0.135 mM DPPH was prepared in methanol. Various concentrations of test samples were mixed with 2.5 ml of DPPH solution. The absorbance of these solutions was measured at 517 nm and ascorbic acid as standard(13).

The capability of test samples to scavenge DPPH radical and control was calculated from the following formula:

% DPPH inhibition = [(OD of control - OD of test)/(OD of control)]×100

Preparation of test solution For MTT assay, serial two fold dilutions (3.125-50µg) were prepared from this assay.

Cell lines and culture medium Cell lines used for cytotoxicity screening were Rat skeletal muscle cell line (L6), Ehrlich's Ascites Carcinoma cell line (EAC), Human cervical cell line (He La), Human Breast cancer cells (MCF 7), and human hepatocellular carcinoma cells (Hep G2), and the cell lines acquired from National Centre for Cell Science (NCCS), Pune, India. Stock cell lines were cultured in a medium added with 10% inactivated New born calf serum, penicillin (100 IU/ml), and streptomycin (100 µg/ml) in a moistened atmosphere of 5% CO_2 at 37°C till confluent.

Procedure By using MTT assay measure cytotoxicity (loss of viable cells). This assay is founded on the metabolic reduction of the soluble MTT salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide which reflects the normal function of mitochondria dehydrogenase activity and cell viability, into an insoluble colored formazan product, which was measured spectrophotometrically. The activity of mitochondrial dehydrogenase of living cells rightly and proportionately represents the number of viable cells(14).

100µl of cell suspension of density 1 × 10^4 cells/well was positioned into each well of 96-well plates and incubated for 24h. After 24h, when a part of monolayer was formed, the supernatant was flicked off, with the help of a medium the monolayer was washed, add 100µl of different concentrations of test sample onto the partial monolayer in microtiter plates. Incubate the plate at 37°C for 24h in a 5% CO_2 atmosphere. After incubation, the test solutions in the wells were discarded and 100µl of MTT (1mg/ml of MTT in PBS) was added to each well. The plate was incubated for 4h at 37°C in a 5% CO_2 atmosphere. The supernatant was removed instead of that add 100µl of DMSO, the plate was gently shaken to solubilize the formed formazan. The absorbance was measured by means of a microplate reader at a wavelength of 570 nm (4,6).

The percentage of viability was calculated using the following formula:

% of viability = Sample abs/Control abs x 100

Where

Abs= Absorbance value

Isolation of active constituents Methanol used to dissolve the plant extract and adsorbed in silica gel 60 – 120. Evaporate the solvent, which was loaded into a silica gel column (100 – 200 mesh size), prepared in hexane. Elute the column with hexane followed by gradually increasing polarity with Hexane, Chloroform, Ethyl acetate, and Methanol. Absolutely 106 fractions were collected and examined under TLC. Related fractions were combined and the solvent was evaporated under reduced pressure. The resultant crude materials were refined by using activated charcoal in hot ethanol and crystallize the fractions (15). The acquired solid was submitted for Melting point, Mass spectra, FT-IR spectra, ^{13}C-NMR, and ^{1}H-NMR analysis.

Results and Discussion

Preliminary phytochemical studies of hydroalcoholic extract proven the presence of flavonoids, alkaloids, tannins, saponins, triterpenoids, and phenols, etc. Flavonoids, tannins, saponins and triterpenes have all been stated to possess antitumor activity (16,17,18,19). Flavonoids anticancer action has been connected with several mechanisms such as the variation of cell cycle arrest at the G1/S phase, Initiation of cyclin-dependent kinase inhibitors, anti-apoptotic gene downregulation, cell-survival kinase inhibition and inhibition of inflammatory transcription factors, and generation of Ca2+dependent apoptotic mechanism (20).

Results of the study presented that the phenolic compound of the tested extracts (PE, CH , EA, HA) differ from 5.62 to 33.53 GAE/g . Utmost phenolic compounds was identified in hydroalcoholic extract of *L.spinosa* at a 33.53 GAE/g. The amount of total flavonoid compounds was determined as the quercetin equivalent using an equation obtained from a standard quercetin graph (y = 0.003x + 0.2161, R^2 = 0.9709). The flavonoid content of the extracts varied from 0.74 to 19.63mgof quercetin. As shown in Table 1, excellent flavonoid content was found in the hydroalcoholic extract of

Table 1: Quantitative estimation of phytoconstituents present in various extracts of *Lepidagathis spinosa* wight ex Nees

Extract	Total phenol content (mg of GAE/gm of the plant extract)	Total flavonoids content (mg of QE/g of the plant extract)
LSPE	5.623188	0.744444
LSCH	14.25673	3.077778
LSEA	15.49896	6.744444
LSHA	33.53209	19.63333

Table:2 *Invitro* antioxidant activity of various extracts of*L.spinosa wight* ex Nees, whole plant

Extract	DPPH IC_{50} value	Total antioxidant capacity (mg/g)
LSPE	>320	4.03
LSCH	>320	16.23
LSEA	>320	21.73
LSHA	262.02	24.9

Table No:3. Assessment of cytotoxicity and cell viability for Hydroalcoholic extract of Lepidagathis spinosa whole plant on L6 cell line with MTT assay

S.No	Conc. (µg/ml)	% cell viability	% Cytotoxicity	IC_{50} Value (µg/ml)
	Control	100	0	>100
	3.125	98.772±007	1.228±0.024	
	6.25	97.299±005	2.711±0.032	
	12.5	94.879±0.004	5.120±0.048	
	25	91.898±0.005	8.120±0.039	
	50	87.969±0.006	12.030±0.059	

Table No:4 Assessment of cytotoxicity and cell viability for Hydroalcoholic extract of Lepidagathis spinosa whole plant on EAC cell line

S.No	Conc.(µg/ml)	% cell viability	% Cytotoxicity	IC_{50} Value (µg/ml)
1	Control	100	0	28.95
2	3.125	95.96±0.017	4.01±0.038	
3	6.25	82.92±0.012	17.08±0.025	
4	12.5	70.63±0.028	29.37±0.068	
5	25	56.54±0.009	43.46±0.083	
6	50	34.16±0.042	65.84±0.076	

Table No:5. Assessment of cytotoxicity and cell viability for Hydroalcoholic extract of Lepidagathis spinosa whole plant on MCF7 cell line using MTT assay

S.No	Conc.(µg/ml)	% cell viability	% Cytotoxicity	IC_{50} Value (µg/ml)
	Control	100	0	41.44
	3.125	95.28±0.011	4.72±0.058	
	6.25	86.92±0.012	13.08±0.045	
	12.5	71.50±0.012	28.50±0.028	
	25	63.75±0.006	36.25±0.059	
	50	42.458±0.006	57.54±0.072	

Table No:6. Assessment of cytotoxicity and cell viability for Hydroalcoholic extract of Lepidagathis spinosa whole plant on HEPG2 cell line

S.No	Conc.(µg/ml)	% cell viability	% Cytotoxicity	IC_{50} Value (µg/ml)
	Control	100	0	39.73
	3.125	96.168±0.0226	3.832±0.074	
	6.25	87.151±0.0226	12.849±0.026	
	12.5	76.201±0.0411	23.799±0.037	
	25	61.899±0.0133	38.101±0.014	
	50	41.173±0.0181	58.827±0.060	

Table No:7. Assessment of cytotoxicity and cell viability for Hydroalcoholic extract of Lepidagathis spinosa whole plant on HeLa cell

S.No	Conc.(µg/ml)	% cell viability	% Cytotoxicity	IC_{50} Value (µg/ml)
	Control	100	0	64.63
	3.125	98.208±0.00961	1.792±0.064	
	6.25	94.560±0.0106	5.44±0.088	
	12.5	80.424±0.009	19.576±0.032	
	25	65.196±0.008	34.804±0.046	
6.	50	53.266±0.0186	46.734±0.079	

L.spinosa.

In general, the phenolic content of the hydroalcoholic extract was extensively high, which could be a most important contributing factor to the strong antioxidant activity. Phenolic components are potential antioxidants and free radical terminators. They might decrease the risk of cardiovascular disease, and protect against urinary tract infections and cancer. They are also assumed to have an inhibitory effect on carcinogenesis. Flavonoids as one of the greatest and well-known groups of natural compounds are undoubtedly the most important natural phenolics. Phenolic compounds and flavonoids have a broad spectrum of chemical and biological actions, associated with antioxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals (20,21,22).

Total antioxidant capacity The assessment of total antioxidant contents between the different extracts was revealed. Total antioxidant capacity in the test samples (PE, CH, EA, HA) by means of the calibration curve, was found to be 4.03, 16.23, 21.73, 24.96mg/g ascorbic acid. The principle of this assay comprises the activity of an antioxidant compound which leads to the reduction of the hexavalent form of

molybdenum [Mo (VI)] to pentavalent form [Mo (V)], and the development of a green phosphate/ Mo (V) complex at acidic pH and at a higher temperature. This is spectrophotometrically measured at 695 nm (13).

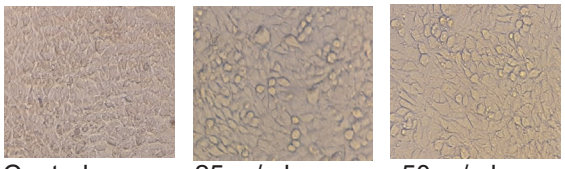
DPPH assay DPPH is a constant nitrogen-centered free radical, generally intended for testing radical scavenging activity of the compound or plant extracts. The violet color of the DPPH radical was reduced to yellow-colored diphenylpicrylhydrazine radical when they accept an electron from an antioxidant compound which was measured colorimetrically. Constituents which are able to make this type of reaction can be considered antioxidants and then radical scavengers. Hydroalcoholic extract of *L.spinosa* exhibited good antioxidant activity compared to others (21). The reports were presented in table 2. In a concentration-dependent manner, DPPH scavenging was increased and compared to ascorbic acid used as the positive antioxidant control. Medium inhibitory concentration (IC_{50}) value for standard ascorbic acid was 21.06µg/ml, in plant extracts hydro alcohol extract of *Lepidagathis spinosa* (LPHA) had the lesser percentage inhibition (IC_{50}-262.02 µg/ml).)

MTT assay Hydroalcoholic extract of the whole plant of *Lepidagathis spinosa* was screened for cytotoxic properties on L6, EAC, MCF, He La and Hep G2 cells lines by MTT Assay. The hydroalcoholic extract did not expose any cytotoxicity against the normal L6 cell line. Among EAC, Hep G2, MCF 7, and He La cell lines exposed significant cytotoxicity with the IC_{50} value of 28.95 µg/ml, 39.73µg/ml, 41.44 µg/ml, and 64.63 µg/ ml. Hydroalcoholic extract shows good activity against EAC (Experimental tumor model), among the human cell lines the hydroalcoholic extract showed significant cytotoxicity against Hep G2 cell line followed by moderate activity against MCF 7, He La cell lines as shown in Tables and Figures. Flavonoids possess good antimutagenic and antimalignant result. Furthermore, they have a chemopreventive character in cancer through their effects on signal transduction in cell proliferation in addition to inhibition of neovascularization (23,24).

The hydrpalcoholic extract of *Lepidagathis spinosa* wight ex Nees., has more significant anticancer activity, it is mainly because of the phytoconstituents present in the extract. The phytochemical study revealed the presence of various phytoconstituents like flavonoids, phenolic compounds, alkaloids, triterpenoids, etc.

Compound isolation Since plant extracts normally occur as a mixture of different types of bioactive compounds or phytochemicals with different polarities, their separation remains a major challenge for the bioactive compound identification and characterization process. In isolating these bioactive compounds, it is common practice to use a variety of different separation methods, such as TLC, column chromatography, etc., to obtain pure compounds.

Figure No:1. Effect of hydroalcoholic extract of Lepidagathis spinosa whole plant on L6 cell line.



Control 25µg/ml 50µg/ml

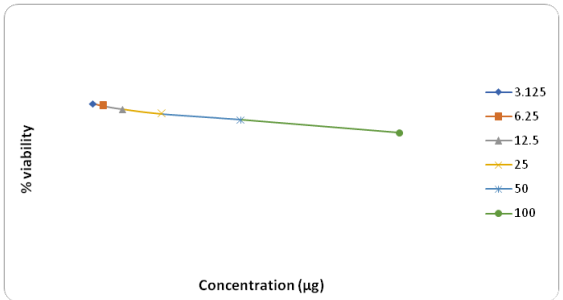
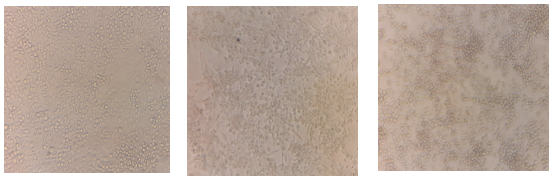


Figure No: 2. Assessment of cytotoxicity and cell viability for hydroalcoholic extract of Lepidagathis spinosa whole plant on L6 cell line

Figure No:3. Effect of hydroalcoholic extract of Lepidagathis spinosa whole plant on EAC cell line



Control 25 µg/ml 50 µg/ml

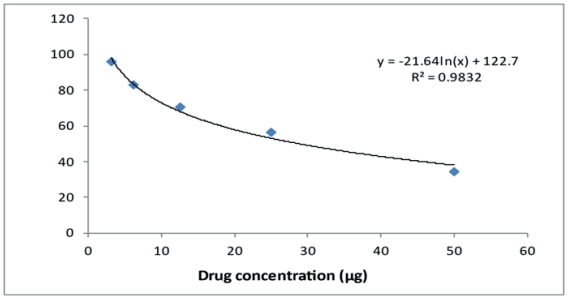
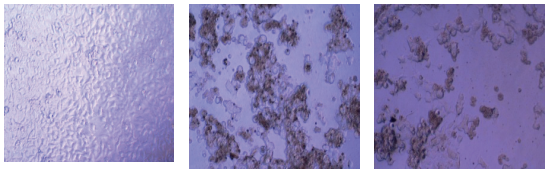


Figure No:4. Assessment of cytotoxicity and cell viability for Hydroalcoholic extract of Lepidagathis spinosa whole plant on EAC cell line



Control 25 µg/ml 50 µg/ml

Figure No:5. Effect of hydroalcoholic extract of Lepidagathis spinosa whole plant on MCF7 cell line using MTT assay

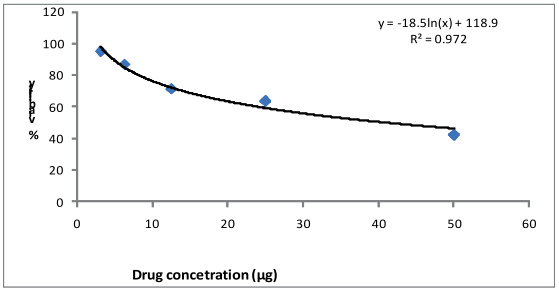
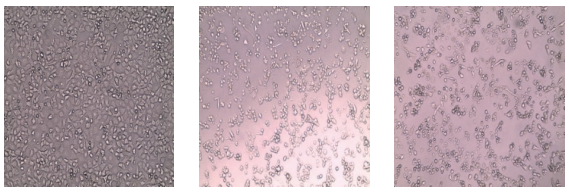


Figure No: 6. Assessment of Hydroalcoholic extract of Lepidagathis spinosa whole plant on MCF7 cell line

Figure No: 7. Effect of hydroalcoholic extract of Lepidagathis spinosa whole plant on HEPG2 cell line



Control 25 µg/ml 50 µg/ml

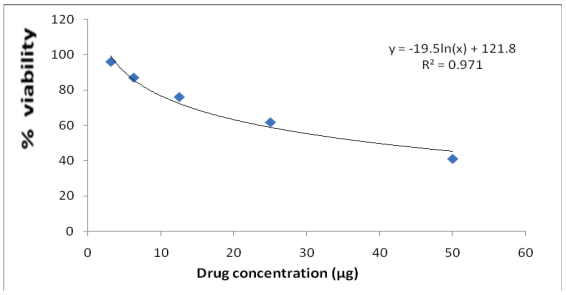
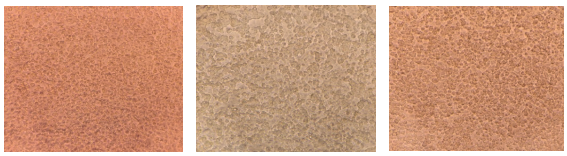


Figure No: 8. Assessment 0f cytotoxicity and cell viability of hydroalcoholic extract of Lepidagathis spinosa whole plant on HEP G2 cell line.



Control 25 µg/ml 50 µg/ml

Figure No: 9. Effect ofhydroalcoholic extract of Lepidagathis spinosa whole plant on HeLa cell line

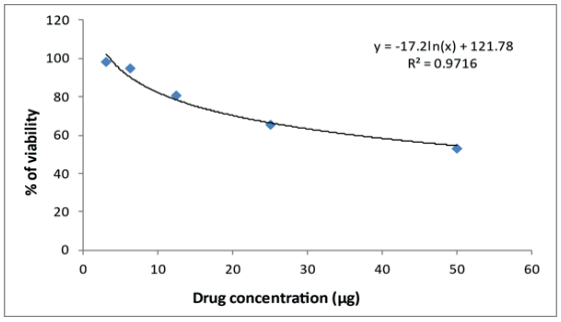


Figure No: 10 Assessment of cytotoxicity and cell viability for hydroalcoholic extract of Lepidagathis spinosa whole plant on HeLa cell line

The pure compounds are then used for structure and biological activity determination.

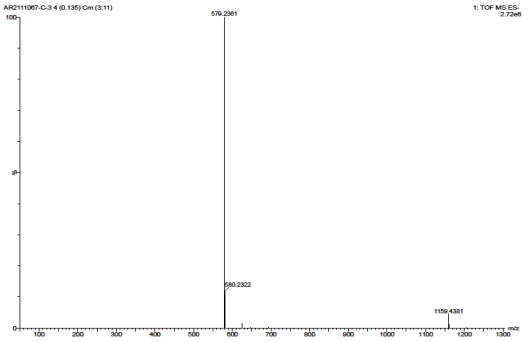


Figure No: 11 Mass spectrum of isolated compound

Fourier-transform infrared spectroscopy (FTIR) can also be used to achieve and promote the detection of bioactive compounds in addition to this phytochemical screening assay. Data from a broad range of spectroscopic techniques such as Nuclear Magnetic Resonance (NMR), Mass spectroscopy andInfrared (IR) are used to determine the structure of natural products. Spectroscopy's basic concept is to transfer electromagnetic radiation through an organic compound that absorbs some, but not all, of the radiation. A spectrum can be generated

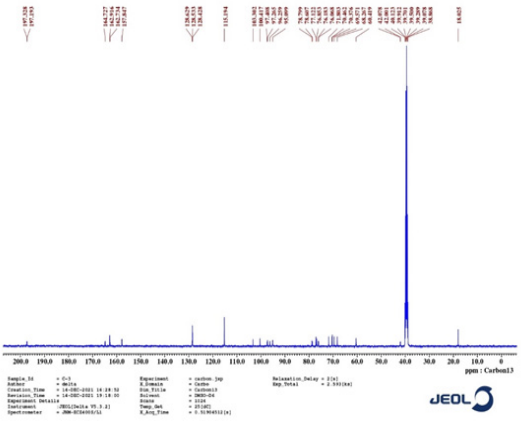


Figure No: 12 ^{13}C-NMR spectrum of isolated Compound

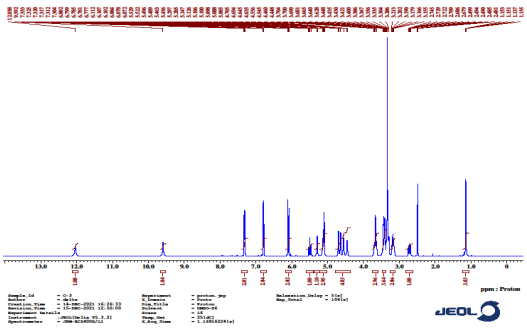


Figure No: 13 ^{1}H NMR spectrum of isolated compound

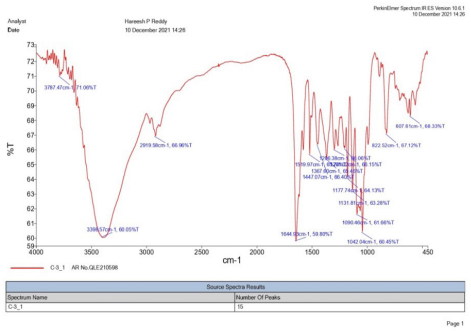


Figure No: 14 IR spectrum of isolated compound

by measuring the amount of absorption of electromagnetic radiation. The spectrum in a compound is unique to such bonds. The composition of the natural compound can be defined based on these spectrums.(24).

Pale yellow colour solid, m.p. 163 - 164 °C; C27H32O14 ;EI-MS m/z: 579.23 (M-H)-

1H-NMR (DMSO-d6): δ ppm: δ 12.03 (s, 1H, -OH), 9.59 (s, 1H, -OH), 7.30 -7.33 (m, 2H, H-2' and H6'), 6.77 – 6.80 (m, 2H, H-3' & H-5'), 6.07 – 6.11 (m, 2H, H-8 H-6), 5.45 – 5.52 (m, 1H, H-2), 5.28 – 5.29 (d, 1H, H-1'"), 5.08 – 5.14 (d, 1H, H-1"), 4.69 – 4.70 (d, 1H, Ha-6"), 4.63 – 4.64 (d, 1H, Hb-6"), 4.54 – 4.55 (d, 1H, H-5'"), 4.44 – 4.46 (d, 1H, H-5"), 3.62 – 3.71 (m,2H, H-3'", H-3"), 3.42 – 3.47 (m,2H, H-2", H-2'"), 3.15 – 3.21 (m,2H, H-4'", H-4"), 2.67 – 2.75

(m,2H, Ha-3, Hb-3), 1.13 – 1.15 (dd, 3H, CH3-6'").

13C-NMR (DMSO-d6):δ 197.32 (C-4,), 164.72 (C-7), 162.91 (C-5), 162.73 (C-9), 157.84 (C-4'), 128.62 (C-1'), 128.53, 128.42 (C-2' and C-6'), 115.19 (C-3' and C-5'), 103.30 (C-10), 100.41 (C-1"), 97.40 (C-1'"), 96.25 (C-6), 95.09 (C-8), 78.79 (C-2), 78.60 (C-2"), 77.12 (C-5"), 76.18 (C-3"), 71.80 (C-4'"), 70.46 (C-2'" and C-3'"), 69.57 (C4"), 68.26 (C-5'"), 60.41 (C-6"), 42.07 (C-3), 18.02 (C-6'").

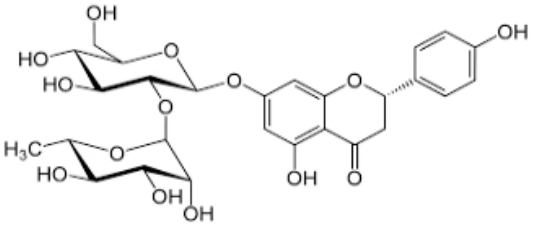
FT-IR (KBr cm-1):3398 cm^{-1} (-OH stretching) ; 1296 cm^{-1} (-C-O bending);644 cm^{-1} (-C=O stretching); 1519 cm^{-1} (-C=C stretching); 1447 cm^{-1} (-CH bending) ; 1367 cm^{-1} (-CH bending), , 1090 cm^{-1} (-C-O stretching). 2919 cm^{-1} (-CH stretching).

The ^{1}H-NMR spectrum revealed signals at *δ* 12.03 and 9.59 showing the presence of aromatic hydroxyl group. A multiplet of protons at δ 6.11 to 7.30 were allotted to aromatic protons declaring a flavonoid ring system. Protons from 1.13 to 4.63 typical signal observed and suggesting the presence of sugar moiety.

The ^{13}C-NMR spectrum showed 27 carbons which included carbon at 164.72 and 162.91 was a characteristic of carbon attached with hydroxyl moiety. Carbon from 18.02to100.41 representing the presence of sugar moiety.

FTIR spectrum showed absorption band at wave number region 3398 cm^{-1} for the shifting vibration of hydroxyl group (OH), wavelength region of 2919 cm^{-1} was a C-H group of aromatic ring, wave length region of 1519 cm^{-1} shows the C=C bond of the aromatic ring,the wavelength region of 1447 cm^{-1} was a C-H bond in the CH_2 group, then the absorption band number in the wave length region 1367 cm^{-1} was the C-H bond of the CH_3. The wavelength region of 1090 cm^{-1} is the C-O-C bonds of the ether. Based on these data it can be estimated that the tested compound contains a hydroxy group (−OH), aromatic cyclic groups (C=C), CH_2, CH_3, and ether groups.

The molecular weight of the separated compound ascertained by high-resolution



mass spectrometry and the molecular ion peak appearing at m/z 579.23 (M-1).

This pattern of the spectrum was identical with that of compound naringin [1]. The structure of compound-1 as given below

Conclusion

In the system of folk and clinical medicine of different countries, herbal plants have always been played a major role in prevention and treatment of cancer and other diseases. Hydroalcoholic extract of *Lepidagathis spinosa* has more significant anticancer activity, it is mainly because of the phytoconstituents present in it. Also, it is rich in flavonoid and phenolic content, and possess good antioxidant activity in the experimental and human cancer cell line. Using column chromatography the active compound was isolated and elucidated it is similar to Naringin. In the future, the investigation can be extended using *invivo* on a particular cell line and also need to study the mechanism of action of isolated compound.

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