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Isolation and Characterization of a New Anticancer Compound from Aerial Parts of Tanacetum dolicophyllum (Kitam.) Kitam, Ladakh, India

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ABSTRACT:

Introduction: Tanacetum dolicophyllum (Kitam.) Kitam is recognized for its pharmacacological and ethnomedical significance. The compound derived from T. dolicophyllum demonstrated important antioxidant activity, indicating its advanced antioxidant potentials. Objectives: To examine the bioactive component isolated from T. dolicophyllum, specifically focuses on its antioxidant and anticancer characteristics. Methods: The compound isolation process commenced with column chromatography and thin layer chromatography techniques which was then characterized by FT-IR, 1H NMR, 13C NMR, and HRMS to elucidate its chemical structure and functional groups. Then their biological activity was evaluated which involves antioxidant and anticancer assays. Results: FT-IR, NMR, and HRMS studies of the compound showed the characteristics peaks of functional groups like alkanes, alkynes, ketones, peroxides and esters which show anticancer and antioxidant properties. The anti-cancer activity was investigated by the MTT assay whose IC50 value of the HeLa cell line is 2 µM/ml. Conclusion: This research represents the characterization of the bioactive compound derived from T. dolicophyllum highlighting its antioxidant, anticancer, and pharmacologically active properties. These findings may pave the way for the creation of novel cancer therapeutics.

Keywords: Tanacetum dolicophyllum, MTT assay, Pharmacological activity,

1. Introduction

Plants are the source of vital components for human beings and also provide compounds which biologically active and are useful for human's well-being and in the treatment of several diseases [1]. Plants contain pharmaceutics and phytochemicals, which have been used in pharmaceutical industries, etc. The practice of ancient medicines is the main basis of the well-being system [2]. Medicinal plants showed the presence of compounds that have anticancer, antimicrobial, and antioxidant potential [3,4]. Many plants serve as a vast reservoir of secondary metabolites, but only a rare have been researched and confirmed to be significant sources of bio active components [5]. The purification and characterization of these bio active components have led to the development of exact medications [6]. FTIR technique is used to detect the occurrence and identification of functional groups present in numerous bio active compounds existing in plants [7,8]. The Tanacetum genus consists of nearly 150 flowering plant species that belong to the family Asteraceae. These plants have medicinal significance and may act as better alternatives to artificial drugs that frequently cause harmful health effects [9].

Tanacetum dolicophyllum (Kitam.) Kitam. (*T. dolichophyllum*) is a hairy herb characterized by vibrant floral heads and basal leaves. Typically grows in alpine environments, it blooms from August to October. Locals uses *T. dolichophyllum* in the preparation of incense and aromatic material in the Ladakh and Uttarakhand regions [10]. Very little research on *T. dolicophyllum* has been described so far [11]. There is no description showing the occurrence of several bio-active compounds in parts of *T. dolicophyllum*. Thus, the recent study concentrated on the identifying compounds from ethyl acetate extracts of *T. dolicophyllum* by column and thin-layer chromatography. The research also showed the presence of the antioxidant and anticancer properties of the compound. The isolated compound was identified by using various spectroscopy methods such as Fourier Transform Infra-Red spectrometry (FTIR), High-Resolution Mass Spectrometry (HRMS), and Nuclear Magnetic Resonance (NMR). This will aid in predicting the structure and formula of active compounds present in *T. dolicophyllum* which could be vital for the exploration and development of new drugs.

2. Materials and Methods

Isolation and Purification of Compounds

Column chromatography

Ethyl acetate extract (3.8gm) was assorted with silica gel to make an admixture. 2.4 dia column was packed mixed with hexane. The column was eluted by increasing solvent polarity from hexane to ethyl acetate. Activated silica gel was filled into a glass column using n-hexane as the solvent. *T. dolicophyllum*'s extract was collected and then subjected to column chromatography, with silica gel serving as the stationary phase. A solvent system consisting of mixtures of n-hexane (100:0), n-hexane, and ethyl acetate in the ratio of (95:5), (90:10), (80:20), (75:25), (70:30), and ethyl acetate (0:100 v/v).

Thin-layer chromatography (TLC)

The fractions were ladened onto activated silica gel TLC plates, which were then developed using varied ratios of hexane: ethyl acetate including 90:10, 75:25, 50:50, and 25:75. Spot detection was achieved by exposing the plate to vanillin and iodine fumes [12,13].

Elucidation of chemical structure

The plant compound isolated was identified through various spectroscopic techniques, including High-Resolution Mass Spectrometry (HRMS), Nuclear Magnetic Resonance (NMR), and Fourier Transform Infra-Red spectrometry (FTIR) [14]. The elucidation of the chemical structure of the isolated compound was based on the data obtained from these spectroscopic methods and comparision with available information on the internet.

Fourier Transform Infrared Spectroscopic Analysis (FT-IR)

FT-IR spectrum was transcribed in the absorption range from 400 to 4000 cm^{-1}. All research were approved using a Shimadzu FT-IR spectrometer [15-17].

Identification of functional groups

To identify the functional groups dependent on the peak values, the FT-IR spectrum was used [18,19]. The plant compound's functional groups were parted based on the ratio of their peak [20].

Nuclear Magnetic Resonance Spectroscopy

One-dimensional ^{1}H and ^{13}C NMR spectrum were transcribed with a BRUKER NMR ADVANCE spectrometer working at 500 MHz for ^{1}H and 125MHz for ^{13}C [21,22]. The plant compound was dissolved in chloroform ($CDCl_3$). Chemical shifts reported in ppm expressed in δ units [23].

Biological activity

Antioxidant assays

The reducing power assay (RPA) was conducted by following the standard protocol [24] and dependent on ferric ion to ferrous ion alteration in the occurrence of the solvent fractions. The total antioxidant capacity (TAC) of the plant compound was determined by the phosphomolybdate with ascorbic acid as a reference [25]. The FRAP assay was carried out using the improved method described by Benzie and Strain (1996) [26]. The scavenging activity of the plant compound was accomplished by using the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) method using ascorbic acid as a reference. [27].

Cell Culture

HeLa cell lines were grown in RPMI medium comprising 2% penicillin, 10% FBS, and 1% amphotericin; which was brought from NCCS, Pune. Cells were kept up with 5% saturated CO_2 at 37°C [28]. The statistical results were evaluated in triplicates.

Cytotoxicity Assay

MTT activity was done to examine the anti-cancer activity of the compound with few modifications [29].

Propidium iodide staining

For about 72 hours, the cells were treated with varied concentrations of plant compound. At the end of incubation period, the culture media in tubes were removed and clean with cold PBS. Then, 1 ml of cold PBS was poured to each culture tube. For cell staining, 100 µl of Calcein and 25 µl PI solution were added to each tube and then incubated for 15 min. Under Leica DMI6000B fluorescent microscope the cells were observed [29].

Statistical analysis

The antioxidant assays were conducted in duplicate; except RPA and MTT assays (triplicate). Results are presented as mean values with standard deviation (SD).

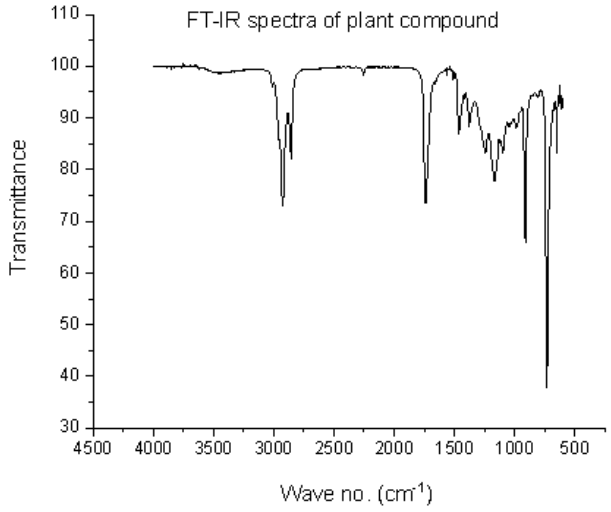
3. Results and discussion

Isolation of compound

The hexane and ethyl acetate fractions of *T. dolicophyllum* were combined and underwent a phytochemical analysis, resulting in the isolation of the compound. About 150 fractions were collected from column chromatography and subsequently concentrated by means of the rotary evaporator. Based on their TLC profiles, using the mobile phase n-hexane/ethyl acetate (80:20) first compound was isolated.

FT-IR Spectrum analysis

The FT-IR spectroscopy discovered the existence of ketones, alkenes, alkanes, and esters. [Fig. 1; Table 1]. Because of the methyl C-H stretch existing in the compound; the absorption band is shown at 2926.5 cm^{-1}. The band at 2855cm^{-1} is because of CH_3 bending; at 1734cm^{-1} presented ester group; at 1462.5cm^{-1} showed methyl C-H bending; at 1168cm^{-1} showed



Aromatic C-O stretch; at 909 cm^{-1} showed C-O peroxides; at 731.5 cm^{-1} presented methylene bend; and at 648 cm^{-1} showed alkene C-H bend.

Figure 1: FT-IR spectrum of plant compound

Table 1: Functional groups present in FT-IR spectrum

Peak no.	Group frequency	Origin	Functional groups
1.	2926.5	CH_2	Methyl C-H asymmetrical/symmetrical Stretch
2.	2855	CH_3	CH_3 bending
3.	1734	R-COO-R	Ester
4.	1462.5	C-H	Methyl C-H asymmetrical/symmetrical Bend
5.	1168.5	C-O	C-O stretch (Ketones)
6.	909	C-O-O	Peroxides
7.	731.5	C-H	Methylene
8.	648	C-H	Alkene C-H bend

Nuclear Magnetic Resonance (NMR) Spectroscopy

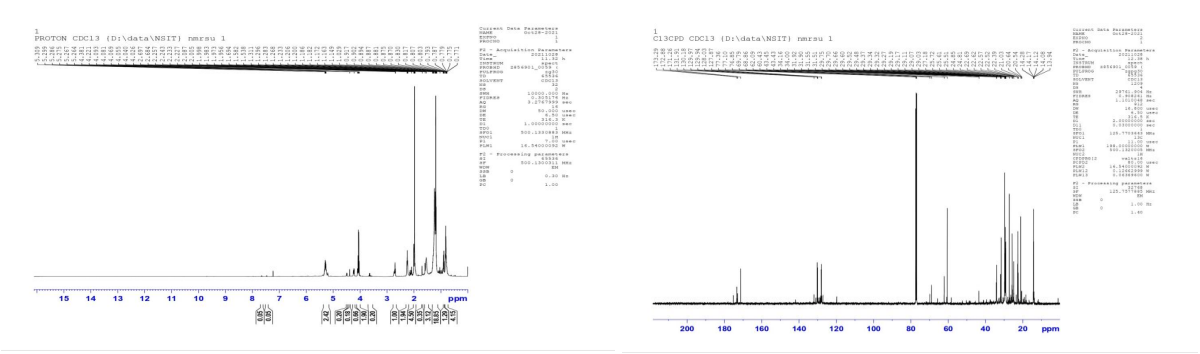
^{1}H NMR spectroscopy

^{1}H NMR spectrum showed the presence of groups which can be divided into 6 main categories: 1) free methyl (CH_3) group (0.771-1.311ppm), 2) methyl group from double bond (1.694ppm), 3) CH_3 from acetyl group (1.998ppm), 4) CH_2 protons in a cyclic ring (2.005-2.697ppm, 5) protons from tertiary carbon (4.026-4.381ppm), 6) unsaturated protons (5.264-5.309ppm) (7.1 ppm) [figure 2].

Figure 2: ^{1}H NMR of plant compound

^{13}C NMR spectroscopy

^{13}C NMR spectrum showed the presence of 7 groups in the structure shown in the figure. They are 1) the free methyl group (13.94-21.03ppm), 2) all cyclic CH_2 group (22.39-42.45), 3) quaternary carbon (60.43ppm), 4) C-O in the epoxy link (69.79ppm), 5) tertiary carbon



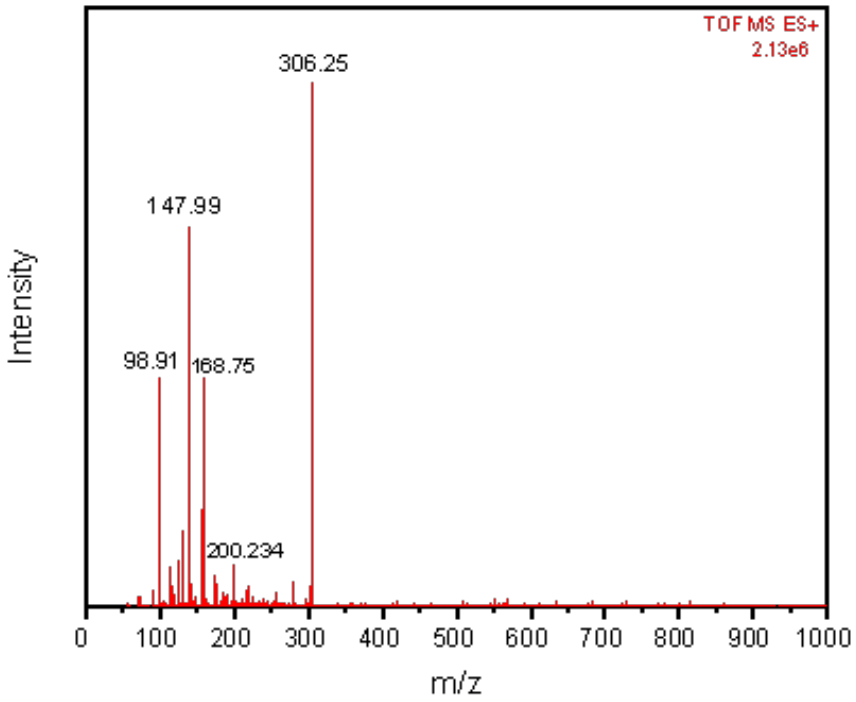
linked to O (76.85-77.36ppm), 6) alkene group C=C (127.87-131.91ppm), 7) C=O group (171.26-173.29ppm) [figure 3].

Figure 3: ^{13}C NMR of plant compound

High-Resolution Mass Spectrometry (HRMS)

HRMS calculated for the isolated compound '[(Z)-1a,5-dimethyl-9-methylene-8-oxo 1a,2,3,6,6a,8,9,9a,10,10a-decahydro-11-oxa-bicyclo (8.1.0) undeca-1(10),4-dieno(7,8-b) furan-10-yl acetate]' is C17H22O5 , Mass (m/z) 306. [Figure 4]

Figure 4: HRMS of plant compound



Structure of compound isolated from *T. dolicophyllum*

The isolated compound was structurally characterized with the help of HRMS and NMR (^1H, ^{13}C) spectral data. The compound isolated was [(Z)-1a,5-dimethyl-9-methylene-8-oxo 1a,2,3,6,6a,8,9,9a,10,10a-decahydro-11-oxa-bicyclo (8.1.0) undeca-1(10),4-dieno(7,8-b) furan-10-yl acetate] and the structure of the compound is shown in figure 5.

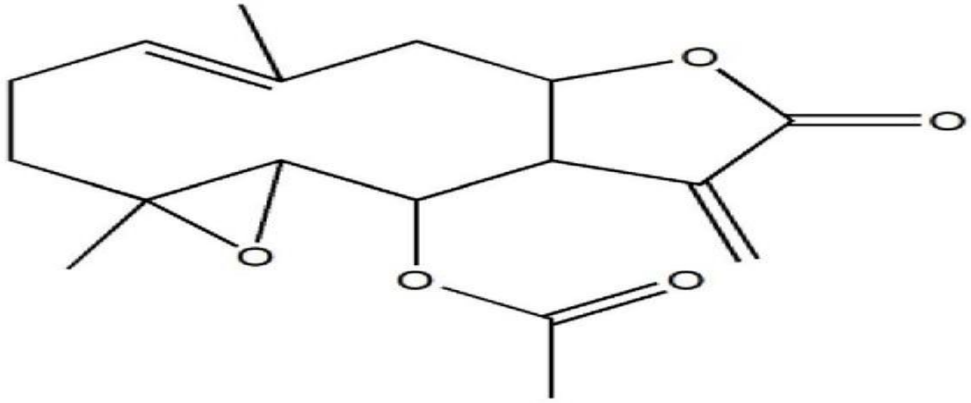


Figure 5: Structure of the compound [(Z)-1a,5-dimethyl-9-methylene-8-oxo 1a,2,3,6,6a,8,9,9a,10,10a-decahydro-11-oxa-bicyclo (8.1.0) undeca-1(10),4-dieno(7,8-b) furan-10-yl acetate] isolated from *T. dolicophyllum*.

Antioxidant Tests

Reducing Power Assay

The RPA of the plant compound of *T. dolicophyllum* was evaluated using the standard graph of ascorbic acid as shown in figure 6(a). With an increase in concentration, the absorbance also increases; with a SD of 0.1 for ascorbic acid and 0.13 for plant compound. The p-value is < 0.05 hence it is statistically significant.

Phosphomolybdenum Assay

The phosphomolybdenum assay was conducted by using a standard curve of ascorbic acid with *T. dolicophyllum*'s plant compound as shown in figure 6(b). With an increase in concentration, the absorbance also increases. The R^2 value is 0.997 for ascorbic acid and 0.999 for plant compound.

Ferric Reducing Antioxidant Power (FRAP)

The FRAP activity was conducted by constructing a curve using $FeSO_4$ at varying concentrations ranging from 100 to 1000 µM. The FRAP activity is evaluated on the reduction of Fe^{3+} iron to Fe^{2+}. The FRAP value of the plant compound is measured at 173.45 µM/g whereas the value of quercetin and ascorbic acid is 752.1 µM/g 11 and 645 µM/g, correspondingly shown in figure 6(c).

DPPH Radical Scavenging Capacity

The DPPH activity of the plant compound of *T. dolicophyllum* was evaluated across various concentrations [figure 6 (d)]. Table 2 shows the inhibition percentages of plant compounds on DPPH along with their IC_{50} values. A lower IC_{50} value suggests greater antioxidant activity of the plant compound. The plant compound shows 112.46 (µg/ml) of IC_{50} values. These findings prompt the further exploration of plant compound's anticancer properties.

Table 2: The inhibitory effects of various concentrations of plant compound on DPPH radical

Concentrations of plant compound(µg/ml)	**% RSA of Plant compound**
50	33.529
100	41.764
150	50.784
200	61.372
250	70.392

300	82.745

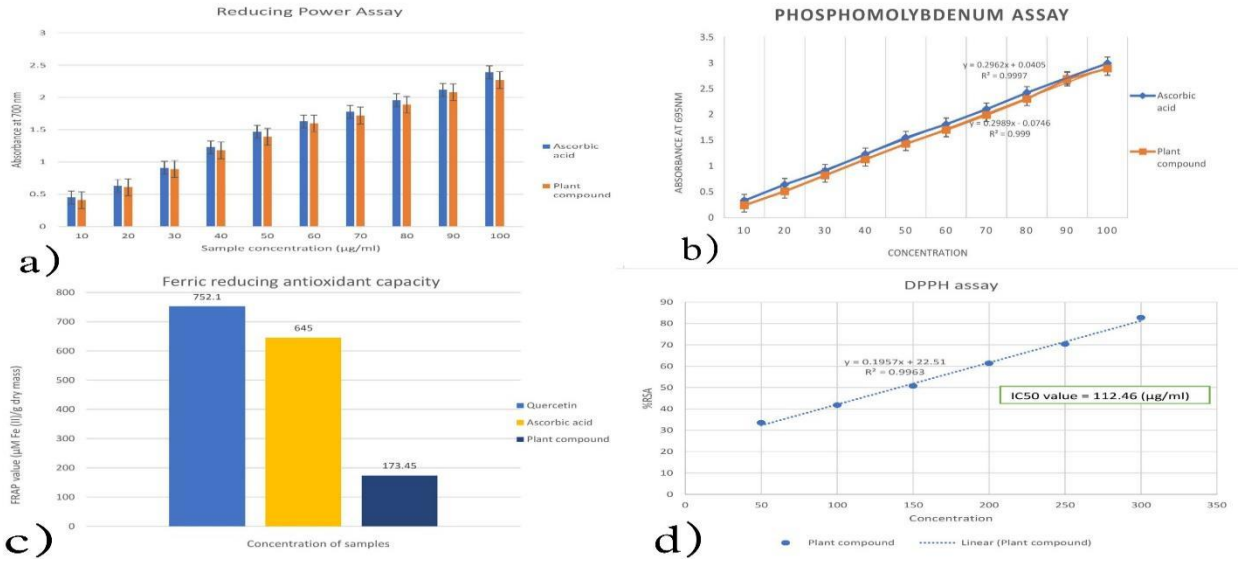


Figure 6: a) Reducing ability of standard and compound with an increase in concentration
b) Phosphomolybdenum assay of ascorbic acid and plant compound with an increase in concentration.
c) Assessment of the overall antioxidant potential of *T. dolicophyllum*'s plant compound and standard controls (quercetin and ascorbic acid) via FRAP assay.
d) Determination of DPPH scavenging activity, along with the crorresponding IC50 value

Anticancer Activity

The anticancer investigation was assessed via MTT assay. The MTT assay showed a IC_{50} value of 2 µM/ml for HeLa cell lines [figure 7(a)].

PI staining

HeLa cells were subjected to staining with PI and Calcein/EthD-1 nuclear dyes to monitor changes in the nuclei morphology following treatment. Initially the nuclei of untreated HeLa cells appeared blurred but consistently stained with calcein. Post treatment, the DNA in the nucleus of apoptotic cell was much fragmented, condensed, and luminous. High concentration led to a greater abundance of late apoptotic cells, observed by their red staining with PI dyes due to the high membrane permeablility.

Morphological examinations revealed characteristic sign of cell death, including cell separation, cell size shrinkage, decreased cell count, nuclear fragmentation, creation of apoptotic bodies, and permeable cell membrane. The initiation of apoptosis revealed some characteristic changes in potential of mitochondrial membrane in cells following treatment [figure 7(b)].

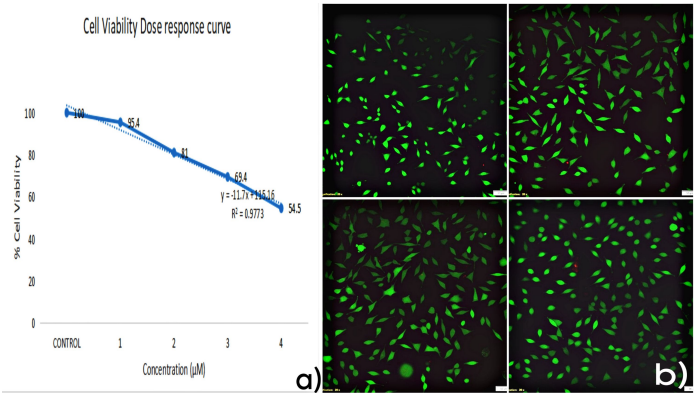


Figure 7: a) Anti-cancer activity of plant derived compound on cancer cell line (HeLa)
b) Propidium iodide/calcein double staining screening nuclear morphological differences at 20× magnification between unprocessed cells (0 µM) and processed cells (50 µM) for 72 hours.

4. Conclusion

The recent study was mainly focused on the purification of a bioactive compound isolated from *T. dolicophyllum* by CC and TLC. The compound is responsible for many pharmacological and therapeutic properties. It also tested the biological activity and shows the anticancer and antioxidant properties. The identification of the isolated compound '[(Z)-1a,5-dimethyl-9-methylene-8-oxo 1a,2,3,6,6a,8,9,9a,10,10a-decahydro-11-oxa-bicyclo (8.1.0) undeca-1(10),4-dieno(7,8-b) furan-10-yl acetate]' was performed using several analytical methods like NMR, HRMS, and FTIR. This structure elucidation suggests that the novel compound could be valuable in drug discovery and advanced cancer treatment.

5. Acknowledgement

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6. Conflict of interest

The authors state no conflict of interest.

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