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Rohina Bashir
Department of Biochemistry, University of Kashmir- Srinagar, Jammu and Kashmir, India

Ovais Zargar
Department of Biochemistry, University of Kashmir- Srinagar, Jammu and Kashmir, India

Qazi Parvaiz
Microbial Biotechnology Division, CSIR-IIIM, Sanat Nagar Srinagar India

Rabia Hamid
Department of Biochemistry, University of Kashmir- Srinagar, Jammu and Kashmir, India

Correspondence
Rabia Hamid
Department of Biochemistry, University of Kashmir- Srinagar, Jammu and Kashmir, India

In vitro antiproliferative activity of *Thymus linearis* essential oil from five ecozones of Kashmir valley

Rohina Bashir, Ovais Zargar, Qazi Parvaiz and Rabia Hamid

Abstract
The purpose of the current study was to evaluate the comparative antiproliferative activity of *Thymus linearis* essential oil on a panel of cancer cell line. The oil was extracted from *Thymus linearis* collected from five ecozones using hydrodistillation and screened individually for their cytotoxicity on the cell lines using MTT assay. The TDR essential oil sample exhibited the growth inhibition of the cell lines under study to varying degrees. Compared to other cell lines the two breast cancer cell lines MCF-7 and T47D were more sensitive to the inhibitory effects of TDR essential oil, displaying cytotoxicity maximally against MCF-7 cells; with an IC_{50} of 79.55µg/mL. Further, a dose dependent colony forming restraint was observed both in terms of size and the number of colonies in comparison to untreated group. TDR essential oil at lower concentration of 80µg/mL showed significant ($*p \leq 0.05$) inhibitory effect on colony formation of the order of 29% relative to untreated. The comparative antiproliferative profile of essential oil of *Thymus linearis* growing wild in Kashmir suggests its chemical polymorphism and study expands its scope to be developed as antitumor agent.

Keywords: thymus linearis, antiproliferative activity, MCF-7, cancer, essential oil, colony forming assay

1. Introduction
The existence of mankind is intimately interconnected to the use of medicinal plants as a dependable source of new drugs and active ingredients. The phytochemical interventions are based on essential oils, formulations and extracts of plants and form the basis for the discovery of bioactive phytochemicals [1]. Essential oils being complex mixtures of volatile odoriferous secondary metabolites, monoterpenes, and sesquiterpenes, along with carbohydrates, alcohols, ethers, aromatic compounds aldehydes, and ketones, have been successful used as anti-microbial, anti-inflammatory, antioxidant, and anti-carcinogenic properties [2].
The word "essential" comes from the term "essence" denotating smell or taste. Essential oils occur in a limited plant families such as Cyperaceae, Lamiaceae, Annonaceae, Apiaceae, Compositae, Burseraceae, Cupressaceae, Lauraceae, Leguminosae, Poaceae, Myrtaceae, Rutaceae, Pinaceae, and Zingiberaceae. On the other hand, phtyo-compounds from extracts belong to phenolics, flavonoids, alkaloids, coumarins, phenylpropanoids, terpenoids, tannins, carotenoids and other metabolites having diverse biological activities like antioxidant, anticancer, anticoagulant, antiparasitic, anti-inflammatory, immunosuppressant and hormone regulatory effects [3, 4]. The use of herbal medicines continues to expand throughout the world and exploring the rich repertoire of phytochemicals, is enticing the attention of researchers all over the world, so as to produce bioactive compounds of novel or known structures as lead compounds for semi synthesis to produce patentable entities of higher activity and/or lower toxicity [5]. In fact, it has been estimated that about 28% of new chemical entities introduced in the market are natural products or their fine-tuned forms. Out of the 252 drugs from basic and essential list of the World Health Organization (WHO), 11% are exclusively of plant origin. In the scenario of these attributes, medicinal plants have been extensively used for the preparation of standardized formulations and medicines in the pharmaceutical industry. It is assessed that 60% of anti-cancer and anti-infectious drugs currently in the market or under clinical trial are of natural origin [6].
Cancer is a comprehensive term used to define diseases that is caused when cells in the body propagate in an uncontrolled manner often culminating in death. Cancer has become one of the main causes of mortality and morbidity round the globe and by 2030, the number of cancer patients is estimated to rise to 21 million [7]. In 2019, about 606,880 deaths are expected in America only which translates to 1,660 deaths per day [8].

Besides, many difficulties and deficiencies in current chemotherapeutic drugs prevail, most importantly, drug resistances that usually occurs in cancer chemotherapy. This demands for new anticancer agents with more effectiveness and specificity. The anticancer drug zone has the greatest impact of plant derived drugs to avert, hamper, defer, or cure cancer [9].

Paclitaxel isolated from the bark of Pacific yew tree, effective against ovarian and breast cancer, is the best example to benchmark the importance of medicinal plants for curing cancer. Other plant derived drugs like vinblastine, vincristine, taxol, and camptothecin have revolutionized the chemotherapy of some cancers [10].

In recent years, antiproliferative aspect for some phytochemical compounds have been unveiled such as, resveratrol isolated from grapes, apigenin from parsley, phytoestrogens from soybean, EGCG from green tea polyphenols, curcumin from turmeric etc., Thus yet again signifying the fact that enduring hunt for new anticancer compounds in plant medicines is a realistic and promising strategy against battle of cancer [11].

Thymus linearis from Lamiaceae family is a small aromatic shrublet, about 15-30 cm high, with a lot of tiny oblong leaves and dense whorls of pinkish blue weakly 2-lipped flowers, sometimes crowding over the leaves. The plant grows on rocky slope in the Himalayas, at altitudes of 1500-4300 m. Traditionally aerial parts of *Thymus linearis* have been extensively used as herbal tea, antitussive tonic, antiseptic and against eczema and psoriasis [12]. A number of pharmacological activities have been attributed to *Thymus linearis* like antimicrobial [13], anti-hypertensive, hepatoprotective activity [14]. *Thymus linearis* is an essential source of thyme oil. In addition, the augmented demand for natural products as supplements and clinical use as an alternative of synthetic chemicals, has enthused research into many medicinal and aromatic plants among which *Thymus linearis* holds an important niche [15].

In this study, we have investigated the comparative antiproliferative activity of *Thymus linearis* collected from five different ecozones.

2. Material and Methods

2.1 Collection of *Thymus linearis* and preparation of extracts

Thymus linearis was procured in the month of May from five different ecozones of Kashmir Valley viz Sonamarg (TS), Pahalgam (TPG), Naranag (TNN), Dreng Tangamarg (TDR) and Wanihama Srinagar (TBW). The plants material was cleared of unwanted substances and chopped into smaller pieces for further use.

2.2 Extraction of essential oil

The extraction of Essential oil of *Thymus linearis* from all the five ecozones was individually carried out by hydrodistillation method using Clevenger type apparatus. The fresh I kg whole plant of *Thymus linearis* was submerged in sufficient quantity of water into a extraction flask. The extraction flask was then connected to the Clevenger apparatus and extraction process continued for 4-5h. The condensed mixture containing oil got collected in a Florentine flask due to their immiscibility and difference in density. The oil samples were collected in a pre weighed glass vial and the weight of oil was determined. The traces of water in the oil samples was gotten rid off using anhydrous sodium Sulphate. The pure oil samples were labelled and stored at 4°C till analysis.

2.3 *In vitro* antiproliferative activity

2.3.1 Maintenance of cell line

The Human cancer cell lines Breast (MCF and T47D), Colon (HCT-116, HT-29 and SW-620), Lung (A-549), Prostrate (PC-3), Pancreas (Mia-Pc-Ca2), Myeloid Leukemia (HL-60) and Lymphoblastic Leukemia (MOLT-4) acquired from National Cancer Institute (NCI), Bethesda, USA [16] were used for appraising the *in vitro* cytotoxicity of *Thymus linearis* essential oil. The cell lines were maintained in the recommended DMEM or RPMI-1640 medium supplemented with 10% heat inactivated fetal calf serum (FCS) and penicillin (100Units/mL) and streptomycin (100µg/mL). The cells were reared incubator at 37°C with 5% CO_2environment and 98% relative humidity.

2.3.2 Cell viability evaluation by MTT assay

The cytotoxic activity was assessed using MTT assay [17] in 96-well plates. The exponentially growing cells at required densities were seeded in 96-well plate with 100µl of media and with 10% FCS and incubated for 24h. After incubation, the oil samples at mentioned concentration (100-300µg/mL) were added to the wells and incubated at 37°C with 5% CO_2 for 48 hours. After incubation, 20µl of MTT dye was added to each well (5mg/mL in PBS) and incubated further for 4h at 37°C. The experiment was terminated by the addition of 150µl of absolute DMSO to each well. The plates were kept on shaker until the purple color of formazan was obtained and the absorbance was recorded at 570 nm using an absorbance plate reader (Bio-Rad Micro plate Reader). The experiment was performed in triplicates.

2.3.3 Calculation of IC_{50} values

The IC_{50} value is defined as the test compound concentration that causes 50% decrease in the reference point of proliferation. The reference point for calculation of IC_{50} value is the absorbance of the corresponding controls in the assay plate. The percent inhibition for each concentration of test agent is calculated by the below equation:

$$A = \frac{(OD_{Control} - OD_{Treated})}{OD_{Control}} \times 100$$

The % inhibition for each concentration of oil samples and standard were plotted on the graph for interpolation of IC_{50} value.

2.4 Colony formation assay

Since TDR essential oil was most potent against MCF-7 cell line, we next examined its long term inhibitory effects on reproductive capacity of the MCF-7 through colonogenic assay. MCF-7 cells from logarithmically growing cultures were seeded at a concentration $2x10^5$/mL and kept for overnight incubation so that cells attain their adherent morphology. The cells were then treated with the indicated concentrations of TDR essential oil and Paclitaxel for 48h. After treatment, cells were trypsinised and $1x10^3$ cells were counted from each well and seeded in a fresh 6 well culture plate with fresh media. The MCF-7 cells were incubated for 15 days at 37°C in CO_2 incubator. The media was changed on alternate days. Afterwards the media was thrown away and the colonies washed with PBS buffer. Subsequently, the

colonies were fixed in 4% formaldehyde and stained with 0.1% crystal violet for 1 h to permit adequate staining. The staining solution was aspirated slowly and plates were washed gently several times with tap water to get rid of excess stain. The plates were air dried at room temperature. The colonies in each well were counted manually from three independent experiments.

3. Results

3.1 Isolation of oil

Whole plant *Thymus linearis* was subjected to hydrodistillation to isolate the essential oil. The oil obtained had a characteristic aromatic odor and was pale yellow in color. The average yield of 0.27% on dry weight basis was acquired (Table 1).

Table 1: *Thymus linearis* essential oil yield (%v/w by hydrodistillation) from different ecozones of Kashmir Valley

Location	Essential oil yield (%v/w)
Sonamarg (TS)	0.27
Pahalgam (TPG)	0.30
Naranag (TNN),	0.21
Dreng Tangmarg (TDR)	0.39
Wanihama Batpora (TBW),	0.20

3.2 Cytotoxicity by MTT assay

Table 2 represents the cytotoxicity profile of *Thymus linearis* essential oil collected from five ecozones. The essential oils from all five ecozones exhibited inhibitory effects on cancer cell lines to various extents. We found the *Thymus linearis* essential oil from Dreng ecozones to be most potent against the cell lines under study, while the breast cancer cell lines were found to be most sensitive to its inhibitory effects. Among the two breast cancer cell lines under study, TDR essential oil displayed cytotoxicity maximally against MCF-7 cells; inhibiting their proliferation by 81% at 300µg/mL after 48h treatment. We further calculated the IC_{50} values of essential oil against the two breast cancer cell line as represented by figure 1. TDR essential oil has the minimum IC_{50} of 79.55µg/mL and 86.20µg/mL for MCF-7 and T47D breast cancer cell lines respectively.

Table 2: Cytotoxic potential (% inhibition) of *Thymus linearis* essential oil on panel of Cancer cell lines by MTT assay.

Tissue	Cell line	Concentration µg/mL	TS	TPG	TNN	TDR	TBW
Colon	HCT-116	100	29.30±1.20	34.55±1.50	44.20±2.00	49.80±1.45	29.30±1.00
		200	36.45±2.21	46.30±2.00	51.20±1.55	55.12±1.00	36.40±2.10
		300	42.30±1.50	51.25±1.75	60.12±2.50	66.30±2.00	46.50±2.00
Colon	HT-29	100	31.50±2.00	25.50±2.00	36.70±2.00	44.60±1.50	30.20±1.50
		200	38.20±1.20	36.20±2.45	49.20±1.55	51.20±1.25	34.12±1.75
		300	46.20±1.50	46.50±2.00	59.50±1.20	62.12±2.00	42.20±2.00
Colon	SW-620	100	30.20±1.45	32.45±1.45	36.79±2.00	48.12±1.45	29.20±2.45
		200	36.42±1.55	38.20±2.00	49.20±1.55	54.20±1.20	36.40±2.00
		300	42.20±2.20	49.12±1.20	55.12±1.20	60.12±2.00	41.50±2.55
Lung	A549	100	29.55±2.00	26.25±2.00	32.20±2.00	44.45±2.00	30.20±2.00
		200	34.50±1.50	34.20±1.50	39.40±2.00	51.50±1.20	36.90±1.75
		300	41.20±2.50	42.20±2.00	49.20±2.75	59.20±2.45	42.50±2.00
Prostrate	PC-3	100	36.20±1.20	38.20±1.45	36.25±2.00	44.15±2.00	27.75±2.45
		200	42.63±1.50	44.20±2.00	49.20±1.20	52.20±1.55	32.40±2.00
		300	49.70±1.20	54.12±2.45	55.50±2.00	60.12±2.00	39.99±1.55
Pancreas	Mia-Pc-Ca2	100	28.50±2.00	30.50±2.00	29.30±2.00	44.60±2.45	36.20±1.20
		200	36.40±2.50	39.90±1.25	38.20±1.75	52.20±1.50	40.12±1.00
		300	42.20±2.00	46.20±2.00	42.20±2.45	59.60±1.00	46.20±2.50
Breast	MCF-7	100	36.20±1.55	44.20±1.25	49.12±1.00	60.20±2.00	38.50±2.00
		200	44.75±1.50	49.25±2.00	59.20±2.00	76.20±2.45	42.20±1.45
		300	52.30±1.45	52.30±2.50	66.12±2.40	81.20±2.00	51.12±1.20
Breast	T47D	100	39.70±2.00	45.25±1.20	44.12±2.00	52.12±1.75	42.50±2.00
		200	49.20±2.50	56.30±2.50	51.50±1.75	68.12±2.00	49.75±1.50
		300	53.25±2.15	66.20±2.55	60.25±2.00	74.20±1.25	56.50±2.00
Myeloid Leukemia	HL-60	100	38.20±1.50	38.20±2.00	36.50±2.45	44.20±1.00	29.30±1.00
		200	46.30±1.20	46.20±1.5	42.50±2.00	49.60±1.25	36.42±1.20
		300	49.20±2.00	50.12±2.5	49.25±1.55	60.12±1.45	44.50±2.20
Lymphoblastic Leukemia	MOLT-4	100	36.40±1.55	30.20±2.00	29.12±2.45	38.20±2.00	25.42±1.45
		200	42.55±1.45	39.20±2.00	36.20±2.00	42.80±1.75	36.20±2.00
		300	49.20±2.00	46.50±1.55	42.20±2.40	50.12±1.20	41.20±1.75

3.3 TDR hampers the reproductive capacity in MCF-7 cells

To unveil the antiproliferative ability of new antitumor therapeutics, tumor colony forming assay is very helpful in relations to its validity. Colony forming assay determines the capability of a single drug treated cancer cell to produce acolony for defining the long term effects of chemo or radiotherapy. For such events, we inspected the colony formation ability of MCF-7 after 48h treatment with different concentrations of TDR (80µg/mL and 160µg/mL) essential oil and Paclitaxel (100nM). As represented by figure 2 (a-b) a dose dependent colony forming restraint was observed. The reduction was detected in size as well as the number of colonies in comparison to untreated group. TDR essential oil

at lower concentration of80µg/mL showed significant (*p≤0.05) inhibitory effect on colony formation of the order of 29%.

However, highly significant (**p≤0.01) restrainment in colony number of the order of 46% was observed at 160µg/mL. Notably, paclitaxel established 74% colony inhibition at 100nM under similar conditions relative to untreated.

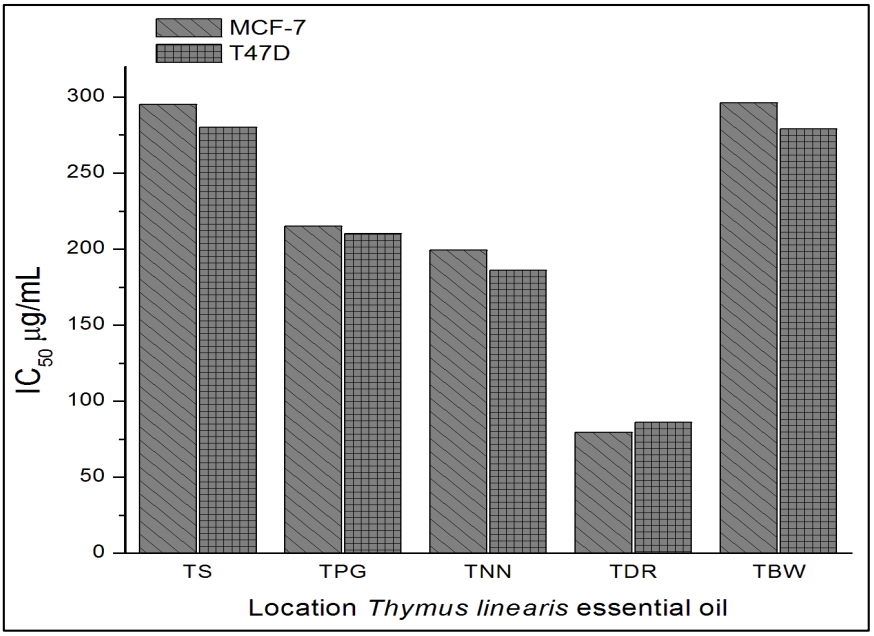

Fig 1: IC_{50} values of *Thymus linearis* essential oil from various locations on breast cancer cell lines (MCF-7 and T47D) by MTT assay.

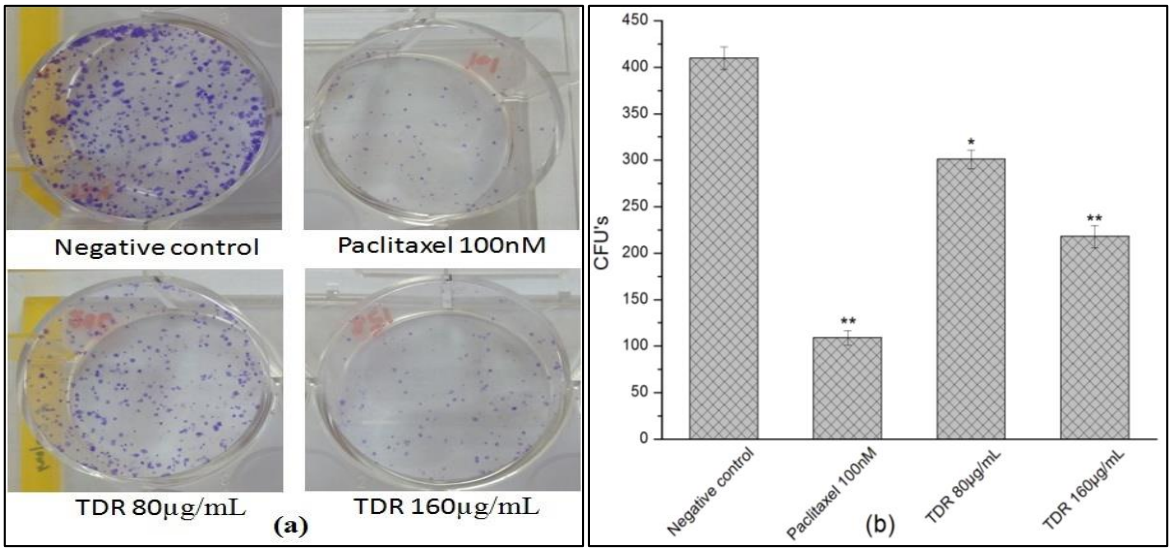

Fig 2: Cells treated with indicated concentration of TDR inhibits the colony formation of MCF-7cells. A representative result of three independent experiments is shown (a). Data were presented (b) as mean ± SD of three independent experiments and statistical significant (*P < 0.05 and **P < 0.01). Paclitaxel (100nM) was used as reference standard drug.

4. Discussion

Medicinal plants have become the richest bio resource of drugs of traditional systems of medicine, nutraceuticals, modern medicines, pharmaceutical intermediates and chemical scaffolds for synthetic drugs. Nearly, a quarter of all Food and Drug Administration (FDA) and/or the European Medical Agency (EMA) approved drugs are plant based, with a long list of well-known drugs [18]. The aromatic and therapeutic plants are the main cradles of herbal formulations and new drugs. Estimated figures point out that plant kingdom comprises about 250,000 species of which 10 percent have been scrutinized for pharmacological applications. Medicaments from plants have been used in various forms like extracts, ointments, potions and essential oils.

The pharmacological importance of plants lie in their component phytochemicals such as alkaloids, terpenes, tannins, flavonoids, phenolics and other secondary compounds, that have the caliber to elicit a definite physiological response in animal cells [19]. Essential oils extracted from medicinal plants, have been shown to possess more than 300 compounds such as limonene, α-pinene, β-myrcene, caryophyllene, β-elemene, humulene, α-farnesene, kaurene, eucalyptol, camphor, carveol,, caryophyllene oxide, farnesol, humulene epoxide, α-bisabolene oxide, carvacrol, catechol, eugenol, isopropyl alcohol, etc., that have been largely classified as monoterpenes, sesquiterpenes and hydrocarbons. Essential oils protect the plants from herbivores and also act against microbes and insects. They

find use in perfumes, dentistry make-up products, sanitary products, agriculture, and aromatherapy. Owing to their antimicrobial properties, essential oils are replacing synthetic antimicrobial agents and chemical food preservatives due to consumer concerns toward chemical preservatives [20]. Essential oils being rich in phenolic compounds have been explored extensively to appraise their activity as antioxidants or free radical scavengers. Reactive oxygen species damage lipids, proteins, and nucleic acids instigating oxidative stress and molecular alterations allied with cancer, cardiovascular disease, and neurodegenerative disorders [21].

In this study, we evaluated the essential oil of *Thymus linearis* from various locations of Kashmir valley for the cytotoxic potential against a panel of cancer cell lines. Among all the tested essential oil samples TDR essential oil was found to be potent against all the cell lines under study to varying degrees. Most effective inhibitory activity was observed against breast cancer cell lines MCF-7 and T47D. TDR inhibited the proliferation of MCF-7 and T47D by 81% and 74% at 300µg/mL after 48h treatment having the IC_{50} of 79.55µg/mL and 86.20µg/mL respectively. These results can be attributed to the intraspecific variability of the essential oil found in the genus *Thymus*. These chemical differences have been mainly ascribed to their monoterpene and sesquiterpenes [22, 23]. Our study also serves as a preliminary support to the chemical polymorphism found in the *Thymus linearis* species growing wild in Kashmir as is evident from the behavior of the essential oil from different ecozones on various cell lines.

Since MCF-7 cell line was most sensitive to the inhibitory effects of TDR essential oil, we next investigation the long term effects of TDR on its proliferation. Colony formation assay is an *in vitro* cell survival assay portraying competency of a distinct cell to establish into a colony after drug treatment. The colony is defined to encompass at least 50 cells. This assay represents a technique of choice to describe the reproductive death after treatment with ionizing radiation and is well used to assess the efficacy of cytotoxic gents on a cells [24]. Our findings established that TDR essential oil reduced colony number in MCF-7 cancer cells. The decrease was detected in size and the number of colonies in comparison to untreated group. TDR essential oil at lower concentration of 80µg/mL showed significant ($*p \leq 0.05$) inhibitory effect on colony formation of the order of 29%.The effects observed, were dose dependent where the most prominent effect was observed at higher concentration, thus revealing long term antiproliferative effect of TDR essential oil by hindering reproductive capability of MCF-7 cells to form colony. The exact mechanism by which these phytochemicals perform anticancer functions is still a topic of research because phytochemical exercise eclectic and complex range of actions on various nuclear and cytosolic factors of a cancer cell. A phytomolecule can quash malignant transformation of an initiated pre-neoplastic cell either by obstructive the metabolic conversion of the pro-carcinogen or modulating cellular signaling events involved in growth, invasion and metastasis of cancer cell. Neutralization of the reactive oxygen species (ROS) by phytochemicals has been shown to promote activities of antioxidant enzymes in a transformed cell [25]. These phytomolecules also have epigenetic elements such as DNA methylation, histone modifications and miRNAs expression as their important targets [26]. In nutshell in this study, we report the comparative antiproliferative profile of essential oil of *Thymus linearis* growing wild in Kashmir suggestive of its chemical polymorphism the study expands its scope and potential to be developed as antitumor agent.

5. Conclusion

In conclusion, our findings showed *Thymus linearis* essential oil has the potential to be explored further to identify the anticancer or compounds. The present results represent a preliminary basis for selection of appropriate plant species for advanced investigation, exclusively in isolating new bioactive compounds.

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