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INVESTIGATION ON THE *IN VITRO* ANTIOXIDANT, ANTIMUTAGENIC AND CYTOTOXIC POTENTIAL OF *THYMUS VULGARIS* L. HYDRO-ALCOHOLIC EXTRACT

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ABSTRACT:

Aim: To evaluate the *in vitro* antioxidant, antimutagenic and cytotoxic property of hydro-alcoholic extract of *Thymus vulgaris* L. by subjecting it to free radical scavenging, Ames and MTT assay respectively.

Methods: Powdered Thyme leaves were extracted in a Soxhlet apparatus using 70% ethanol to obtain a dry extract. The extract was assessed for its total phenolic, tannin, flavonoid and flavonol content using quantitative assays. The antioxidant potential of Thyme was determined using $DPPH^{\bullet}$, $ABTS^{\bullet+}$, FTC and metal chelating assays. The *in vitro* inhibitory effects of Thyme on lipid peroxidation of liver and kidney along with erythrocyte hemolysis were performed following standard procedures. Further, the antimutagenic property of Thyme was investigated using Ames assay against TA 98 and TA 100 mutant strains of *Salmonella typhimurium*. The *in vitro* cytotoxicity assay of Thyme was examined following the MTT assay against Hep 2 and Hep G2 cell lines.

Results: The hydro-alcoholic extract of Thyme registered 91.42 ± 0.90, 63.23 ± 1.02, 55.01 ± 0.42 and 65.69 ± 0.43 mg/g extract of total phenolics, tannins, flavonoids and flavonols respectively. The Thyme extract effectively scavenged the $DPPH^{\bullet}$ (IC_{50} 147.02 ± 1.84 µg/ml), $ABTS^{\bullet+}$ (IC_{50} 1.50 ± 0.39 µg/ml) and metal ions (IC_{50} 878.38 ± 5.14 µg/ml) in a concentration dependent manner. It also significantly inhibited the *in vitro* lipid peroxidation in liver and kidney and also AAPH induced erythrocyte hemolysis. The percentage inhibitions on the last day of the FTC assay for the hydro-alcoholic Thyme extract and BHT were found to be 51.05% and 76.84% respectively. Depletion in the sodium azide induced His^{+} revertants was observed which justified the antimutagenic property of Thyme. A decrease in the viability of cancer cells were observed with increasing concentrations of Thyme extracts supporting its cytotoxic potential.

Conclusion: This study reveals that the common Thyme could serve as a potential antioxidant and chemotherapeutic culinary herb which may be justified by further *in vivo* studies.

INTRODUCTION: Cancer is a grievous disease which ends up fatal to the humankind at most of the time amidst the tremendous advancements in the field of chemotherapy. The reason behind is the highly expensive therapeutics along with their worse after effects. Moreover, free radical generated oxidative stress is one of the primary reasons for the pathogenesis of cancer. Hence, the current research is focused on screening the economical, easily accessible and natural source which could cure cancer without any secondary effects. Plants take up a very important place in the treatment of cancer due to their high antioxidant potential against most of the free radical generated pathogenesis.

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Thymus species are predominantly found in Mediterranean region, Asia, Southern Europe and North Africa which comprises around 300 species of perennial, aromatic herbs and sub shrubs. They are used as herbal tea, tonic, carminative, antitussive, antiseptic and in treatment of cold [1].

They are known for their medicinal uses due to their pharmacological and biological properties. Though these plants are very well known for their traditional medicinal uses, only very scarce scientific reports are available for their antioxidant properties.

Thymus vulgaris L. (Thyme) belonging to the Lamiaceae family is an aromatic culinary herb used throughout the world for its invaluable medicinal properties [2]. Thyme contains 1-2% of ethereal essential oil among which monoterpenes are the major constituents with 40% of thymol and carvacol which are known for their antimicrobial, spasmolytic and expectorant effects [3]. The other essential oils of Thyme include geraniol, α-terpineol, thuyanol-4 and linalool [4].

Owing to its aroma, Thyme essential oil is well defined for the use in perfumery and cosmetics[5]. The earlier pharmacological reports on Thyme include its chemical composition and antioxidant activity [6], antimicrobial activity of its essential oil [1], antibacterial activity [7], antifungal activity of essential oil [8], *in vitro* antibacterial effect against swine strains of *Escherichia coli* [9], antiradical activity [10], antileishmaniasis [11], anticancer activity against HeLa cell line [12], antispasmodic activity [13] and immunomodulatory activity [14]. The different organic crude extracts of *Thymus vulgaris* have recently been reported for its chemical composition by Al Hashmi *et al* [15].

MATERIALS AND METHODS:

Chemicals: 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), 2,2-Diphenyl-1-picryhydrazyl (DPPH), 3-(2-pyridyl)-5, 6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine (Ferrozine), 2,4,6-tripyridyl-S-triazine (TPTZ), linoleic acid, α, α′-Azodiisobutyramidine dihydrochloride (AAPH), Butylated hydroxytoluene (BHT), sodium azide, Dulbecco's Modified Eagle Medium (DMEM) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetra zolium bromide (MTT) were purchased from Sigma or Himedia. The *Salmonella typhimurium* strain used in the antimutagenic assay was obtained from Microbial Type Culture Collection and Genebank (MTCC), Chandigarh, India. The cytotoxicity assay used Hep2 (human oral carcinoma) and HepG2 (human liver carcinoma) cell lines which were retrieved from National Centre for Cell Sciences (NCCS), Pune, India. All other chemicals used in the study were of analytical grade.

Experimental animals: Male albino Wistar rats (150-200 g) used in the present study were procured from the small animals breeding station, Mannuthy, Kerala, India. They were housed in polypropylene cages (38 x 23 x 10cm) with not more than six animals per cage and maintained under standard environmental conditions (14h dark /10h light cycles; temp 25±2°C; 35-60% humidity, air ventilation) and were fed with standard pellet diet (M/s. Hindustan Lever Ltd., Mumbai, India) and fresh water *ad libitum*.

The animals were acclimatized to the environment for two weeks prior to experimental use. Animals were fasted over night before the experimental schedule, but have free access for water *ad libitum*. The experiment was carried out according to the guidelines prescribed by Animal Welfare Board and animal ethic committee.

Preparation of plant extract: The fresh Thyme leaves were collected during the month of May from Ooty, Tamil Nadu, India. The leaves were cleaned to remove adhering dust, shade dried and powdered in a Willy mill to 60- mesh size and used for solvent extraction. The Thyme powder was then extracted in a soxhlet apparatus using 70% ethanol. The hydro-alcoholic extract was concentrated in a rotary evaporator (Yamato BO410, Japan) and then dried completely. The extract obtained was weighed and the yield percent was calculated. This extract was dissolved in 70% ethanol at required concentrations and used for the following *in vitro* assays.

Determination of Secondary Metabolites:

- **Determination of total phenolics and tannins:** The total phenolic content of Thyme extract was determined by Folin-Ciocalteu method. Using the same extract, the tannins were estimated after treatment with polyvinyl

polypyrrolidone (PVPP). The amount of total phenolics and tannins were calculated as the tannic acid equivalents (TAE) following the method described by McDonald *et al* [16].

- **Determination of Flavonoids and Flavonols:** The flavonoid content was determined according to the method described by Barreira *et al* [17] and the flavonol content was measured following the method detailed by Grubesic *et al* [18].

Radical Scavenging Assays:

- **Determination of DPPH$^{\bullet}$ Radical Scavenging Assay:** The DPPH$^{\bullet}$ radical scavenging efficacy of Thyme hydro-alcoholic extract along with the standard antioxidant BHT was measured according to the method of Blios [19]. Concentration of extract required to decrease the initial concentration of DPPH by 50%, IC_{50} was calculated.

- **Determination of ABTS$^{\bullet+}$ Radical Scavenging Assay:** The total antioxidant activity of the hydro-alcoholic extract of Thyme and BHT was measured by ABTS radical cation decolorization assay according to the method of Re *et al* [20]. The results were expressed in terms of IC_{50}.

Determination of Metal Chelating Activity: The iron (II) chelating activity was measured by determining the efficiency of the hydro-alcoholic extract of Thyme to inhibit the formation of iron (II)-ferrozine complex following the method of Dinis *et al* [21]. The chelating activity of the standard antioxidant BHT was also determined.

Determination of *in-vitro* Antilipid peroxidation: The efficiency of the Thyme extract to inhibit lipid peroxidation in liver and kidney tissue of rat was performed by the modified method of Ohkawa *et al* [22] described by Adefegha *et al* [23]. The inhibition percentage was calculated for five different concentrations of extract (100 – 500 µg) and the results were expressed as the IC_{50}, concentration of extract required to inhibit 50% of lipid peroxidation.

Determination of *in vitro* Antihemolytic activity: The effect of the Thyme hydro-alcoholic extract to inhibit the *in vitro* hemolysis of erythrocytes generated by 50 mM AAPH in the blood drawn from rats was assessed following the method of Jimenez *et al* [24]. Results were expressed as IC_{50}.

Determination of Antioxidant activity by Ferric thiocyanate (FTC) test: This test was carried out according to the linoleic acid emulsion system described by Ismail *et al* [25].

Determination of Antimutagenic Activity: The antimutagenic effect of Thyme against sodium azide, a direct acting mutagen was determined according to the method of Maron and Ames[26]. For this 2 ml of top agar containing 0.2 ml of 0.5 mM histidine–biotin was mixed with sodium azide at a concentration of 0.0025 mg/plate. 1-5 mg/ml of Thyme extract dissolved in dimethyl sulfoxide (DMSO) and 0.1 ml of freshly grown *Salmonella typhimurium* culture (1×109 cells/ml approximately) were poured onto minimal agar plates and incubated at 37^{o}C for 48 h. After the incubation, the reverting colonies were counted using a colony counter.

Determination of *In vitro* Cytotoxicity Assay: The *in vitro* cytotoxic effect of Thyme extract was tested against Hep2 (human oral carcinoma) and HepG2 (human liver carcinoma) cell lines. Cells were cultured in DMEM growth medium supplemented with 10% Fetal Bovine Serum, 100 U/ml of penicillin and 100 µg/ml of streptomycin sulphate. The cells were maintained in logarithmic phase under a humidified atmosphere of 95% air and 5% CO_2 at 37º C [27]. For experiments, cells were plated in 96-well plates (105 cells/well for adherent cells or 0.3×106 cells/well for suspended cells in 100 µL of medium).

The stock solution of Thyme extract was prepared in 1% DMSO at a concentration of 50 µg/ml and four more concentrations were prepared by serially diluting the stock in the ratio of 1:1, 1:2, 1:3 and 1:4. After 24 h of incubation, the well with the monolayered cancer cells were loaded with the different concentration of extracts and incubated for 3 days (72 h). Control groups received the same amount of DMSO. Growth of tumor cells was quantified by the ability of living cells to reduce the yellow dye (MTT) to a blue formazan product [28]. At the end of 72 h incubation, the medium in each well was replaced by fresh medium (200 µL) containing

0.5 mg/ml of MTT. Three hours later, the formazan product of MTT reduction was dissolved in DMSO, and absorbance was measured using a multi-plate reader (DTX 880 Multimode Detector, Beckman Coulter Inc., Fullerton, CA, USA) at 550 nm. The results were expressed as the percent of cancer cells viable after its treatment with the plant extract.

RESULTS:

Percent yield and secondary metabolites: The percent yield of extractable compounds in 70% ethanol of Thyme leaves was found to be 8.7%. The antioxidants namely phenolics, tannins, flavonoids and flavonols were estimated and given in **table 1**. The total phenolic content of the hydro-alcoholic extract was found to be 91.42 ± 0.89 mg TAE/g extract whereas the flavonoid was 55.01 ± 0.42 mg RE/g extract. Total phenolics and tannins were expressed in terms of tannic acid equivalents whereas flavonoids and flavonols were expressed in terms of rutin equivalents.

TABLE 1: PHYTOCHEMICAL CONTENTS OF HYDRO-ALCOHOLIC EXTRACT OF *THYMUS VULGARIS*

Sample	Phenolics (mg TAE/g extract)	Tannins (mg TAE/g extract)	Flavonoids (mg RE/g extract)	Flavonols (mg RE/g extract)
Hydro-alcoholic Thyme extract	91.42 ± 0.89	63.23 ± 1.02	55.01 ± 0.42	65.69 ± 0.43

TAE – Tannic acid equivalents; RE – Rutin equivalents. Values are means of three independent analyses ± standard deviation (n = 3)

Radical Scavenging Assays: The hydro-alcoholic extract of Thyme effectively scavenged free radicals like $DPPH^{\bullet}$ and $ABTS^{\bullet+}$. The scavenging potential of the Thyme extract was in direct proportion with its concentration. The IC_{50} values of Thyme extract obtained from these radical scavenging assays are depicted in **table 2**. The IC_{50} values obtained for the $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and metal chelating assay were 147.02 ± 1.84 µg/ml, 1.50 ± 0.39 µg/ml and 878.38 ± 5.14 µg/ml respectively. The IC_{50} values rendered by the standard antioxidant BHT against $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and metal chelating assay were found correspondingly to be 14.36 ± 0.49 µg/ml, 0.06 ± 0.002 µg/ml and 128.43 ± 1.28 µg/ml.

TABLE 2: IC_{50} VALUES OF HYDRO-ALCOHOLIC EXTRACT OF *THYMUS VULGARIS* FOR RADICAL SCAVENGING ASSAYS

Sample	IC_{50} (µg/ml)		
	$DPPH^{\bullet}$	$ABTS^{\bullet+}$	Metal chelating assay
Hydro-alcoholic Thyme extract	147.02 ± 1.84	1.50 ± 0.39	878.38 ± 5.14
BHT	14.36 ± 0.49	0.06 ± 0.002	128.43 ± 1.28

BHT – Butylated hydroxyl toluene; Values are means of three independent analyses ± standard deviation (n = 3)

The extract inhibited *in vitro* lipid peroxidation of liver and kidney with an IC_{50} of 130.99 ± 1.57 µg/ml and 260.65 ± 1.70 µg/ml respectively. AAPH induced lysis of erythrocytes was inhibited by Thyme extract in a dose dependent manner whose IC_{50} was found to be 114.79 ± 1.81 µg/ml. The results are shown in **table 3**.

TABLE 3: IC_{50} VALUES OF HYDRO-ALCOHOLIC EXTRACT OF *THYMUS VULGARIS* AGAINST *IN VITRO* LIPID PEROXIDATION AND ERYTHROCYTE HEMOLYSIS

Sample	IC_{50} (µg/mL)		
	In vitro Lipid peroxidation		*In vitro* erythrocyte hemolysis
	Liver	Kidney	
Hydro-alcoholic Thyme extract	130.99 ± 1.57	260.65 ± 1.70	114.79 ± 1.81

Values are means of three independent analyses ± standard deviation (n = 3).

The hydroperoxides formed in the linoleic acid emulsion system was effectively inhibited by the hydro-alcoholic thyme extract whose results are given in **figure 1**. The percentage inhibitions of the thyme extract and BHT on the last day were 51.05% and 76.84% respectively.

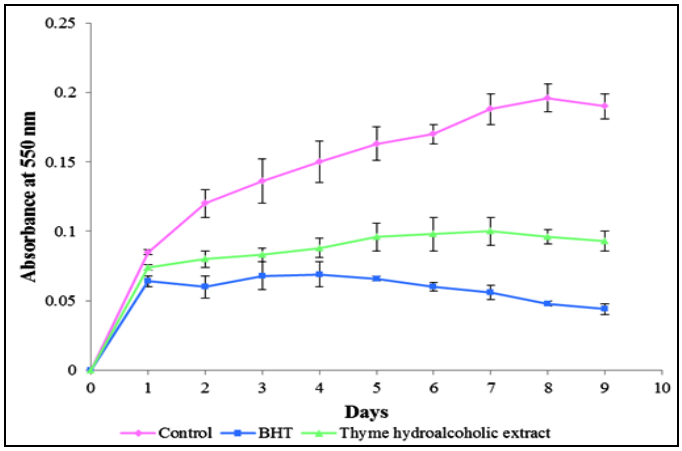

FIGURE 1: ABSORBANCE OF HYDRO-ALCOHOLIC EXTRACT OF *THYMUS VULGARIS* AND BHT AT 550 nm

Antimutagenic activity: The antimutagenic activity of hydro-alcoholic extract of Thyme was assessed at five different concentrations (1 – 5 mg) and the results obtained are shown in **table 4**. The extract reduced the number of sodium azide induced revertants in a concentration dependent manner. The extract at a concentration of 5 mg/plate exhibited the maximum inhibition percentage against both TA 98 (88.64 ± 0.86%) and TA 100 (90.95 ± 0.41%) strains. Control showed the maximum number of revertants (930.00 ± 18.00 for TA 98 and 1010.00 ± 15.00 for TA 100) compared to the plates treated with the plant extract.

TABLE 4: ANTIMUTAGENIC ACTIVITY OF HYDRO-ALCOHOLIC EXTRACT OF *THYMUS VULGARIS* (TV) AGAINST SODIUM AZIDE (NaN_3)

Concentration (mg/plate)	Average number of revertants/ plate		Percentage inhibition (%)	
	TA 98	TA 100	TA 98	TA 100
NaN_3 (0.0025)	930.00 ± 18.00	1010.00 ± 15.00	-	-
NaN_3 + TV (5)	105.67 ± 8.02	91.33 ± 4.16	88.64 ± 0.86	90.95 ± 0.41
NaN_3 + TV (4)	206 ± 8.54	234.33 ± 6.03	77.85 ± 0.92	76.80 ± 0.60
NaN_3 + TV (3)	271.33 ± 6.11	366.33 ± 9.29	70.82 ± 0.66	63.73 ± 0.92
NaN_3 + TV (2)	347.67 ± 9.60	425.67 ± 8.33	62.61 ± 1.03	57.85 ± 0.82
NaN_3 + TV (1)	462 ± 9.16	536.33 ± 11.24	50.32 ± 0.99	46.90 ± 11

Values are means of three independent analyses ± standard deviation (n = 3)

***In vitro* Cytotoxicity assay:** The cytotoxic effect of hydro-alcoholic Thyme extract on Hep2 and HepG2 cell lines was assessed following MTT assay and the results are given in figure 2. The results are expressed as the percent of viable cancer cells present after its treatment with different concentrations of extract. The viable Hep2 and HepG2 cells present after its treatment with 50 µg/ml of Thyme extract was found to be 37.73% and 23.83%. The viability of both the cancer cell lines decreased with increasing concentrations of the Thyme extract.

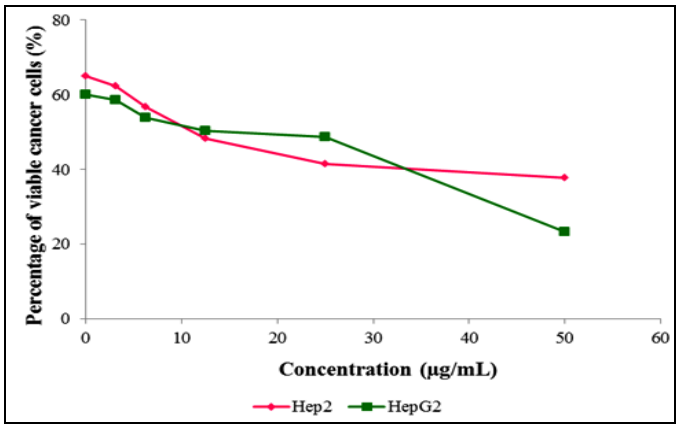

FIGURE 2: MTT ASSAY OF HYDRO-ALCOHOLIC EXTRACT OF *THYMUS VULGARIS* ON Hep2 AND HepG2 CELL LINES

DISCUSSION: Leaves of *Thymus vulgaris* are known quite well for their inevitable chemical composition which includes flavonoids, tannins, triterpenes, α- pinene, p- cymene, limonene, thymol, carvacrol and germacrene-D [29]. Though the essential oil of *Thymus vulgaris* has already been validated for many of its properties, there are only few researches published in the crude extract. Based on the reports of Marghitaş *et al* [7], the present study extracted the compounds using 70% ethanol and the percent yield was found to be 8.7%.

The hydro-alcoholic extract contained appreciable amounts of phenolics, tannins, flavonoids and flavonols. The presence of phenolics may exert antioxidant effects as free radical scavengers, as hydrogen donating sources or as singlet oxygen quenchers and metal ion chelators [30].

$DPPH^{\bullet}$ is a commercially available nitrogen centered free radical which produced a deep purple color when dissolved in methanol. This purple color decreased with increasing concentrations of the Thyme extract whose absorbance was read at 517 nm. The Thyme extract scavenges the free radical by functioning as a proton radical or hydrogen

donor [31]. $ABTS^{\bullet+}$ is a blue colored cationic radical which gets converted to a colorless neutral form on reacting with antioxidants like phenolics, thiols and vitamin C [32]. The hydro-alcoholic Thyme extract decolorized the $ABTS^{\bullet+}$ radical quite effectively even at a very lower concentration range like 0.5-2.5 µg/ml which could be attributed to the presence of phenolic compounds.

The metal ion chelating activity increased with increasing concentrations of Thyme extract. This chelating agent may serve as secondary antioxidant because it reduces the redox potential thereby stabilizing the oxidized form of the metal ions [33]. Though the Thyme extract did not exhibit a strong antioxidant effect as that of the standard antioxidant BHT, it did have an activity that reveals Thyme as a potential electron donor which can react with the free radicals and convert them to a stable product.

Lipid peroxidation refers to the process in which the free radicals react with the lipid molecules of cell membranes resulting in cellular damages. It is also believed that lipid peroxidation is one of the causes of cardiovascular diseases and cancer. Plant phenolics are known for their ability to delay the onset of lipid peroxidation and decompose hydroperoxides in living tissues [34].

The Fe^{2+} induced lipid peroxidation in rat liver and kidney was inhibited by the hydro-alcoholic Thyme extract in a dose dependent manner. This activity of the extract may be due to the phenolic phytoconstituents which could have formed complexes with the Fe^{2+} thereby preventing them from catalyzing the lipid peroxidation [23]. AAPH induces hemolysis by oxidation of lipid molecules and degradation of protein in the erythrocyte membrane [35]. The cytoprotective effect of Thyme was evident from the diminishing AAPH derived peroxyl radicals which was measured at 540 nm. This protective effect could be because of the presence of essential oils and phenolics present in Thyme which would have prevented the oxidation of erythrocyte membrane molecules.

Hydroperoxide inhibition activity of the Thyme extract was assessed following the FTC assay. The Thyme extract inhibited the hydroperoxide formation throughout the experiment with the percentage inhibition of 51.05% on the last day which was lesser than that of BHT (76.84%).

The Thyme extract would have retarded the formation of hydroperoxides by acting aggressively over the hydroxyl radicals generated in the linoelic acid emulsion system. The flavonoids in addition to the phenolic compounds function as effective scavengers of these free radicals rendering a protective effect [36]. The reactive oxygen species act either as direct or indirect initiators as well as promoters of mutagenesis and carcinogenesis. Thus the free radical scavenging efficacy of a plant may also play an important role in the antimutagenic activity [37]. Since the hydro-alcoholic extract ensured good antioxidant activities, the present study also investigated the antimutagenic activity of Thyme against sodium azide induced mutagenicity in TA98 and TA100 strains of *Salmonella typhimurium*.

The Thyme extract at a concentration of 5 mg/ml demonstrated to be a potential antimutagenic agent against TA98 and TA100 by decreasing the number of revertants to a greater extent (88.64% and 90.95% respectively). As the recent reports of Boubaker *et al* [38] suggests, the flavonoids present are most likely to be involved in the antimutagenic activity.

The cytotoxic effect of the hydro-alcoholic Thyme extract against the Hep2 and HepG2 cell lines were assessed by the MTT assay. The Thyme extract at a concentration of 50 µg/ml, remarkably reduced the percentage of viable Hep2 and HepG2 cells to 37.73% and 23.38% respectively. This validates the use of *Thymus vulgaris* in the treatment of cancer due to its apoptotic activity on human cancer cell lines. Though the mechanism behind this antiproliferative property of Thyme extract needs to be analyzed in depth, our results obtained were in good agreement with the recent reports of Debnath and Hussain [39] who justified the cytotoxic potential of *Ocimum sanctum* which also belongs to the family Lamiaceae.

CONCLUSION: The results of the present study reveal that the hydro-alcoholic Thyme extract serves as an economical natural source with antioxidant, antimutagenic and antiproliferative activities. Further, it is required to assess the mechanism behind the above mentioned activities and their relationship with the phytochemical constituents and essential oils present in *Thymus vulgaris* by *in vitro* and *in vivo* studies.

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