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Research Article

Cytotoxic activity of *Thymus capitatus* collected from Hail region in Saudi Arabia with mechanistic study via induction of caspase-dependent apoptosis and S-phase arrest

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Abstract

Thymus capitatus is a plant grows in Mediterranean area and some Arab countries such as Saudi Arabia. It possesses numerous medicinal values. Its common name is Zaatar and it belongs to family Lamiaceae *Thymus capitatus* leaves and stem were collected from Hail region, Saudi Arabia. Then both leaves and stem were extracted with ethanol. This study was performed to evaluate cytotoxic activity of *Thymus capitatus* leaves and stem ethanolic extract in details. Doxorubicin was used as a standard and the relevant half maximal inhibitory concentration (IC_{50}) values were computed for each cell line by 3-(4,5- diemthylthiazole-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. In addition, further mechanistic study was carried out by using Apoptosis assay to explore cytotoxic activity of plant extract. Both leaves and stems extracts were screened against HepG2, A-549, HCT-116 and MCF-7 cancer cell lines. It was found that leaves' extract shows high and moderate cytotoxic activity against both A-549 and HepG2 cancer cell lines, respectively (with IC_{50} = 13.6 and 21.5 µg/ml, respectively), while stem's extract exerted moderate cytotoxic activity against A-549 cancer cell lines (with IC_{50} = 21.38 µg/ml). Further mechanistic study was carried out on A-549 cells by using apoptosis assay. It showed that leaves' extract resulted in arrest of S-phase and caused apoptosis *through* activation of caspase-3, p53 and Bax, in addition to down regulation of Bcl-2.

Keywords: apoptosis; cytotoxic; extract; mechanism; *Thymus capitatus*

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Introduction

Plants have been used as a treatment for many diseases, and became an important source of various natural compounds that possess important biological activities. Many aromatic plants possess medicinal properties and one of these plants is thymus genus that belongs to Lamiaceae family. This wild-growing plant was traditionally used in medicine for its antispasmodic, antiseptic and antimicrobial properties (Van Den Brouck and Lemli, 1981; Pannizi *et al*., 1993; Smith-Palmer *et al*., 1998; Cosentino *et al*., 1999).

One of the most important species is *Thymus capitatus* (Tc), which is a short herb that possess strong aromatic odor. Its tall ranges between 20 -50 cm, with upright erect branches, woody, light colored young white felted, often only leafy underarm clumps (Bouyahya *et al*., 2020).

Tc proved to possess various biological activities, such as antioxidant (Ricci *et al*., 2005; Bounatirou *et al*., 2007; Al-Mustafa AH and Al-Thunibat, 2008), antifungal (Grayer and Harborne, 1994; Kalemba and Kunicka, 2003) and antibacterial activities against both gram positive and negative bacteria (Alves TM *et al*., 2000; Fan and Chen, 2001; Al-Tarawneh, 2004; Sienkiewicz *et al*., 2012; Inayatullah *et al*., 2017). In addition, *Thymus capitatus* aqueous extract revealed good activity against *Helicobacter pylori* (Tabak *et al*., 1996) and anthelmintic activity and can be considered as promising alternative to the commercially available anthelmintic such as albendazole (Elandalousi *et al*., 2013).

Few studies were performed on Tc to explore its cytotoxic activity (Kaileh *et al*., 2007; Dzamic *et al*., 2015; Yavuz *et al*., 2017). According to Dzamic *et al*.**,** a study was carried out on Tc using tetrazolium (MTT) colorimetric assay. From this assay, it was observed that Tc possess moderate cytotoxic activity against human cell lines MRC-5, HCT 116 and HT-29 (IC50=30–150l gm.l^{-1}) and it caused a significant reduction of pathogen colonization in colon.

According to Yavuz *et al*., a study was performed on Tc essential oil extract by testing for its biological activity on neoplastic transformed human telomerase reverse transcriptase (h-TERT) immortalized mesenchymal stem cells (hMSC- telo1) and a tumorigenic cell line. It was found that there was a significant effect of the extract on h-MSC- telo1and irradiated h-MSC- telo1cells and it showed higher effect on cells treated with 0.5% (v/v) thyme essential oil. In addition, it was deduced that essential oil of thyme is potential for cell viability.

In addition, another study was carried out on Tc essential oil. This study proved that it has pro-apoptotic and anticancer potential on colon cancer stem cells (Yavuz *et al*., 2021).

Previous studies did not explore cytotoxic mechanism of action; hence, the main objective of this work is to examine the anticancer activity of ethanolic extract of *T. capitatus* leaves and stem from Hail region in Kingdom of Saudi Arabia against various cell lines with elucidating its mechanism of action

Materials and Methods

Chemicals and solvents

All chemicals and solvents used were of high quality and analytical grade from Sigma-Aldrich (St. Louis, MO, USA)

Collection and extraction of plant material

Tc was obtained from Hail region, Saudi Arabia. The plant material was identified, air dried and then powdered. The powdered leaf and stem parts were extracted separately by repeated cold maceration with 95% ethanol. The extracts were then concentrated using vacuum to give residue of leaf extract (TCL) and stem extract (TCS).

Cancer cell lines

MCF-7 cells (human breast cancer cell line), HepG-2 cells (human Hepatocellular carcinoma), HCT-116 (Human colorectal carcinoma) and A-549 (Lung carcinoma) were obtained from Nawah Scientific Inc., (Mokatam, Cairo, Egypt). Cells were maintained in DMEM media supplemented with 100 mg/mL of streptomycin, 100 units/mL of penicillin and 10% of heat-inactivated fetal bovine serum in humidified, 5% (v/v) CO_2 atmosphere at 37 °C.

Cell viability assay

Aliquots of 100µL cell suspension (5x10^3 cells) were in 96-well plates and incubated in complete media for 24 h. Cells were treated with another aliquot of 100 µL media containing drugs at various concentrations. After 72 h of drug exposure, cells were fixed by replacing media with 150µL of 10% TCA and incubated at 4 °C for 1 h. The TCA solution was removed, and the cells were washed 5 times with distilled water. Then the numbers of viable cells were determined by the MTT test.

Apoptosis analysis (Annexin V/PI Assay)

Apoptotic cells were further analyzed by Annexin V/PI assay. A549 (lung carcinoma) cells were cultured to a confluent monolayer then treated with the tested sample at the IC_{50} concentration as described earlier. After treatment for 72 h, A549 cells were then harvested and rinsed twice in PBS (20 min. each) followed by binding buffer. Moreover, cells were re-suspended in 100µL of kit binding buffer with the addition of 1µL of Annexin V followed by 40 min. incubation at 4 °C. Cells were then washed and re-suspended in 150µL of binding buffer with the addition of 1µL of 4′,6-diamidino-2-phenylindole, is a fluorescent stain (DAPI) (1µg/mL in phosphate buffer solution (PBS)). Then, the cells were analyzed using the flow cytometer.

Results and Discussion

Cytotoxic activity of T. capitatus ethanolic extract against cancer cell lines

T. capitatus ethanolic extract decreased cancer cell viability and exhibited selectivity for these cells

The anticancer activity of Tc has not been studied in details, hence, we tried to explore the cytotoxic potential of Tc leaves and stem ethanolic extracts and investigate its mechanism of action. The ethanolic extract of Tc was screened against various cancer cell lines such as; HepG2, A-549, HCT-116 and MCF-7. The resulting IC_{50} values were summarized in Table 1.

Table 1. IC50 values of *Thymus capitatus* leaves' extract against HepG2, A-549, HCT-116 and MCF-7 cancer cells

IC50 (µg/ml)[a]					
		HepG-2	**A-549**	**HCT-116**	**MCF-7**
Extract	**Leaves**	21.5 ± 0.53	13.6 ± 0.67	65.4 ± 0.35	58.6 ± 0.46
	Stem	>100	21.38 ± 0.42	>100	66.5 ± 0.38
Doxorubicin		0.64 ± 0.19	0.11 ± 0.35	0.21 ± 0.26	0.36 ± 0.41

a IC50 values are reported as the mean (IC50±SD) of three experiments.

It was found that leaves' extract exhibited high cytotoxic activity against both A-549 and HepG2 cancer cell lines (with IC_{50} value of 13.6 and 21.5 µg/ml, respectively), while it produced mild cytotoxic activity versus HCT-116 and MCF-7 cancer cell lines (with IC_{50} value of 65.4 and 58.6 µg/ml, respectively) (Figure 1). Stem extract demonstrated high and mild cytotoxic activity on both A-549 and MCF-7 cancer cell lines (with IC50 values of 21.3 and 66.5 µg/ml, respectively) (Figure 2).

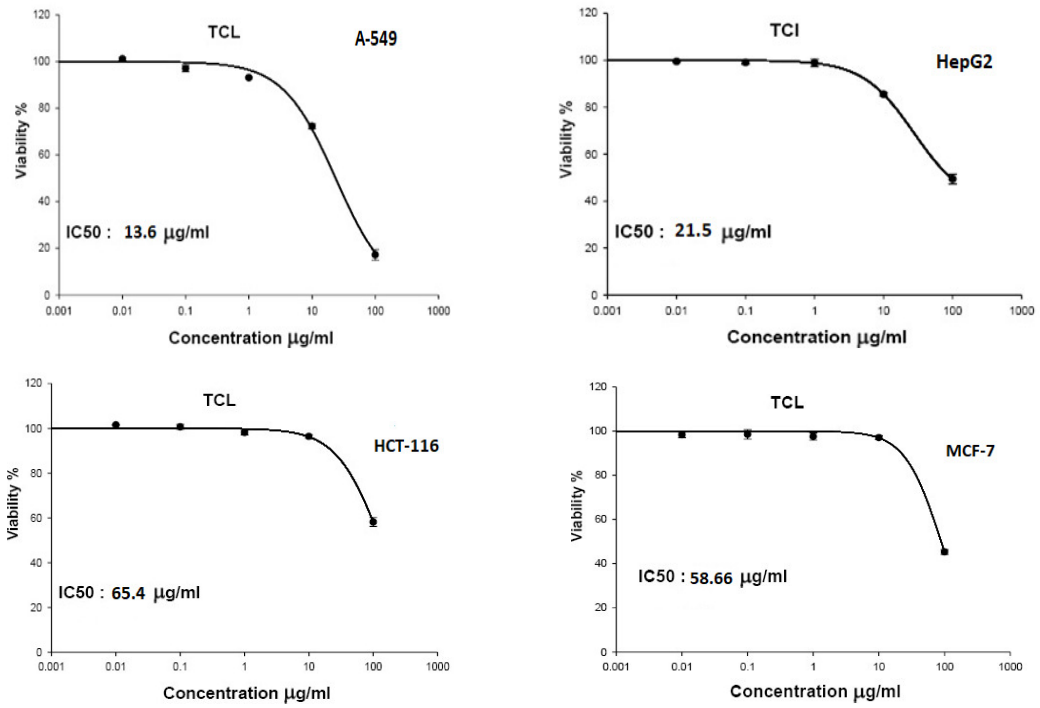

Figure 1. Dose response curve indicating cytotoxic activity of *Thymus capitatus* leaves' extract on the viability of A-549, HepG2, HCT-116, MCF-7 cancer cell lines

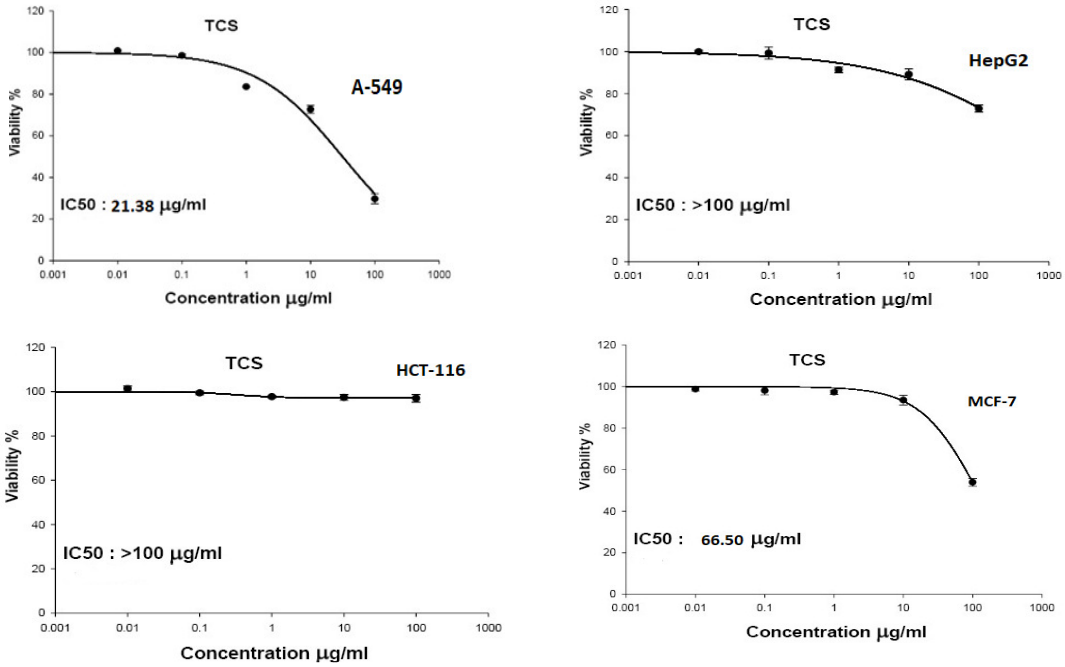

Figure 2. Dose response curve indicating cytotoxic activity of *Thymus capitatus* stem's extract on the viability of A-549, HepG2, HCT-116 and MCF-7 cancer cell lines

Moreover, leaves' extract showed good selectivity towards A-549 and HepG2 cancer cell lines when compared to the healthy normal MRC-5 cells. Highest selectivity was observed against A549 cells, whereby leaves' extract was about 3 times more selective to A549 cells, Table 2. From this high selectivity, we can deduce that leaves' extract is less toxic against normal cell. These promising results against A549 cells inspired us to elucidate the mechanism of action of Tc leaves' extract on A549 cancer cell lines.

Table 2. IC50 values of *Thymus capitatus* leaves' extract against MRC-5 normal cells

	IC50 (µg/ml)[a]	Selectivity Index (SI)[b]			
Cell Lines	MRC-5	HepG-2	A-549	HCT-116	MCF-7
Leaves' Extract	38.9 ± 0.45	2.2	2.9	0.59	0.66

a IC50 values are reported as the mean (IC50±SD) of three experiments. b SI = (IC50 of MRC5)/(IC50 of cancer cell).

Thymus capitatus leaves' ethanolic extract generated S-phase cell-cycle arrest in A-549 cells

As highest cytotoxic activity was observed against A-549 cancer cell lines by leaves extract, so we decided to characterize bioactivity of this extract *through* demonstrating its effect on the progression of cell cycle. A-549 cancer cells that was treated with leaves' ethanolic extract indicated an increase in cells' fraction present in S-phase (48.31% compared to 36.17% in untreated cells) (Figure 3).

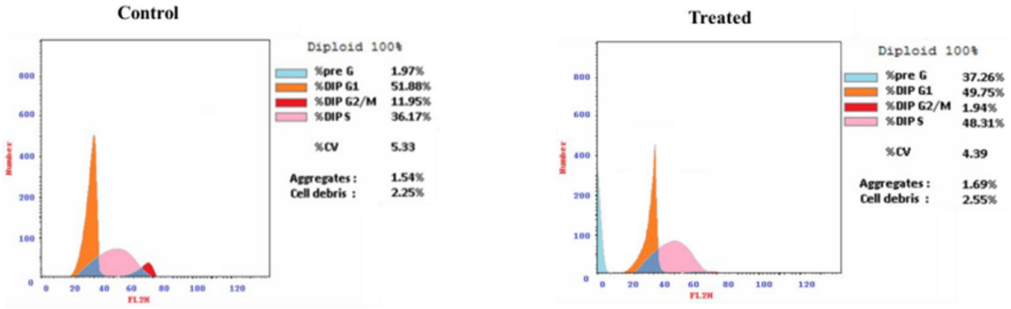

Figure 3. Cell-cycle histograms showing effect of *Thymus capitatus* leaves' ethanolic extract on cell-cycle progression in A549 cancer cells after 72 h of treatment at IC50 concentration

Furthermore, cells' fractions in the pre-G1 phase have been increased upon treating them with leaves' ethanolic extract (37.26% compared to 1.97% in the untreated cells). These results indicate that leaves' ethanolic extract causes apoptosis in A-549 cells. Consequently, cell cycle analysis showed S-phase arrest and apoptosis induction in A-549 cells.

Thymus capitatus leaves' ethanolic extract generated caspase-dependent and A-549 cells apoptosis mediated through p53

Upon cell-cycle analysis, it was found that ethanolic extract of leaves caused apoptosis in A-549 cells. Consequently, to ensure apoptosis induction by the extract, an Annexin V/propidium iodide (PI) apoptosis assay was carried out, by which A-549 cell lines were treated with leaves' extract. The assay results indicated that leaves' ethanolic extract caused early (1.69% compared to 0.55% in the untreated cells) and late (21.99% compared to 0.18% in the untreated cells) apoptosis, Figure 4. Also, it was observed that number of necrotic cells has been increased after that treatment (13.58% compared to 1.24% in untreated cells). Hence, we can deduce that leaves' ethanolic extract resulted in death of cancer cells mostly *via* apoptosis induction with low amount of cells that undergone necrosis.

Apoptosis induction by the leaves' ethanolic extract was confirmed *via* testing levels of expression for some proteins related to apoptosis, such as caspase-3 and p53. Caspases' activation is usually considered the main mark of apoptosis. p53 is a protein responsible for tumor suppression and it affects many anti-proliferative

processes including apoptosis. This protein activation is critical for suppressing tumorigenesis (Khazaei *et al.*, 2017). Results obtained from western blot analysis exhibited high levels of protein expression for cleaved caspase-3 upon treating A549 cancer cells with leaves' ethanolic extract. This explains apoptosis' induction in the treated A-549 cancer cells and matches up the Annexin V/PI assay data, Figure 5.

Furthermore, p53 expression levels were increased upon treatment with leaves' ethanolic extract, which indicates that p53 mediates probably the induced apoptosis. Therefore, it can be conducted that Tc leaves' extract cause cell death through activating caspase-3 and p53 in A-549 cells.

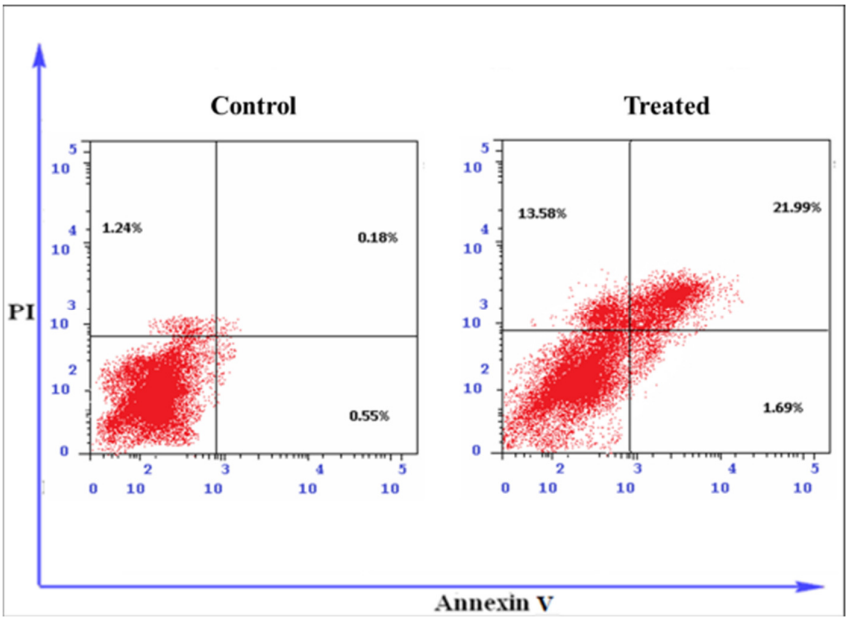

Figure 4. Apoptosis quadrant plots showing the apoptotic effects of *Thymus capitatus* leaves' extract on A549 cancer cells upon treatment with extract at IC50 concentration for 72 h

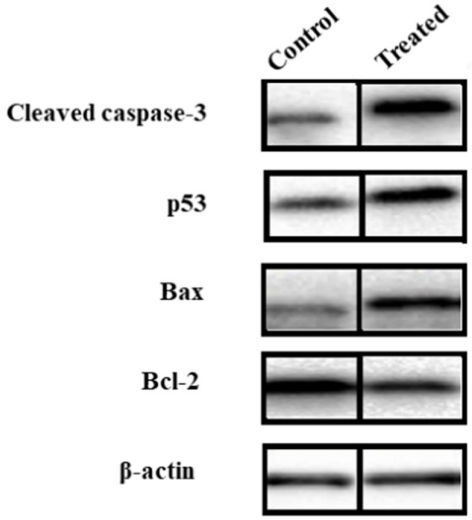

Figure 5. Western blot analysis of cleaved caspase-3, p53, Bax and Bcl-2 in A549 cancer cells, upon treatment with *Thymus capitatus* leaves' extract for 72 h at IC50 concentration. β-actin was used as an internal control

Bax is considered as p53 target and it is responsible for the activation of caspase during apoptosis, but the anti-apoptotic protein Bcl-2 can prohibit these Bax's effects (Chen *et al*., 2008; Al-Salman *et al*., 2020). Hence, testing these proteins might emphasize the apoptosis mechanism activated by leaves' extract. From western blot analysis results, it was found that leaves' extract led to high level of Bax protein expression, while it resulted in reduction of Bcl-2 expression level in A-549 cancer cells (Figure 5), that ensures pro-apoptotic effects of leaves' extract and matches results of other apoptosis-related assays.

Upon extraction of Tc leaves and stem with ethanol and investigating its cytotoxic activity of on different cancer cell lines, it was found that a good activity was obtained on A-549 lung cancer cells. Further apoptotic study was carried out on these cancer cells to explore extract's mechanism of action. It was observed that leaves' extract induces S-phase cell cycle arrest through caspase dependent apoptosis.

Conclusions

The current study investigated anticancer effect of Tc leaves and stem ethanolic extract in details. It was found that leaves' extract has high cytotoxic activity against some cancer cell lines and mild activity against others, with highest activity observed against A-549 cancer cells. Additional studies demonstrated that leaves' extract caused caspase-dependent; apoptosis mediated through p53 in A-549 cancer cells that led to S-phase cell cycle arrest. We expect that this study may direct others to isolate the relevant bioactive constituents with performing in vivo studies.

Authors' Contributions

Conceptualization: KMY, BH, and ASA; Data curation: KMY and BH; Formal analysis: KMY and ASA; Funding acquisition: KMY, BH and ASA; Investigation: KMY, KOA and ZMA; Methodology: AFA and NOA; Project administration: KMY and BH; Resources: KOA, ZMA and AFA; Software: KMY and ASA; Supervision: KMY; Validation: KMY and BH; Writing original draft: KMY and ASA.

All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

References

Al-Mustafa AH, Al-Thunibat OY (2008). Antioxidant activity of some Jordanian medicinal plant used traditionally for treatment of diabetes. Pakistan Journal of Biological Sciences 11(3):351-358.

Al-Salman HNK, Ali ET, Jabir M, Sulaiman GM, Al-Jadaan SAS (2020). 2- Benzhydrylsulfinyl-N-hydroxyacetamide-Na extracted from fig as a novel cytotoxic and apoptosis inducer in SKOV-3 and AMJ-13 cell lines via P53 and caspase-8 pathway. European Food Research and Technology 246(8):1591-1608. *https://doi.org/10.1007/s00217-020-03515-x*

Al-Tarawneh AA (2004). Study on *Pseudomonas aeruginosa* isolated from infected patients: copper uptake, hematological findings and effect of some medicinal plants. M.Sc. Thesis, Sudan University for Science and Technology, Sudan. *http://repository.sustech.edu/handle/123456789/6053*

Alves TM, Silva AF, Brandao M, Grandi TS, Samania EF, Junior AS, Zani CL (2000). Biological screening of Brazilian medicinal plants. Chemotherapy 95(3):367-373. *https://doi.org/10.1590/S0074-02762000000300012*

Bouyahya A, Chamkhi I, Guaouguaou FE, Benali T, Balahbib A, El Omari N, ... El Menyiy M (2020). Ethnomedicinal use, phytochemistry, pharmacology and food benefits of *Thymus capitatus*. Journal of Ethnopharmacology 259:112925. *https://doi.org/10.1016/j.jep.2020.112925*

Bounatirou S, Smiti S, Miguel MG, Faleiro L, Rejeb MN, Neffati M, ... Pedro LG (2007). Chemical composition, antioxidant and antibacterial activities of the essential oils isolated from Tunisian *Thymus capitatus* Hoff. et Link. Food Chemistry 105:146-155. *https://doi.org/10.1016/j.foodchem.2007.03.059*

Chen C-Y, Liu T-Z, Tseng W-C, Lu F-J, Hung R-P, Chen Chi-Hung, Chen C-H (2008). (−)-Anonaine induces apoptosis through Bax- and caspase-dependent pathways in human cervical cancer (HeLa) cells. Food Chemical Toxicology 46(8):2694-2702. *https://doi.org/10.1016/j.fct.2008.04.024*

Cosentino S, Tuberoso CIG, Pisano B, Satta M, Mascia V, Arzedi E, Palmas F (1999). *In-vitro* antimicrobial activity and chemical composition of Sardinian *Thymus* essential oils. Letters in Applied Microbiology 29(2):130-135. *https://doi.org/10.1046/j.1472-765x.1999.00605.x*

Dzamic A, Nikolic B, Giweli A, Mitic-Culafic D, Sokovic M, Ristic M, ... Marin P (2015). Libyan thymus capitatus essential oil: anti-oxidant, anti-microbial, cytotoxic and colon pathogen adhesion-inhibition properties. Journal of Applied Microbiology 119(2):389-399. *https://doi.org/10.1111/jam.12864*

Fan M, Chen J (2001). Studies on antimicrobial activity of extracts from thyme. Wei Sheng Wu Xue Bao (Acta Microbiologica Sinica) 41(4):499-504.

Grayer RJ, Harborne JB (1994). A survey of antifungal compounds from higher plants (1982-93). Phytochemistry 37(1):19-42. *https://doi.org/10.1016/0031-9422(94)85005-4*

Inayatullah S, Shafee M, Shafiq M, Asif M, Parveen S, Kakar K, Hayat-Khan Y, Amir M, Abdul Q, Yakoob M, Baloch A (2017) Antimicrobial activity of thyme essential oil cultivated in Quetta, Balochistan. Pakistan Journal of Biosciences 10(2):105-110. *https://doi.org/10.12692/ijb/10.2.105--110*

Kaileh M, Vanden Berghe W, Boone E, Essawi T, Haegeman G (2007). Screening of indigenous Palestinian medicinal plants for potential anti-inflammatory and cytotoxic activity. Journal of Ethnopharmacology 113(3):510-516. *https://doi.org/10.1016/j.jep.2007.07.008*

Kalemba D, Kunicka A (2003). Antibacterial and antifungal properties of essential oils. Current Medicinal Chemistry 10(10):813-829. *https://doi.org/10.2174/0929867033457719*

Khazaei S, Abdul Hamid R, Ramachandran V, Mohd Esa N, Pandurangan AK, Danazadeh F, Ismail P (2017). Cytotoxicity and proapoptotic effects of *Allium atroviolaceum* flower extract by modulating cell cycle arrest and caspase dependent and p53-independent pathway in breast cancer cell lines. Evidence Based Complementary and Alternative Medicine 2017:1468957. *https://doi.org/10.1155/2017/1468957*

Panizzi L, Flamini G, Cioni PL, Morelli I (1993) Composition and antimicrobial activity of essential oils of four Mediterranean Lamiaceae. Journal of Ethnopharmacology 39(3):167-170. *https://doi.org/10.1016/0378-8741(93)90032-z*

Elandalousi RB, Akkari H, B'chir F, Gharbi M, Mhadhbi M, Awadi S, Darghouth MA (2013). *Thymus capitatus* from Tunisian arid zone: chemical composition and in vitro anthelmintic effects on *Haemonchus contortus*. Veterinary Parasitology 197(1-2):374-378. *https://doi.org/10.1016/j.vetpar.2013.05.016*

Ricci D, Fraternale D, Giamperi L. Bucchini A, Epifano F, Burini G, Curini M (2005). Chemical composition, antimicrobial and antioxidant activity of the essential oil of *Teucrium marum* (Lamiaceae). Journal of Ethnopharmacology 98(1-2):195-200. *https://doi.org/10.1016/j.jep.2005.01.022.*

Sienkiewicz M, Lysakowska M, Denys P, Kowalczyk E (2012). The antimicrobial activity of thyme essential oil against multidrug resistant clinical bacterial strains. Microbial Drug Resistance 18(2):137-148. *https://doi.org/10.1089/mdr.2011.0080*

Smith-Palmer A, Stewart J, Fyfe L (1998) Antimicrobial properties of plant essential oils and essences against five important food-borne pathogens. Letters in Applied Microbiology 26(2):118-122. *https://doi.org/10.1046/j.1472-765x.1998.00303.x*

Tabak M, Armon R, Potasman I, Neeman I (1996). *In-vitro* inhibition of *Helicobacter pylori* by extracts of thyme. Journal of Applied Bacteriology 80(6):667-672. *https://doi.org/10.1111/j.1365-2672.1996.tb03272.x*

Van Den Broucke CO, Lemli JA (1981) Pharmacological and chemical investigation of Thyme liquid extracts. Planta Medica 41(2):129-135. *https://doi.org/10.1055/s-2007-971689*

Yavuz DÖ, Mavis M, Ateş G, Hanoğlu A, Hanoğlu DY, Başer KHC, Serakıncı N (2017). Identification of potential therapeutic role of *Thymus capitatus* essential oil using cellular imaging. Procedia Computer Science 120:961-966. *https://doi.org/10.1016/j.procs.2017.11.332*

Yavuz DO, Becer E, Kendirci R, Vatansever HS (2021). Proapoptotic and anticancer potentials of *Thymus capitatus* essential oil on colon cancer stem cells. Cyprus Journal of Medical Sciences 6(4):319-324. *https://doi.org/10.3923/pjbs.2008.351.358*