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ASSESSMENT OF CYTOTOXIC AND APOPTOTIC EFFECTS OF EXTRACTS OF TANACETUM ARGENTEUM SUBSP. ARGENTEUM IN LIVER AND BREAST CANCER CELL LINES

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ABSTRACT:
THE STUDY AIMED TO INVESTIGATE THE ANTICANCER EFFECTS OF HEXANE, CHLOROFORM, METHANOL, AND AQUEOUS METHANOL EXTRACTS OF TANACETUM ARGENTEUM SUBSP. ARGENTEUM (TA) AGAINST BREAST CANCER CELL LINE (MCF-7) AND HUMAN LIVER CANCER CELL LINE (HEPG2) IN VITRO. ANTICANCER, ANTI-INFLAMMATORY, ANTIOXIDANT AND ANTIMICROBIAL PROPERTIES OF TANACETUM SPECIES HAVE BEEN ATTRACTED SCIENTISTS FOR YEARS. CELL VIABILITY WAS DETECTED BY MTT ASSAY AT DIFFERENT CONCENTRATIONS (100, 50, 25, 10, 2.5, AND 1 µG/ML). DRIED AERIAL PARTS OF TA WERE EXTRACTED WITH METHANOL (TAM) AND PARTITIONED BETWEEN WATER AND SUBSEQUENTLY N-HEXANE (TAH) AND CHLOROFORM (TAC). TAC WITH AN IC50 VALUE OF 13.66 MG/ML EXTRACT ON HEPG2 CELLS AND TAM WITH AN IC50 VALUE OF 48.4 MG/ML EXTRACT ON MCF-7 CELLS SHOWED THE MOST POTENT ANTIPROLIFERATIVE ACTIVITY IN A CONCENTRATION-DEPENDENT MANNER AT 24H TIME. THEREFORE, THESE GROUPS ARE USED FOR FURTHER IMMUNOHISTOCHEMICAL ANALYSES. AFP EXPRESSION HAS DETECTED A MEANINGFUL (P<0.05) DECREASE BETWEEN CONTROL GROUPS AND HEPG2 TAC IC50. P53 EXPRESSION DEMONSTRATE SIGNIFICANT (P<0.05) INCREASE IN HEPG2 TAC IC50 GROUP COMPARED TO THE CONTROL. THUS, THE RESULTS OF OUR STUDY SUGGEST THAT TAH AND TAC EXTRACTS ON HEPG2 CELLS ALSO TAH AND TAM EXTRACTS ON MCF-7 CELLS MIGHT HAVE HIGH ANTICANCER EFFECTS.

KEY WORDS: TANACETUM ARGENTEUM, HEPATOCELLULAR CARCINOMA BREAST CANCER, ANTICANCER ACTIVITY, ANTIPROLIFERATIVE ACTIVITY

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INTRODUCTION

Cancer is a pathological condition when the balance between cell proliferation and cell death is disrupted by excessive cell proliferation or decreased apoptosis. It is reported that suppressed or decreased apoptosis plays an essential role in the cancer formation process.

Cell cycle arrest is an effective strategy in eliminating cancer cells.\(^6\) Induction of apoptosis involves activation of the cell death pathway, which is a p53 dependent apoptotic chemo preventive process.\(^7\)

Both hepatocellular carcinoma (HCC) and breast cancer are among the most prevalent cancers around the globe.\(^8\)\(^9\) Since HCC is a complex disease, diagnosis and treatment methods have not been fully enlightened.\(^10\) Research related to breast cancer has led to spectacular advances in our perception of breast cancer, introducing us to more advanced and efficient treatment options.\(^11\) Consciously evolved research about HCC and breast cancer mechanisms is required to develop efficient treatments.\(^12\)\(^13\)

The development of new generation anticancer agents has become a trendy field of research due to the development of resistance to anticancer drugs used against cancer cells and the chronic use of these drugs also showing toxicity in normal cells. For this purpose, both synthetic and natural products are often tested in in vitro and in vivo studies in new generation anticancer agent research.\(^14\)

Numerous studies about the cytotoxic activity of different Tanacetum species on various cancer cell lines\(^15\)\(^16\)\(^17\)\(^18\)\(^19\)\(^20\)\(^21\)\(^22\)\(^23\)\(^24\). The genus Tanacetum L., which belongs to the


\(^14\) Goyal S, Gupta N, Chatterjee S, Nimesh S. “Natural Plant Extracts as PotentialTherapeutic Agents for the Treatment of Cancer.” \textit{Current topics in medicinal chemistry}. 2016; 16.


\(^19\) Vasileva AM, Iliev IA, Lozanov VS, Dimitrova MB, Mitev VI, Ivanov IP. “In vitro study on the antitumor activity of Tanacetum vulgare L. Extracts.”. \textit{Bulgarian Chemical Communications}. 2019;51(2):249–55.

Asteraceae family, contains 46 species in Turkey. One of these species is endemic, *Tanacetum argenteum* subsp. *argenteum*.25

The *Tanacetum* species have traditionally been used in rural regions as a medicine for arthritis, fever, aches, menstrual disorders, migraine, insect bites, and toothache.

Additionally, it is reported that *Tanacetum* species have shown that they have antimicrobial, anti-inflammatory, wound healing, and antioxidant effects.26 27 28 29

The primary chemical contents of *Tanacetum* species are sesquiterpenoids and flavonoids metabolites. These components are considered to have anticancer features.30 31

This study aims to investigate how various dosages of different extracts of *Tanacetum argenteum* subsp. *argenteum* aerial affects the proliferation rate and apoptosis in liver and breast cancer. For that purpose, we used HepG2 and MCF-7 cell lines.

**MATERIAL AND METHODS**

**Plant material**

Aerial parts of *T. argenteum* subsp. *argenteum* were collected from the Doğanşehir region of Malatya province of Turkey in 2012 and were identified by Sukran Kultur, PhD, from the Department of Pharmaceutical Botany, Faculty of Pharmacy, University of Istanbul. Several plant specimens were deposited in the Herbarium of the Faculty of Pharmacy, Istanbul University (ISTE No: 98969).

**Extraction**

Aerial parts of *T. argenteum* subsp. *argenteum* (TA) were dried and pulverized. Ten grams of powdered samples were extracted with 100 mL methanol three times (24 h per time).

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at room temperature. After filtration and evaporation, the residue was dissolved in 50 mL 50% aqueous methanol and subjected to solvent-solvent partition between n-hexane (3×50 mL) and chloroform (3×50 mL). The methanol, n-hexane, chloroform, and aqueous methanol extracts of *T. argenteum* subsp. *argenteum* obtained by these methods were coded as TAM, TAH, TAC, and TAAM, respectively. All extracts were stored at +4°C for further studies.

**Cell Culture**

MCF-7 (American Type Culture Collection, USA) and HepG2 (American Type Culture Collection, USA) cells were cultured in 89% Dulbecco’s Modified Eagle’s Medium (DMEM) and supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (Penicillin+Streptomycin). The cells were cultured at 37°C under a humidified atmosphere containing 5% CO2. Cells were imaged daily under an inverted phase-contrast microscope (Zeiss Primovert).

**MTT Assay**

MCF-7 and HepG2 cells (10,000 cells/well) were cultured in 96-well culture plates. All the extracts were dissolved in DMSO. Cells were treated with six different concentrations (1, 2.5, 10, 25, 50, and 100 µg/mL) of those four different extracts for 24 hours under the same conditions.

After 24 hours, viability and proliferation of the cells were measured by using an MTT assay. The MTT solution 10 µL/well was added to the plates incubated for 4 hours at 37°C. Formazan crystals were solubilized in Crystal Dissolving Solution with 100 µL per well. The absorbance of the formazan solution and the dye were measured spectrophotometrically with the microplate reader (Synergy H1, BioTek) at 570 nm. Cell viability in the medium was determined. According to the result of MTT analysis, the IC50 values, the 50% inhibition concentration at cell proliferation were determined. Each experiment was performed in triplicate.

**Immunohistochemistry**

To detect the AFP and p53 expression, cultivated HepG2 and MCF-7 cells were washed two times 1xPBS after washing cells and fixed in formalin for 15 min at room temperature. Next, the cells were washed three times in PBS, then were incubated in PBS containing 2% bovine serum albumin (BSA) for 15 min at room temperature to block nonspecific bindings. Subsequently, the cells were incubated with AFP (1:100) overnight. The next day, cells were washed and labeled with Alexa Fluor 488 conjugated anti-rabbit IgG for AFP (1:200, Thermo Fisher, US) and mouse for p53 (1:200, Thermo Fisher, US) expression for 30 minutes at RT. After three washing steps, nuclei were counterstained with 1 mg/ml in Hoechst solution (Sigma, Germany) and mounted on microscope glass slides.

**Statistical Analysis**

Final data statistically analyzed by Graph-Pad Prism 9.0 (GraphPad Software, San Diego, CA, USA) program. Statistical analysis was evaluated by One-way analysis of variance (ANOVA) and Tukey/Bonferroni post Hoc test, student t-tests.
RESULTS

Detection of Cell Viability and Antiproliferative Activities

Fig. 1. MTT proliferation test results of HepG2 cells under the treatment of TAM, TAH, TAC, TAAM in doses of 1, 2.5, 10, 25, 50, 100 μg/mL. *Significant from the control group. (*: p<0,05, **:p<0,01, ***: p<0,001) Methanol, hexane, chloroform and aqueous methanol extracts of the aerial parts of Tanacetum argenteum subsp. argenteum were shown as TAM, TAH, TAC and TAAM, respectively.

Fig. 2. MTT proliferation test results of MCF-7 cells under the treatment of TAM, TAH, TAC, TAAM in doses of 1, 2.5, 10, 25, 50, 100 μg/mL.*significant from the control group. (*: p<0,05, ***: p<0,001). Methanol, hexane, chloroform and aqueous methanol extracts of the aerial parts of Tanacetum argenteum subsp. argenteum were shown as TAM, TAH, TAC and TAAM, respectively.
All the extracts of TA with different solvents (methanol, hexane, chloroform, and aqueous methanol extracts) showed dose-dependent antiproliferative activity on both HepG2 and MCF-7 cells (Figure 1,2).

As displayed in Figure 1, TAH and TAC extracts of TA inhibited the growth of HepG2 cells in all doses. The IC50 values of TAH and TAC extracts were 54.9 μg/mL and 13.66 μg/mL, respectively. TAAM and TAM extracts also inhibited growth of HepG2 cells in concentrations of 2.5, 10, 25, 50, 100 μg/mL (*: p<0.05, **: p<0.01, ***: p<0.001). However, the IC50 values of TAAM and TAM could not be calculated.

In doses of 2.5, 10, 25, 50, 100 μg/mL of all extract groups of MCF-7 cells, there was a meaningful decrease in cell proliferation (Figure 2 *: p<0.05, ***: p<0.001). The IC50 values of TAH and TAM were 65.11 μg/mL and 48.4 μg/mL, respectively. Even though TAAM and TAC possessed strong cytotoxic activity against MCF-7 cells, the IC50 values of these groups could not be determined.

TAC on HepG2 and TAM on MCF-7, the most effective groups, were followed in the immunohistochemical analyses for further investigations after 24-hours treatment with IC50.

**Fig.3.** Phase-contrast micrographs of HepG2 cells. A: Control untreated HepG2 cells B: HepG2 cells under the treatment of TAC (IC50). Scale Bars: 50 μm

*TAC: Chloroform extract of the aerial parts of Tanacetum argenteum subsp. argenteum.*

**Fig.4.** Phase-contrast micrographs of MCF-7 cells. A: Control Untreated MCF-7 cells B: MCF-7 cells under the treatment of TAM (IC50). Scale Bars: 100 μm

*TAM: Methanol extract of the aerial parts of Tanacetum argenteum subsp. argenteum.*
After 24 hours, treated groups of TAC (IC50) on HepG2 cells and TAM (IC50) on MCF-7 cells indicated a significant decline of cell viability in comparison to the control groups under examination of the phase-contrast microscope (Figure 3,4).

**Expression of AFP and P53**

![Confocal microscopic images of HepG2 cells labeled with the AFP antibody.](image)

**Fig.5.** Confocal microscopic images of HepG2 cells labeled with the AFP antibody. DyLight® 488 was used as the secondary antibody for the groups (A) control and (B) TAC (IC50) treated HepG2 cells. Nuclei were counterstained blue with Hoechst 33258. Images merged (right) groups showed a significant decrease of AFP expression on TAC (IC50) treated HepG2 cells when compared with the control group.

Scale bars: 100 μm.

*TAC: Chloroform extract of the aerial parts of Tanacetum argenteum subsp. argenteum.*
As represented in Figure 5 and Figure 6 (A), there was a significant (p<0.05) decrease of AFP fluorescence intensity on TAC (IC50) treated HepG2 cells when compared with the control group. At the microscopic level, labeling of the cells with the specific immunofluorescent antibody demonstrated a significant (p<0.05) difference in P53 expression in the presence of TAC (IC50) treated HepG2 cells (Fig. 6 B, Fig. 7).

P53 expression was also declined in TAM (IC50) treated MCF-7 cells. However, this incline was statistically insignificant (Fig. 6 C, Fig. 8).

p53 Expression on HepG2 cells

Fig.7. Confocal microscopic images of HepG2 cells labeled with the p53 antibody. DyLight® 488 was used as the secondary antibody for the groups (A) control and (B) TAC (IC50) treated HepG2 cells. Nuclei were counterstained blue with Hoechst 33258. Images merged (right) groups showed a significant increase of p53 expression on TAC (IC50) treated HepG2 cells when compared with the control group.

Scale bars: 100 μm.

TAC: Chloroform extract of the aerial parts of Tanacetum argenteum subsp. argenteum.
p53 Expression on MCF-7 Cells

Fig. 8. Confocal microscopic images of MCF-7 cells labeled with the p53 antibody. DyLight® 488 was used as the secondary antibody for the groups (A) control and (B) TAM (IC$_{50}$) treated MCF-7 cells. Nuclei were counterstained blue with Hoechst 33258. Images merged (right) groups showed no significant change.

Scale bars: 100 μm.

TAM: Methanol extract of the aerial parts of Tanacetum argenteum subsp. argenteum.

Briefly, our research concludes that all extracts of Tanacetum argenteum subsp. argenteum (TAM, TAH, TAC, TAAM) inhibited the growth of MCF-7 and HepG2 cells. The most effective extracts were TAC on HepG2 and TAM on MCF-7. The results of this present study underpin the data about the therapeutic activity of TA extracts in human liver and breast cancer cells.

DISCUSSION

Chemotherapy, radiation therapy, and surgery are used as a standard for cancer treatment. However, these treatments are not fully effective, and they have severe side effects. Natural products are one of the sources that have been considered vital resources for cancer therapeutics. Plant extracts and traditional foods have been included in new strategies related to the prevention and treatment of cancer. Tanacetum species have evidenced widespread use among people living in rural areas with previously published ethnomedical data.

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Various studies were conducted on the anticancer activity of different *Tanacetum* species. In this study, we examined TA extracts’ cytotoxic and apoptotic potential (TAM, TAH, TAC, TAAM) on both HepG2 and MCF-7 cell lines by using MTT and immunohistochemistry. We confirmed the reduction of cell viability by TA extracts through the MTT assay. TA extracts decreased cell viability in both HepG2 and MCF-7 cell lines in a dose-dependent manner (Fig.1, Fig 2.). American National Cancer Institute (NCI) reported that extracts with 30 µg/mL or less IC50 value have significant anticancer activity, and they are considered potential anticancer agents. According to criteria based on NCI, TAC extract against HepG2 cells presented a robust cytotoxic activity with an IC50 value of 13.66 µg/mL. Also, TAH extracts on HepG2 cells and TAH and TAM extracts on MCF-7 cells exhibited good anticancer activity with IC50 values of 54.9, 65.11, and 48.4 µg/mL, respectively. Previous studies revealed that methanol extracts from *TA* leave inhibited the proliferation of the MCF-7 cancer cell line. Also, Şen *et al.* reported that n-hexane and chloroform extracts of TA have a substantial anticancer effect against MCF-7 and HT-29 cancer cell lines. Likewise, it is reported that n-hexane extract of *T. argentum* subsp. argentum

possessed high cytotoxic activity on MDA-MB-231, HEK-293, MPANC-96, CaCo-2, and PC3 cell lines and studied the anti-inflammatory activity of this species.\textsuperscript{48}

Previous phytochemical studies have reported sesquiterpene lactones in this species. It was also observed in these studies that some of these sesquiterpene lactones had cytotoxic activity.

In addition, sesquiterpene lactones are known as essential compounds with cytotoxic activity.\textsuperscript{49} \textsuperscript{50} Therefore, especially sesquiterpene lactones found in the plant may be responsible for the activity of the extracts.

The p53 protein, the guardian of the genome, is primarily responsible for ensuring the adequate functioning of cells. The p53 protein halts the cell cycle and recruits DNA repair enzymes to sustain the genomic integrity when detected damage.\textsuperscript{51} Furthermore, if the damage is beyond repair, p53 induces apoptosis and senescence to exclude flawed cells to protect the body against tumorigenesis by inhibiting anti-apoptotic genes. As a result, the end product of TP53, the p53 protein, is either suppressed or mutated in more than %50 cancers.\textsuperscript{52}

The expression level of p53 was found meaningfully increased in the high dose \textit{Tanacetum polycephalum} hexane extract group compared to the untreated control in mice and the MCF-7 cell line.\textsuperscript{53} A crude methanol extract prepared from Fenugreek seeds was treated to HepG2 cell for 48 hours, and it is stated that p53 expression was upregulated for 72 hours, attributable to induced apoptosis triggered by p53 in a dose-dependent manner.\textsuperscript{54} \textit{C. galaticus}, \textit{C. antalyensis}, and \textit{L. candidum} species at different concentrations of methanol extracts have strong cytotoxic effects even in the lowest concentration due to p53-mediated apoptosis on MCF-7 cells.\textsuperscript{55} Several concentrations of hydroalcoholic extract of \textit{C. cretica} were inspected for their p53 expression in the HepG2 cell line. The p53 expression was upregulated in the treatment groups compared to the control group.\textsuperscript{56} Parthenolide is known for \textit{Tanacetum parthenium} (L.) Schultz Bip. with antioxidant and antitumor activities. Parthenolide reduces the cell population on human cervical cancer (SiHa) and breast cancer

\textsuperscript{50} Gören, N., Tahtasakal, E., Pazzuto, J. M., Cordell, G. A., Shwarzt, B., Proksch, P. “Sesquiterpene lactones from \textit{Tanacetum argenteum}.”, \textit{Phytochemistry, 1994;36(2), 389–392.}
(MCF-7) cell lines. The p53 gene was detected upregulated on these treated cells by reverse transcriptase-PCR and DNA fragmentation assay.\textsuperscript{57}

In this study, p53 expression in HepG2 cells significantly increased under TAC (IC50) treatment. In addition, MCF-7 cells under TAM (IC50) treatment displayed increased p53 expression compared to untreated cells, parallel to other studies, even though the increase of p53 expression was not significant.

Alpha-fetoprotein (AFP) is a tumor-associated protein that has a considerable role in diagnosing hepatocellular carcinoma, the most common form of primary liver tumor.\textsuperscript{58} AFP is specific to the mammalian embryo, and it is found in a small amount in healthy adults straight after birth.\textsuperscript{59}

In a previous study in which eight different compounds isolated from the methanolic extract of \textit{T. hirsuta} showed vigorous antiproliferative activity on HepG2 cells. These compounds have caused an increase in p53 expression; also, a meaningful decrease in serum levels of AFP.\textsuperscript{60}

It is reported that the AFP expression of HepG2 cells, when treated with matrine, was decreased. This suggests that the proliferation of HepG2 cells has been inhibited under the treatment of matrine.\textsuperscript{61} Furthermore, antiproliferative activity examination of methanol (MeOH) extracts of mulberry leaves (\textit{Morus alba} L.) had a promising inhibitory effect on HepG2 cells. The levels of AFP decreased in HepG2 cells compared to the control group by immunohistochemical analyses.\textsuperscript{62}

Consistent with aforementioned studies, AFP expression in HepG2 cells under the treatment of TAC (IC50) extract was decreased in our study. Priorly, a few studies have been performed on \textit{Tanacetum argenteum} subsp. argenteum on MCF-7 cell line.\textsuperscript{63, 64}

To our knowledge, we present the first data related to the changes at the expression of AFP and p53 in liver cancer cell lines under the \textit{Tanacetum argenteum} subsp. argenteum extracts into the literature.

Recently, the anticancer effects of \textit{Tanacetum} species on several human cancer cell lines have been examined and proceed to illuminate. Our study was carried out to examine the reported cytotoxic activity of TA on MCF-7 and HepG2 cell lines. The results showed


that TAC and TAH extracts on HepG2 cells and TAM and TAH extracts on MCF-7 cells had a promising antiproliferative effect.

The possible mechanism of TAC, which is the most effective extract that we determined among the 4 extracts by MTT method in HepG2 cells, may be the induction of apoptosis with the increase in p53 expression in the cells. The increase in the expression of p53 protein, which plays a role in the regulation of the cell cycle, may also decrease the elevated AFP expression in cancer cells.

Although the TAM extract, we used for MCF7 cells tended to increase p53 expression, insignificance of the increase may be due to the duration of treatment we applied. This effect needs to be investigated further with longer duration experiments and examination of other apoptosis-related proteins.

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