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ORIGINAL ARTICLE

In Vitro Cytotoxic and Anti-inflammatory Activities of *Tanacetum argenteum* (Lam.) Willd. subsp. *argenteum* Extract

Tanacetum argenteum subsp. *argenteum* Ekstrelerinin *In Vitro* Sitotoksik ve Anti-inflamatuvar Etkileri

Gökay ALBAYRAK[1], Ayşe NALBANTSOY[2], Şüra BAYKAN[1]*

[1]Ege University, Faculty of Pharmacy, Department of Pharmaceutical Botany, İzmir, Turkey
[2]Ege University, Faculty of Engineering, Department of Bioengineering, İzmir, Turkey

ABSTRACT

Objectives: The objective of this study was to examine the anti-inflammatory and cytotoxic potential of n-hexane, ethyl acetate, and methanolic extracts of *Tanacetum argenteum* subsp. *argenteum*.

Materials and Methods: *Tanacetum* L. is the third largest genus of *Asteraceae* family and is represented by 60 taxa in Turkish flora. Sesquiterpene lactones and pyrethrins are the main chemical groups of the genus. *T. argenteum* subsp. *argenteum* is an endemic taxa that is distributed in the Central and South Anatolia.

Results: *In vitro* anti-inflammatory activity was assayed using iNOS and NF-κB inhibition tests on RAW264.7 and HeLa cells. The cytotoxic activities were tested against ten cell lines using MTT assays.

Conclusion: As a result, the n-hexane extract was found more active than the positive control parthenolide in iNOS test (IC_{50}: 0.627±0.16 µg/mL) and cytotoxic experiments against PC3 and MPANC-96 cell lines (IC_{50}: 2.85±0.51 µg/mL and 5.35±1.24 µg/mL, respectively).

Key words: *Tanacetum argenteum* subsp. *argenteum*, anti-inflammatory, NF-κB, iNOS, cytotoxicity, MTT

ÖZ

Amaç: *Tanacetum argenteum* subsp. *argenteum* bitkisinin hekzan, etil asetat ve metanol ekstrelerinin anti-inflamatuvar ve sitotoksik potansiyellerini incelemektir.

Gereç ve Yöntemler: *Tanacetum* L. *Asteraceae* familyasının en büyük 3. cinsidir ve Türkiye florasında 60 taksonla temsil edilir. Seskiterpen laktonlar ve piretrinler cinsin ana kimyasal gruplarıdır. *T. argenteum* subsp. argenteum İç ve Güney Anadolu'da yayılış gösteren endemik bir taksondur.

Bulgular: *In vitro* anti-inflamatuvar aktivite iNOS ve NF-κB inhibisyon metodlarıyla RAW264.7 ve HeLa hücrelerine karşı test edilmiştir. Sitotoksik aktivite MTT metoduyla 10 hücre hattına karşı denenmiştir.

Sonuç: Sonuç olarak, n hekzan ekstresi iNOS testinde (IC_{50}: 0.627±0.16 µg/mL) ve PC3 ve MPANC-96 hücreleri (IC_{50}: 2.85±0.51 µg/mL ve 5.35±1.24 µg/mL, sırasıyla) üzerinde yapılan sitotoksik ölçümlerde pozitif kontrol partenolitten daha etkili bulunmuştur.

Anahtar kelimeler: *Tanacetum argenteum* subsp. *argenteum*, anti-inflamatuvar, NF-κB, iNOS, sitotoksisite, MTT

INTRODUCTION

The genus *Tanacetum* L. consists of around 160 species in the world[1] and 60 taxa of the genus exist in Turkey, 26 of which are endemic.[2] The genus is distributed in Europe and West Asia and all over Turkey, excluding the Aegean side.[3,4] *Tanacetum* sp. are used as insecticides, tonic, appetizers, anthelmintics, diuretics, carminatives, stimulants, emmenagogues, antipyretics, and antimigraine agents in Turkey.[5] *Tanacetum parthenium* (L.) Schultz Bip. (feverfew) is the most prominent species. It is known as having antimigraine properties and is used to relieve menstrual pain in traditional medicine, and standardized capsules of leaf extract are available on the market.[3,6] Eudesmane sesquiterpenes are the main components of the genus. *Tanacetum argenteum.* (Lam.) Willd., subsp. *argenteum* is an endemic perennial plant

*Correspondence: E-mail: sura.baykan@ege.edu.tr, Phone: +90 505 640 48 68 ORCID-ID: orcid.org/0000-0002-3624-4811
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with 20-30 cm stem length and deeply divided or 2-pinnatisect leaves. The involucre is 3-4 (-5) mm broad and campanulate. Its flowers are yellow, female flowers are absent, and achenes are brown, 2-2.25 mm. It grows on rocks and limestone cliffs, ranging from 990 to 2500 m. The plant is mainly distributed in Inner and South Anatolia.[4] The main chemical constituents of *T. argenteum* subsp. *argenteum* are sesquiterpenoids and flavonoids. Phytochemical studies on this species also resulted with the isolation of β-sitosterol and β-amyrin, desacetyllaurenobiolide, spiciformin, tatridin-A, tatridin-B, desacetyl–β-cylclopyrethrosin, desacetyltulipinolide-1β, 10α-epoxide and 8α-angeloyloxycostunolide.[7] Sesquiterpenoids are thought to be the bioactive constituents of this taxon.[3] The major compounds of the essential oil of the plant are reported as monoterpenes; α-pinene, β-pinene and santolinatriene by previous studies.[7-9] Caryophyllene oxide and α-thujone were found as the main constituents of the oil of *T. argenteum* subsp. *canum* var. *canum* in Gören and Tahtasakal[10] study.

Recently, Orhan et al.[11] measured parthenolide levels using liquid chromatograph-mass spectrometry and total flavonoid contents of three subspecies of *T. argentum*. Among all *T. argenteum* subsp. *argenteum* has the highest parthenolide and total flavonoid contents. The authors also investigated the cholinesterase inhibitory potential of these plants. Among them, the leaf extract of *T. argenteum* subsp. *flabellifolium* has the strongest cholinesterase inhibitor activity.[11] Gören and Tahtasakal[10] isolated guaian-type sesquiterpene lactones from *T. argenteum* subsp. *canum* var. *canum* and *T. argenteum* subsp. *Flabellifolium*.[12,13]

The aim of this study was to investigate the anti-inflammatory and the cytotoxic activity of *n*-hexane, ethyl acetate, and methanolic extracts of *T. argenteum* subsp. *argenteum*. For this purpose, nuclear factor kappa B (NF-κB) and induced nitric oxide synthesis (iNOS) methods were used as anti-inflammatory assays and MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used for cytotoxic activity. Thin-layer chromatography (TLC) analysis of extracts were also performed to obtain an overview on the chemical compounds of the extract.

EXPERIMENTAL

Plant material

Plants were collected from Nemrut Mountain, 1600 m, Kahta, Adıyaman on May 30th, 2012, and identified by Şüra Baykan. Voucher specimens have been deposited in the Ege University Herbarium, Faculty of Pharmacy, İzmir, Turkey (IZEF 6029) (www.izef.ege.edu.tr).

Extraction

Dried and powdered plant parts (250 g) were extracted sequentially with *n*-hexane, ethyl acetate and methanol (2x300 mL for each), sonicated at room temperature for 6 h, and then filtered. The combined extracts were evaporated using a rotary evaporator to dryness at 40˚C.

Thin-layer chromatography analysis

TLC analysis of n-hexane, EtOAc, and methanolic extracts of the plant was made. A silica gel-coated aluminum plate was used as the stationary phase and *n*-hexane:ethyl acetate (5:5) solvent system was used as the mobile phase. Comparisons was performed using parthenolide as the reference compound.

Cells

All cells were obtained from American Type Culture Collection (ATCC, Rockville, MD).

Chemicals and other materials

MTT, *n*-hexane, parthenolide, Griess reagent, 100 µ/mL penicillin G sodium, 100 µ/mL streptomycin were from Sigma (St. Louis, MO); ethyl acetate and methanol were from Merck, Germany; RPMI 1640, fetal bovine serum (FBS), 1% L-glutamine and 1% gentamicin were obtained from PAA Laboratories GmbH, Cölbe, Germany; DMEM/F12 and RPMI-1640 were from Invitrogen (Carlsbad, CA); 1 mM calcium and magnesium were from Packard Instrument Company (Meriden, CT); bovine calf serum and FBS were from Atlanta Biologicals (Lawrenceville, GA); and the Luciferase Assay Kit was obtained from Promega (E1500).

Assay for in vitro cytotoxicity

n-hexane, EtOAc, and MeOH extracts of *T. argenteum* subsp. *argenteum* were tested against ten cell lines using MTT assays. These cell lines included A549 (human alveolar epithelial cells), CaCo-2 (human epithelial colorectal adenocarcinoma), MCF-7 (human breast adenocarcinoma), MPANC-96 (human pancreatic cancer), MDA-MB-231 (metastatic human breast cancer), 253J-BV (bladder cancer cells), U87-MG (human glioblastoma-astrocytoma, epithelial-like), prostate cancer (PC_3), human cervical carcinoma (HeLa) as cancerous cells and human embryonic kidney (HEK)-293 as noncancerous cells. Parthenolide was used as a positive control. Cells were cultivated in DMEM-F12 medium, passaged twice a week. Cells (10^4 cells/well) were seeded to the wells of 96-well plates and cultivated for 24 h in an incubator. Extracts with different dilutions (0.5, 5, 50 µg/mL) and the positive control were added and cells were incubated for 48 h. After 48 h, the number of viable cells was determined using an MTT assay. For this purpose; in order to count the number of living cells, 25 µL MTT (stock solution of 2.5 mg/mL) physiologic saline (9% NaCl) was added to the wells and incubated for 4 h. After a while, the medium was removed to dissolve the formazan crystals; 150 µL DMSO was added and the absorbance was read at 520 and 620 nm. All measurements were performed in triplets and the half-maximum inhibitory concentration (IC_{50}) was determined using GraphPad Prism 5.

Assay for the inhibition of NF-κB activity

The NF-κB Luciferase Reporter HeLa Stable Cell Line (Signosis, CA) was used for this assay. Cells were cultured in DMEM-F12 supplemented with 10% FBS, 100 U/mL penicillin G sodium and 100 µg/mL streptomycin at 37˚C in an atmosphere of 5% CO_2 and 95% humidity.

Cells ($5x10^5$ cells/well/90 µL) were added to 96-well plates and incubated for 24 h. Then, test samples at different concentrations (0.5, 5, 50 µg/mL) were added to the medium and incubated for 30 min. After that, cells were induced with PMA (70 ng/

mL) for 24 h, and the medium then was removed and the cells were washed with 200 µL PBS. Cells were incubated with 20 µL lysis buffer on a shaker at room temperature for 1 h. Absorbance was measured using a luminometer subsequent to adding 20 µL Luciferase substrate. Parthenolide was used as a positive control.

Assay for inhibition of iNOS activity

The mouse macrophage (RAW264.7) cells cultured in phenol red-free RPMI medium with 10% bovine calf serum and 100 U/mL penicillin G sodium and 100 µg/mL streptomycin. Cells (10^5 cells/well) were seeded in 96-well plates and incubated for 24 h. Test samples at different concentrations (0.5, 5, 50 µg/mL) were prepared from 10 mg/mL stock solution and 20 µL were added for each.

After 60 µL serum-free medium were added and 30 min of incubation, LPS (5 µg/mL) was added to the cells to induce iNOS and cells were incubated for 24 h. The concentration of nitric oxide produced as a result of iNOS activity was determined by measuring the level of nitrite in the cell culture supernatant using Griess reagent. The absorbance was read at 540-630 nm. IC_{50} values were obtained from dose curves. Parthenolide was used as a positive control and DMSO was used in the tests as a vehicle control.[14] Extracts were also evaluated using MTT analyses to detect cytotoxic activity against RAW264.7 cells, in addition to the iNOS inhibition test.

RESULTS AND DISCUSSION

Parthenolide was detected in *n*-hexane and EtOAc extracts of the plant. R_F values of *n*-hexane and EtOAc extracts and parthenolide were 0.69 and their distances from origin of all three spots were equal and all three spots were blue. Based on this results, it was concluded that the major compound of *n*-hexane and EtOAc extracts of *T. argenteum* subsp. *argenteum* was parthenolide. The TLC result is also given as Figure 1.

The results of the cytotoxic activity tests are shown in Table 1. Among the extracts, significant cytotoxic activity of the *n*-hexane extract was observed against CaCo-2, MPANC-96, HEK-293, MDA-MB-231, and PC_3 (IC_{50} values of 3.959±0.62, 5.353±1.24, 1.651±0.43, 4.154±0.18, 2.847±0.51 µg/mL, respectively). Our results showed that the *n*-hexane extract is more effective than parthenolide against MPANC-96 and PC_3. It also had cytotoxic activity as strong as parthenolide against CaCo-2, HEK-293, MDA-MB-231. The viability percentage of *n*-hexane extract is shown in Figure 2. Microscopy images of the cells are shown in Figure 3.

In many studies, cytotoxic activities of various *Tanacetum* species have been reported. Rateb et al.[15] found that hydroalcoholic and aqueous extracts of different parts of *T. parthenium* L. had cytotoxic effects. The alcoholic extracts of the flowers of the plant showed significant cytotoxic effects (15% cell viability) with 50 µg/mL concentration on Ehrlich ascites carcinoma. This activity is considered as a result of synergy of flavonoid and sesquiterpene lactone contents. Rosselli et al.[16] investigated the cytotoxic activity of eudesmanolides isolated from flowers of *Tanacetum vulgare* subsp. *siculum* against an A549 cell line.

This is the first report to demonstrate the cytotoxic activity of *T. argenteum* subsp. *argenteum* against CaCo-2, MPANC-96, MDA-MB-231, 253J-BV, U87-MG, and PC_3 cell lines. In a previous report, the plant was analyzed for its cytotoxic property on MCF-7, the cell viability inhibition value of methanolic extract was found as 52.58% at a concentration of 100 µg/mL, whereas we found no serious activity with MeOH extracts. The difference is likely due to the extraction methods.[17]

Recently, Şen et al.[18] investigated the cytotoxic activity of aerial parts of *T. argenteum* subsp. *argentum* against four cell lines [A549, HeLa, HT-29 (human colorectal adenocarcinoma), MCF-7] using MTT assays. The authors found that an *n*-hexane extract of the plant inhibited growth of A-549 and HT-29 cells, and a chloroform extract of the plant inhibited growth of A-549 and HeLa cells at the concentrations of 30 µg/mL. Both extracts of the plant inhibited more than 50% proliferation of cells. When compared their results with ours on the same cell lines; the stronger results observed in our study with the same extracts are likely associated with the quantitative superiority of secondary metabolites responsible for the cytotoxic activity. Thus, the disparity between the two studies could be attributed to the different localities of the plant materials.

Apolar sesquiterpene lactones with γ lactone moiety pass into the *n*-hexane fraction could be responsible of the effect because sesquiterpene lactones have a broad spectrum of biologic activities including anti-inflammatory and cytotoxic properties. The lactone ring and exocyclic methylene group of sesquiterpene lactones are considered to be the liable units for biologic activities of sesquiterpenoids.[3]

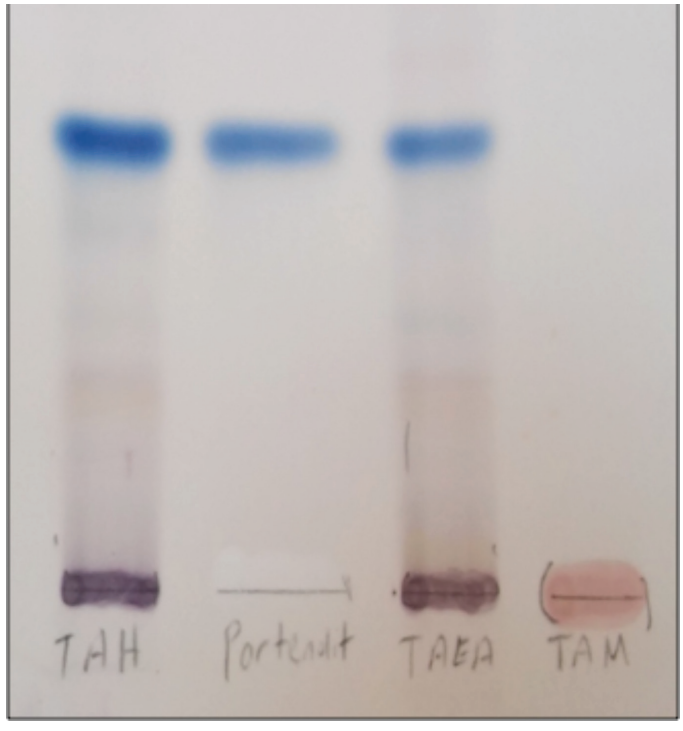

Figure 1. TLC results of parthenolide and *n*-hexane (TAH), EtOAc (TAEA), Methanolic (TAM) extracts of *T. argenteum* subsp. *argenteum*. Mobile phase system: hexane:ethyl acetate (5:5)

It is known that parthenolide, a germacranolide-type sesquiterpene lactone, plays a significant role for anti-inflammatory and cytotoxic activities.[19] Notably *T. parthenium, T. vulgare, Tanacetum densum* subsp. *amani, T. argenteum* subsp. *Flabellifolium*, and *T. argenteum* subsp. *canum* var. *canum* contain parthenolide.[3] The activities of the *n*-hexane extract and the ethyl acetate extract may be associated with the plant's major component parthenolide.

The anti-inflammatory activity of *T. argenteum* subsp. *argenteum* was evaluated using NF-κB and iNOS methods. As the results can be seen in Table 2, n-hexane extract had significant anti-inflammatory activity with an IC_{50} of 6.159±0.45 µg/mL for NF-κB, and 0.627±0.16 µg/mL for iNOS methods, respectively. Anti-inflammatory activities of various Tana*cetum* species using *in vitro* and *in vivo* methods have been reported.[20,21] The anti-inflammatory activities of *Tanacetum* spp. are related to their flavonoid and sesquiterpene contents. In a previous report, Nasri et al.[22] investigated the hydroalcoholic extract of *Tanacetum balsamita* and found anti-inflammatory activity related to quercetin, a flavonoid, and we found the anti-inflammatory activity of the plant through apolar fractions related to sesquiterpene lactones, most probably parthenolide. Bukhari et al.[23] noted that the *n*-hexane extract of *Tanacetum artemisioides* showed stronger *in vivo* anti-inflammatory activity than that of polar aqueous fractions.

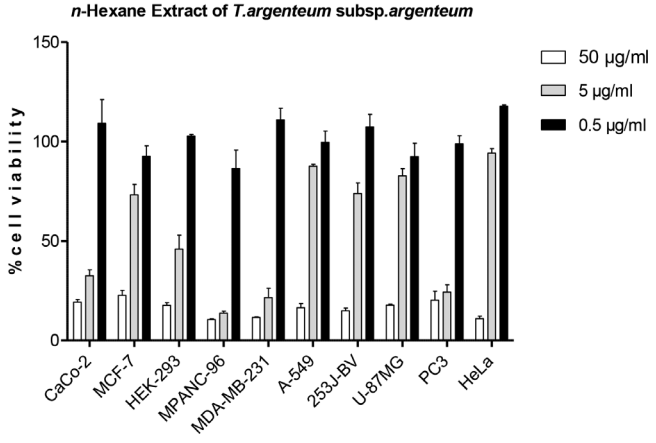

Figure 2. Cell viability of n-hexane extract with different concentrations of *T. argenteum* subsp. *argenteum* on ten cells

As postulated, sesquiterpene lactones are generally known to impair the activity of the NF-κB.[24] Hehner et al.[25] noted that parthenolide, the main compound of *T. parthenium*, is a well-known anti-inflammatory agent that inhibits the NF-κB pathway. López-Franco et al.[26] showed parthenolide's inhibiting role using the NF-κB method against murine cells. The *n*-hexane extract is more effective than parthenolide in iNOS methods, whereas parthenolide is stronger than *n*-hexane extracts with NF-κB. The *n*-hexane extract's potency could be related to a synergistic effect of different secondary metabolites of the extract. The results can also be seen in Figure 4 and 5.

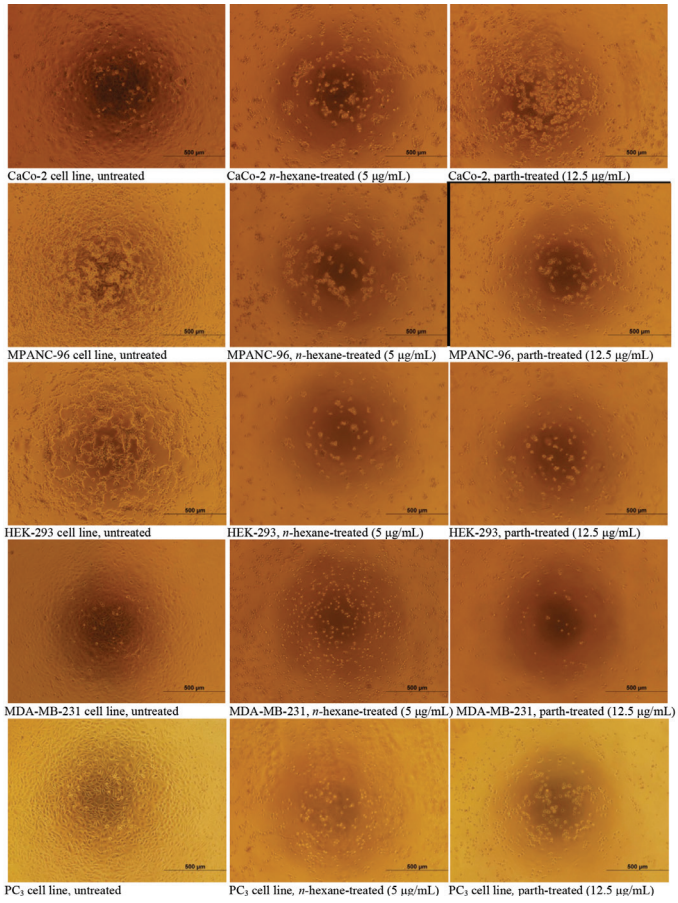

Figure 3. Views of CaCo-2, MPANC-96, HEK-293, MDA-MB-231 and PC_3 cells under a microscope (500 µm). Untreated cells, hexane and parthenolide treated cells

Table 1. IC_{50} values for cytotoxic activities of various extracts of *Tanacetum argenteum* subsp. *argenteum* extracts on different cells, µg/mL

Extract	A549	CaCo-2	MCF-7	MPANC-96	HEK-293	MDA-MB 231	253J-BV	U-87 MG	PC_3	HeLa
n-hexane	17.65±1.52	3.96±0.62	13.99±2.61	5.35±1.24	1.65±0.43	4.15±0.18	12.02±1.19	15.85±1.54	2.85±0.51	18.94±1.00
EtOAc	29.24±5.88	37.54±10.69	38.16±5.10	25.66±3.08	7.39±0.77	16.21±2.58	36.19±10.39	-	18.99±1.53	21.48±4.91
MeOH	-	-	-	-	-	-	-	-	-	-
Partheno-lide	3.26±2.49	3.16±2.93	7.37±0.16	5.51±5.71	1.16±0.09	3.61±3.34	6.35±0.20	3.38±1.97	3.44±0.36	5.79±0.00

Table 2. IC_{50} values for anti-inflammatory activities of various extracts of *Tanacetum argenteum* subsp. argenteum

Extract	iNOS (Raw 264.7) IC_{50} (µg/mL) with SD	NF-κB (HeLa) IC_{50} (µg/mL) with SD
n-hexane	0.627±0.16	6.159±0.45
EtOAc	1.602±0.48	37.505±1.51
MeOH	17.15±1.65	-
Parthenolide	0.674±0.01	1.779±0.14

-: No activity, SD: Standard deviation

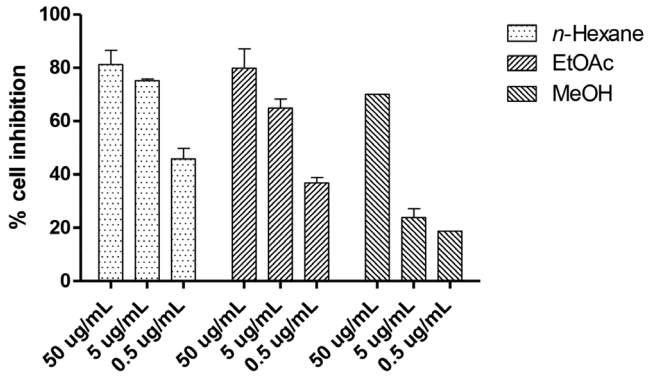

Figure 4. Various extracts of *T. argenteum* subsp. *argenteum* with different concentrations HeLa cell line, NF-κB method

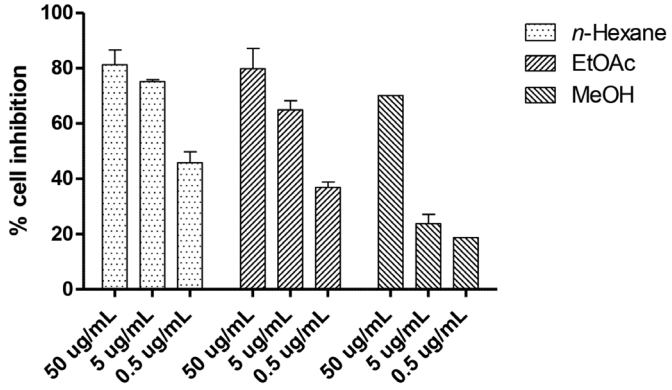

Figure 5. Various extracts of *T. argenteum* subsp. *argenteum* with different concentrations RAW264.7 cell line, iNOS method

CONCLUSION

Our results indicated that the *n*-hexane extract of *T. argenteum* subsp. *argenteum* has promising cytotoxic activity on CaCo-2, MPANC-96, HEK-293, MDA-MB-231, and PC_3 cells, and also anti-inflammatory activity. Parthenolide, a well-known anti-inflammatory and cytotoxic agent was detected in *n*-hexane and EtOAc extracts of the plant using TLC analysis. *n*-hexane extract's activity may be associated with its major component parthenolide and other substances in the plant. Phytochemical experiments are ongoing with *n*-hexane extracts of *T. argenteum* subsp. *argenteum.*

Conflict of Interest: No conflict of interest was declared by the authors.

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