https://doi.org/10.48047/AFJBS.6.14.2024.8734-8740

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

# Cytotoxic Effect of Phytochemicals From *Tanacetum vulgare* L. Flowers on HeLa Cells

Kabanov Dmitry Sergeevich, Baleev Dmitry Nikolayevich, Sidelnikov Nikolay Ivanovich
All-Russian Scientific Research Institute of Medicinal and Aromatic Plants (VILAR),
117216, Grins' street 7/1, Moscow, Russia
kabanovd1@rambler.ru, dbaleev@gmail.com, vilarnii@mail.ru

Volume 6, Issue 14, Aug 2024

Received: 15 June 2024

Accepted: 25 July 2024

Published: 15 Aug 2024

*doi: 10.48047/AFJBS.6.14.2024.8734-8740*

***Abstract*— Using the light microscopy and MTT cytotoxicity assay the pronounced cytotoxic effect of *n*-butanol partitioned phytochemicals from Tanacetum vulgare L. flowers (PhTvF) on viability of human cervical adenocarcinoma HeLa cells has been elucidated. The treatment of HeLa cells with PhTvF for 48 h or 72 h led to changes in cell morphology that had an apoptotic character of cell death. The viability of PhTvF (0.2 mg/ml) exposed HeLa cell for 48 h and 72 h decreased up to 63% ($p \leq 0.05$) and to 48% ($p=0.01$), respectively. The PhTvF dissolved in 0.9% NaCl as vehicle solvent reviled $IC_{50}$~0.1–0.2 mg/ml for HeLa cells. We have concluded that PhTvF exhibit tumor preventing properties in vitro and can be considered as the promising substances for development of a new preparation for antineoplastic therapies. The mechanism and possible leader compound of PhTvF cytotoxicity are now under investigation.***

**Index Terms—anti-neoplastic phytochemicals, caffeoylquinic acids, flavonoid glycosides,mechanism of flavonoids' cytotoxicity, HeLa**

## I. INTRODUCTION

Cervical carcinomas represent the fourth most common cancer type worldwide in women, and the third highest cause of cancer-associated mortality among females. It is an invasive and arises from the pre-existing squamous dysplasia and cervical adenocarcinoma [1]. Current therapeutic strategies of cervical carcinomas include surgery, chemotherapy and radiation therapy that can lead to damage of adjacent or distant normal tissues facilitating cancer cell invasion and metastasis [2]. The exploration of natural plant sources in the hope of finding efficient phytochemicals (Ph) that would help to suppress harmful proliferation and expansion of tumor cells seems to be a promising approach in antineoplastic therapy [3].

Phytochemicals from different organs of *Tanacetum vulgare* (PhTv) have been extensively studied in different laboratories [3]–[6]. *In vitro* cytotoxic effect of PhTv herbs has been assessed by Vasileva *et al*. [3] and Ivanescu *et al*. [4] while sesquiterpene lactones from *T. vulgare* flowers (PhTvF) by Rosselli *et al*. [5]. The viability of HeLa cells exposed to PhTv herbs was decreased in the next order: *T. macrophyllum* > *T. corymbosum* > *T. vulgare* [4] showing species-specific effect. When cytotoxic effects of Ph from *T. vulgare* herbs obtained by aqueous ethanol or chloroform and applied to human breast adenocarcinoma cells MCF-7 have been compared the higher cytotoxicity of the chloroform isolated PhTv was revealed [6]. However, to date, no study has been performed to reveal the cytotoxic effect of *n*-butanol fractionated Ph from *T. vulgare* flowers on human tumor cells. Therefore, the present study was undertaken to

investigate the sensitivity of HeLa human tumor cells to *n*-butanol fractionated PhTvF. The light microscopy and MTT assay were used to achieve this purpose.

## II. MATERIALS AND METHODS

### *A. Plant Material*

*T. vulgare* flowers were harvested during blooming period from VILARs' experimental garden and air-dried for nine days at 28–30°C in a dark well-ventilated room. The dried plant material was ground using electric "Retsch Mixer Mill MM400" (Germany) and stored in air tight containers to protect from oxidation according to The World Health Organization guidelines (2009) on good agricultural and collection practices (GACP) for medicinal plants.

### *B. Preparation of Ph from T. vulgare flowers*

The crude extract of PhTvF was obtained by 90% ethanol at 80°C for 1 h at ratio of plant material to solvent 1/10 and repeated three times. The total hydroalcoholic extract was next centrifuged at 14000 g for 5 min, filtered and concentrated below 40°C under vacuum using rotary evaporator "Heidolph Hei-VAP" (Germany). Then the aqueous phase was treated with dichloroethane and partitioned four times with *n*-butanol. The collected *n*-butanol fraction was concentrated, freeze-dried ("FreeZone" United States) and stored at 4°C protected from light and humidity. The *n*-butanol is no high polar solvent but allows obtaining almost all Ph from plant material [7].

### *C. Composition of n-butanol Fraction*

Ultra Performance Liquid Chromatography (UPLC) High-Resolution Mass Spectrometry (UPLC-MS) was performed using Agilent 1200 series High-Performance Liquid Chromatography (HPLC) gradient system ("Agilent Technology" United States) coupled to a photo diode array detector (PDA/DAD) and MicrOTOF-Q mass spectrometer Bruker (United States) equipped with an electrospray ionization (ESI) source (UPLC-PDA/EST-MS) has been performed in our previous work [8]. Extraction and UPLC-MS analyses were performed in triplicates on the two independent experiments. On the basis of UPLC-MS and UV analyses, seven Ph have been identified in *n*-butanol fraction from *T. vulgare* flowers (see Table I).

Table I. The content of Ph identified in 100 mg of dried *n*-butanol fraction from *T. vulgare* flowers [8]

| No | Compound (phenolic phytochemical) | Content (mg) | References |
|---|---|---|---|
| 1 | 3,5-Dicaffeoylquinic acids | 42.77 | [9] |
| 2 | 4,5-Dicaffeoylquinic acids | 15.99 | [9] |
| 3 | Neochlorogenic acid | 9.81 | [9] |
| 4 | Quercetin-3-glucoside | 6.83 | [9] |
| 5 | Acacetin-7-rutinoside | 6.26 | [10] |
| 6 | Myricetin-3-glucoside | 4.18 | [3] |
| 7 | Acacetin-7-glucoside | 3.97 | [11] |

### *D. PhTvF Working Solutions*

One ml of 0.9% NaCl was added into each tube containing powdered PhTvF from 1.0 mg to 5.0 mg with the increment of 1.0 mg followed by shaking for 1 h at room temperature and vortexing for 5 min. Prepared PhTvF solutions were filtered through sterile syringe 0.45 µm filter ("Merck Millex$^{TM}$-HA" Germany). Then 20 µl of each PhTvF solutions was added into 180 µl of culture medium in cell containing well of 96-well plate ("Costar-Corning" United States) leading to final PhTvF concentrations of 0.1 to 0.5 mg/ml, respectively.

### *E. Reference Compound*

We used doxorubicin hydrochloride (DOX) as the reference compound in the final concentration of 0.02 mg/ml ("Veropharm", Russia) since it is widely used in tumor chemotherapy. It is strong inhibitor of plasma membrane electron transport chain and such essential enzymes as NADH-ferricyanide dehydrogenase and NADH-cytochrome c reductase [12]. DOX is able to penetrate cell membrane and intercalates into DNA strands that is followed by disruption of topoisomerase-II-mediated DNA repair [13], [14].

### *F. HeLa Cells Treatment with PhTvF*

Human epithelial adenocarcinoma HeLa cells were obtained from the Unique Scientific Installation "BioCollection" of N. K. Koltzovs' Institute of Developmental Biology (Russia) then multiplicated and stored as cryopreserved biological cell material in the Unique Scientific Installation "BioCollection" at the VILAR Research Institute.

HeLa cells were grown in DMEM medium with 4.5 g/l supplemented with L-glutamin ("PanEco" Russia), 10% heat inactivated fetal bovine serum ("Dia-M" Russia), penicillin (100 U/ml), streptomycin (100 µg/ml) ("PanEco" Russia) at 37°C, 95% humidity and 5% $CO_2$ (v/v) in $CO_2$-incubator MINI-CELL NB203M ("N-Bioteck" South Korea). Logarithmically growing HeLa cells at the 75–80% confluence were washed by Versenes' solution detached by 0.05% trypsin-EDTA ("PanEco" Russia) and then seeded into a transparent/clear flat bottom 96-well plates ($2.5 \times 10^4$ cells/well) as well as on a glass coverslip [15]. The cell viability at the time of cell seeding was more than 95% as assessed by 0.2% trypan blue ("Dia-M" Russia) exclusion test. Then HeLa cells were left for 24 h at 37°C before cell treatment. After this pre-incubation period the medium was discarded and replaced with the fresh DMEM [16]. Then the cells were exposed to PhTvF, to reference compound and to vehicle control for 48 h and 72 h at 37°C, 95% humidity and 5% $CO_2$ (v/v). The ability of control and treated HeLa cells to reduce yellow tetrazolium salt (MTT-reagent, 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl-2H-tetrazolium bromide) was evaluated by MTT assay [17].

### *G. HeLa Cell Morphology Study*

Light microscopic observation of PhTvF-treated HeLa cells was done by inverted microscope P2-1 ("Lomo Biolam" Russia) and upright Eclipse 80i microscope ("Nikon" Japan) after cell fixing and staining with 0.1% methylene blue ("Dia-M" Russia) according to Hu *et al*. [18], [19].

### *H. MTT Assay*

The MTT assay is an indicator of cell survival (viability) and/or cell growth (proliferation). It determines the presence of living metabolically active cells with functional active mitochondria and other redox enzymes and can be used successfully to detect cytotoxicity of plant derived Ph. The biochemistry of MTT assay is based on the bioreduction of a yellow MTT-reagent into a purple colored MTT-formazan by

intracellular available NAD- or NADP-dependent oxidoreductases / dehydrogenases in metabolically active living cells [20], [21]. At the end of the MTT assay the cells were lysed in DMSO and the sample optical density was recorded by microplate reader SPECTROstar Nano ("BMG LabTech" Germany). The cell viability was then calculated according to the next equation ([OD $A_{540}$–OD $A_{630}$ experimental well] / [OD $A_{540}$–OD $A_{630}$ control well]) × 100%. A decrease of OD in experimental well in comparison to control indicates a reduction in the rate of cell metabolic activity and viability [21], [22]. The further evidence of induced cell death may be inferred from the cell morphological changes assessed by microscopy [18].

*I. Statistical analysis*

The Shapiro-Wilk test was applied to determine whether or not our data come into normal distribution. Statistical significance between the variances was determined using the Mann-Whitney and Kruskal-Wallis signed rank nonparametric tests. Multivariate statistical data R analysis (MVA) was also performed v4.2.2 R Core Team (2022). *P* values less than 0.05 indicate the existence of significant difference between the selected variances while those more than 0.05 are non-significant ones.

## III. RESULTS

*Influence of PhTvF on HeLa cell morphology*

The control untreated HeLa cells (Fig. 1A) were mostly in interphase stage with polygonal cell shape, showing typical features of normal living cells with lamellipodia extensions and clear visible nuclei containing nucleoli. The untreated HeLa cells also showed a high confluence of cells in the monolayer (Fig. 1A).

PhTvF-treated HeLa cells after 48 h (0.1 or 0.5 mg/ml) and 72 h (0.1 or 0.5 mg/ml) of exposure showed typical morphological features of compromised cells (Fig. 1B and 1C). The most pronounced morphological changes were reduction in cell volume and cell rounding followed by cell shrinkage and detachment from the plate bottom (Fig. 1B). Morphological changes increased in dose and time dependent manner (Fig. 1C). All these morphological changes may be attributed to the cell death by apoptosis. The extensive cell shrinkage and detachment with a lot of cell debris were seen when DOX (0.02 mg/ml, 48 h) was added to HeLa cells. The same morphological changes were observed after treatment of HeLa cells by tamoxifen [23], by Ph from *Clinacanthus nutans* (Burm.f) L. leaves [24] or DOX [25].

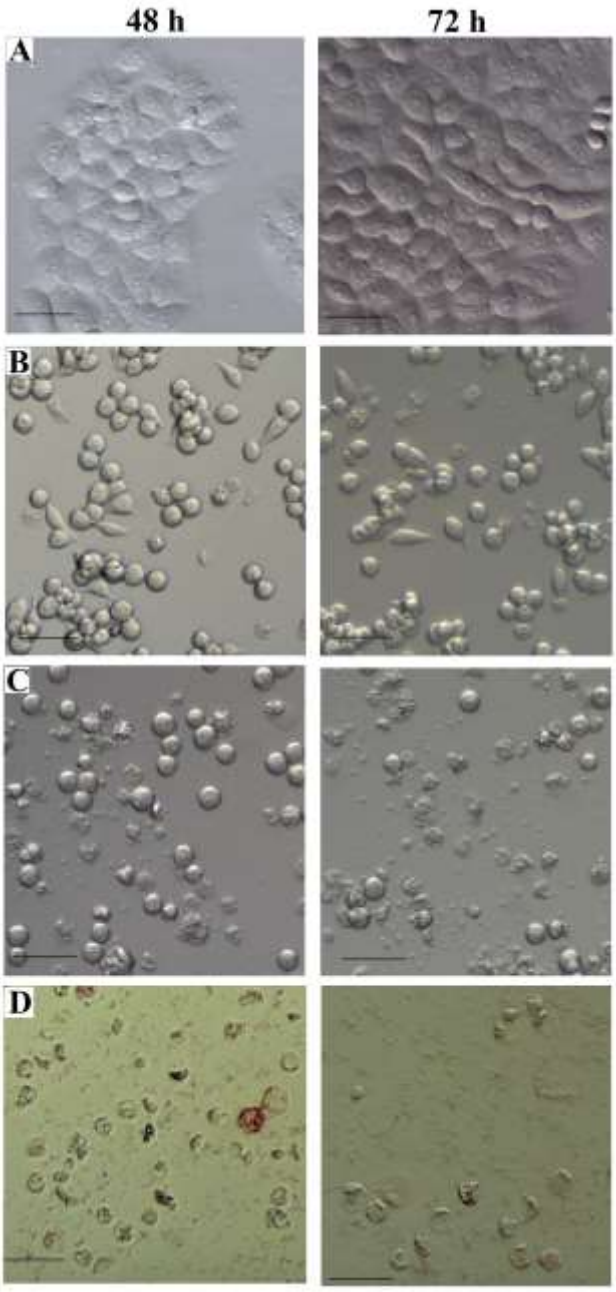


Fig. 1. PhTvF induced changes in HeLa cell morphology after 48 h and 72 h incubations. A) control cells; B) PhTvF (0.1 mg/ml); C) PhTvF (0.5 mg/ml ); D) DOX (0.02 mg/ml). Magnification 20X. Scale bar – 133 µm.

When exposition time was extended the more severe changes in HeLa cells morphology were already observed at the lowest PhTvF concentration (0.1 mg/ml, 72 h). The same changes were seen when PhTvF exposed HeLa cells were stained with methylene blue (Fig. 2). A lost of plasma membrane protrusions, cell rounding with condensation of cytoplasm and occupation of almost all cell interior by dark blue stained nucleus with undistinguishable nucleoli were observed after exposition of HeLa cell to 0.1 mg/ml of PhTvF (Fig. 2B) or 0.5 mg/ml (Fig. 2C) for 48 h. The changes in the cytoplasm / nucleus ratio with huge dense dark stained nucleus have been observed during the pyknosis stage that is the first stage of apoptotic cell death [26], [27]. DOX (0.02 mg/ml) caused extensive cell shrinkage and broken followed by detachment from the coverslip surfaces (Fig. 2 D). Note

that the chemical stress-induced changes in cell morphology such as volume reduction and cell shrinkage have been viewed as the most striking signs of compromised cell in which an intracellular physiological self-destruction pathways are already activated [28], [29].

*PhTvF cytotoxicity for HeLa tumor cells*

After treatment of HeLa cells with 0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml of PhTvF for 48 h their MTT-detected viability decreased to 51%, 63%, 54%, 59% and 53% ($p \leq 0.05$) (Fig. 3). When the same concentrations were applied to HeLa cells for 72 h the cell viability was decreased to 45%, 48%, 55%, 61% ($p=0.01$) and 67% ($p>0.05$). The decrease in cell viability by 20% has already been considered as significant when anticancer potential of Ph is studied [30]. Unfortunately, we did not obtain well concentration dependent results in PhTvF induced decrease of HeLa cell viability that could be attributed mostly to the suboptimal PhTvF concentrations and 0.9% NaCl which was used as the vehicle. When HeLa cells were exposed to DOX for 48 h or 72 h the dramatic decrease of cell viability by 70% was detected.

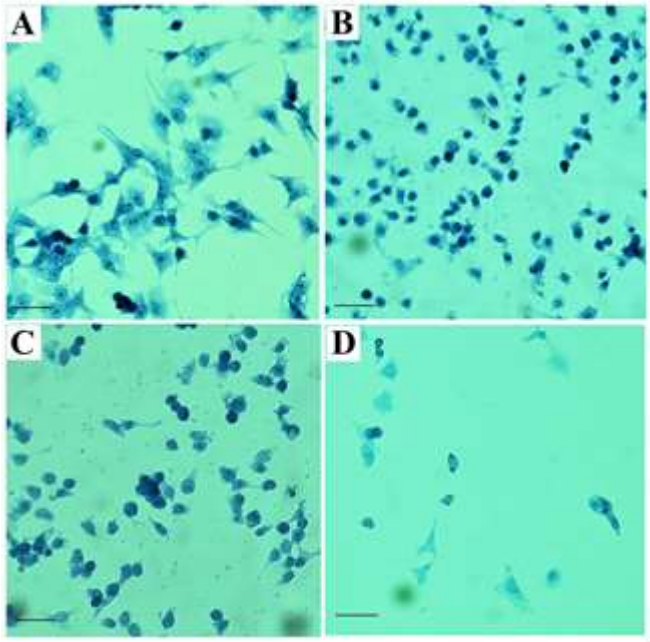


Fig. 2. The representative light microscopy images of control HeLa cell morphology (A) and it changes after cell exposure to PhTvF (0.1 mg/ml) (B) and PhTvF (0.5 mg/ml) (C) or DOX (D) for 48 h and stained by methylene blue The cell rounding and shrinkage as well as cell volume reduction and disappearance of cell to cell contacts can be well observed. Magnification –10X. Scale bar – 80 µm.

We assessed also the influence of PhTvF on MTT reduction without cells. The well correlation between increase in OD values and strengthening of PhTvF concentrations was observed. This finding is in good agreement with results of Talorete *et al*. [20], Chasemi *et al*. [31] and Karakas *et al*. [32]. Taking these results into account we replaced the incubation medium before MTT addition for fresh one.

## IV. DISCUSSION

The present pilot study clearly demonstrates high cytotoxic potential of *n*-butanol partitioned phytochemicals from *T. vulgare* flowers on viability of HeLa tumor cells. The deleterious effects of PhTvF on the viability of HeLa cells were seen well at the level of cell morphology as well as at the biochemical level as decrease in cell metabolic activity. Administration of PhTvF to HeLa cells led to cell volume reduction, lost of cell to cell contacts, cell rounding and shrinkage followed by cells detachment from the plate bottom (Fig. 1 and 2). At the same time we detected time dependent decrease in MTT bioreduction by HeLa cells exposed to PhTvF for 48 h and 72 h (Fig. 3 and 4) that is in accordance with the changes in cell morphology. To date, no study has been done to reveal the influence of *n*-butanol partitioned PhTvF on viability of tumor cells. Moreover, the PhTvF triggered mechanism that orchestrates the changes in HeLa cells morphology and metabolic activity needs further investigations. However, it seems reasonable to assume that cytoskeletal proteins represent appropriate targets for signaling PhTvF initiated pathways for cell death [33].

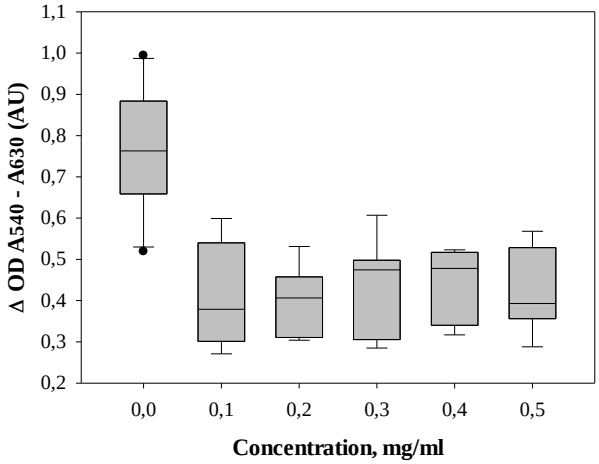


Fig. 3. The decrease in enzymatic MTT bioreduction to MTT-formazan that primary mirrors the rate of glycolytic NADH and NAD(P)H production in HeLa cells exposed to PhTvF for 48 h. $p \leq 0.05$

Whether the cytotoxic effect on HeLa cells is dependent on all or primary ones compounds in PhTvF mixture it is not clear at present. However, the current literature data on the biological activity of phytochemicals [34]–[38] that are detected in our *n*-butanol fraction tend us to assume that viewed PhTvF cytotoxicity is provided by a combined action of all *n*-butanol patitioned Ph on HeLa cells (Table I). Thus, quercetin-3-*O*-β-D-glucoside possess *in vitro* strong anti-tumor activity on HeLa cells, with $IC_{50}$=0.02 mg/ml [34]. Myricetin that is different from quercetin by one extra OH-group binds to epidermal growth factor receptor (EGFR) and suppresses the viability of human lung adenocarcinoma A549 cells [35]. The cell cycle arrest at $G_{0/1}$ phase by neochlorogenic acid in human oral cavity carcinoma cells: A253, HSC4, CAL27 and SCC4 but not in human normal oral keratinocytes (HOK) was shown [36]. Using MTT assay a potent suppressive effect of acacetin (5.7-dihydroxy-4'-methoxyflavone) on proliferation of human oral squamous carcinoma HSC-3 cells was also revealed [37]. The human colorectal adenocarcinoma Caco-2 cells were sensitive to 3.5-dicaffeoylquinic acid *in vitro* while other cells such as

esophageal adenocarcinoma OE-33 cells, lung A549 and urinary bladder T24 carcinoma cells were less sensitive. Moreover, 3.5-dicaffeoylquinic acid caused low cytotoxic effect on fibroblast-like CCD-18Co cells from normal human colon tissues [38]. These data support our suggestion on complex cytotoxicity of all *n*-butanol partitioned compounds from *T. vulgare* flowers on HeLa tumor cells *in vitro*. Although we have observed in our experimental conditions an $IC_{50}$ value of ~0.1 mg/ml PhTvFs' for HeLa cells it needs further investigation using DMSO as the vehicle solvent as well as the lowest working concentrations of PhTvF.

Great progress has been made in recent years to elucidate molecular mechanisms underlying the cytotoxic effect of Ph for human tumor and non-tumor cells *in vitro*. Among the first targets of phenolic phytochemicals are the cell surface proteins in addition to phospholipid bilayer and plasma membrane redox system [14], [37], [39]–[42].

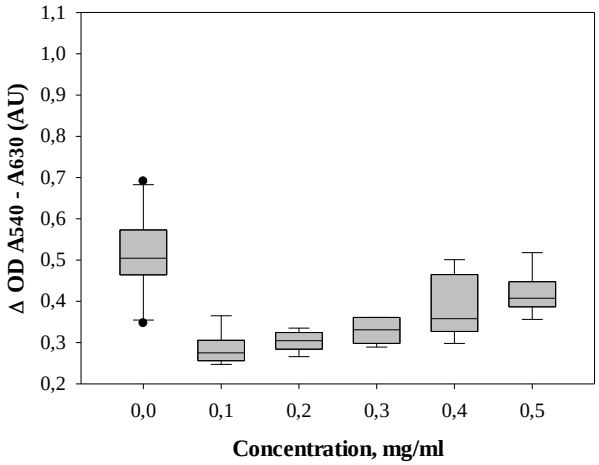


Fig. 4. The further decrease in enzymatic MTT bioreduction to MTT-formazan in HeLa cells exposed to PhTvF for 72 h. $p = 0.01$

It has been found that flavonoids, namely quercetin, myricetin, kaempferol, apigenin, and luteolin, which are lipid-soluble and weakly acidic, can freely diffuse across the cell membrane and specifically accumulate inside K562 leukemic cells [43]. Condensed tannin known as flavonoid (–)-epigallocatechin gallate is also able to penetrate cell membranes and accumulate in the nucleus of human intestinal and hepatic cells [44], [45]. The structure of quercetin allows for hydrophobic-nature-type intercalation of its most hydrophobic segment into the interior of the DNA helix [46]. Flavonoids can alter the conformation of DNA and inhibit DNA amplification, they show impressive induction of cell cycle arrest, and they may promote apoptosis in human hepatocellular carcinoma HepG2, breast MCF-7, and A549 cancer cells [47], [48]. Apigenin and kaempferol directly suppress the interaction between estrogen-related receptor γ (ERRγ) and its coactivator peroxisome proliferators-activated receptor γ coactivator-1α (PGC-1α). In contrast, luteolin suppresses PGC-1α activity through promoting the degradation of PGC-1α, leading to suppression of ERRγ activity in HeLa cells [49], [50].

## V. AUTHOR CONTRIBUTION

N. I. Sidelnikov and D. N. Baleev developed study conception, designed and governed the study, analyzed the data, revised and edited the manuscript content. D. S. Kabanov developed protocol of the study, performed experiments, analyzed the data and drafted the manuscript. All authors read and approved the final submitted manuscript version.

## VI. FUNDING

This study was supported by the Mission of the National Government according to FGUU-2022-0013 and FGUU-2022-0014 scientific ways established by the Ministry of Education and Science of the Russian Federation (Minobrnauka).

## VII. CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

## VIII. CONCLUSION

The high *in vitro* cytotoxicity of *n*-butanol partitioned phytochemicals from *Tanacetum vulgare* L. flowers towards tumor HeLa cells should be further investigated and applied to other cancers cells of different origin to detect their sensitivities and half-maximal inhibitory concentrations.

## IX. ACKNOWLEDGMENTS

We kindly thanks to L. V. Zinnatshina for extraction and characterization of phytochemicals from *T. vulgare* flowers and to all members of VILARs' board commission and to P. G. Mizina and A. I. Morosov namely for supporting and facilitating scientific studies of plant polyphenols' biological activity on human cell lines *in vitro*.

## X. REFERENCES

[1] A. T. Kiddane, M.-J. Kang, T. C. Ho, A. T. Getachew, M. P. Patil, *et al*., "Anticancer and apoptotic activity in cervical adenocarcinoma HeLa using crude extract of *Ganoderma applanatum*," Curr. Issues Mol. Biol., vol. 44, no. 3, pp. 1012–1026, February 2022.

[2] B. Zhao, and M. Hu, "Gallic acid reduces cell viability, proliferation, invasion and angiogenesis," Oncol. Lett., vol. 6, no. 6, pp. 1749–1755, December 2013.

[3] A. M. Vasileva, I. A. Iliev, V. S. Lozanov, M. B. Dimitrova, V. I. Mitev, *et al*., "*In vitro* study on the antitumor activity of *Tanacetum vulgare* L. extracts," Bulg. Chem. Commun., vol. 51, no. 1, pp. 249–255, January 2019.

[4] B. Ivanescu, Ch. Tuchilus, A. Corciova, Ch. Lungu, C. T. Mihai, *et al*., "Antioxidant, antimicrobial and cytotoxic activity of *Tanacetum vulgare*, *Tanacetum corymbosum* and *Tanacetum macrophyllum* extracts," Farmacia, vol. 66, no. 2, pp. 282–288, June 2017.

[5] S. Rosselli, M. Bruno, F. M. Raimondo, V. Spadaro, M. Varol, *et al*., "Cytotoxic effect of eudesmanolides isolated from flowers of *Tanacetum vulgare ssp. sicilum*," Molecules, vol. 17, no. 7, pp. 8186–8195, July 2012.

[6] Z. Gospodinova, N. Bozsity, I. Ocsovszki, O. Orban-Gyapai, M. Krasteva, et al., "Chloroformic fraction of *Tanacetum vulgare* L. induces cell cycle arrest and apoptosis in MCF7 cells," Int. J Pharma Sci., vol. 5, no. 2, pp. 986–990, Mach 2015.

[7] O. R. Alara, N. H. Abdurahman, and C. I. Ukaegbu, "Extraction of phenolic compounds: A review," Curr. Res. Food Sci., vol. 4, pp. 200–214, April 2021.

[8] T. A. Krol, L. V. Zinnatshina, D. N. Baleev, N. I. Sidelnikov, and V. I. Ossipov, "An identification and a quantitative assessment of phenolic compounds in a butanol fraction of extract from cosmary flowers (*Tanacetum vulgare*)," Russian J Bioorganic Chem., vol. 48, pp, 1454–1460, December 2022.

[9] N. Devrnja, B. Andelkovic, S. Arandelovic, S. Radulovic, M. Sokovic, *et al*., "Comparative studies on the antimicrobial and cytotoxic activities of *Tanacetum vulgare* L. essential oil and methanol extracts," South Africa J Botany, vol. 111, pp. 212–221, March 2017.

[10] R. Gevrenova, V. Balabanova, D. Zheleva-Dimitrova, and G. Momekov, "The most promising Southeastern European *Tanacetum* species: A review of chemical composition and biological studies," Pharmacia, vol. 70, no. 4, pp. 1067–1081, October 2023.

[11] M. Mohammadhosseini, and M. Jeszka-Skowron, "A systematic review on the ethnobotany, essential oils, bioactive compounds, and biological activities of *Tanacetum* species," Trends Phytochem. Res., vol. 7, no. 1, pp. 1–29, March 2023.

[12] M. K. Ediriweera, K. H. Tennekoon, and S. R. Samarakoon, "*In vitro* assays and techniques utilized in anticancer drug discovery," J Appl. Toxicol., vol. 39, no. 1, pp. 38–71, August 2018.

[13] C. F. Thorn, C. Oshiro, S. Marsh, T. Hernandez-Boussard, H. McLeod, *et al*., "Doxorubicin pathways: Pharmacodinamics and adverse effects," Pharmacogenet. Genomics, vol. 21, no. 7, pp. 440–446, July 2012.

[14] H. I. Ingolfsson, P. Thakur, K. F. Herold, E. A. Hobart, N. B. Ramsey, *et al*., "Phytochemicals perturb membranes and promiscuously alter protein function," ACS Chem. Biol., vol. 9, no. 8, pp. 1788–1798, June 2014.

[15] M. Gargotti, U. Lopez-Gonzalez, H. J. Byrne, and A. Casey, "Comparative studies of cellular viability levels on 2D and 3D *in vitro* culture matrices," Cytotechnology, vol. 70, no. 1, pp.261–273, February 2018.

[16] H. Mueller, M. U. Kassack, and M. Wiese, "Comparison of the usefulness of the MTT, ATP, and calcein assays to predict the potency of cytotoxic agents in various human cancer cell lines," J Biomol. Screen., vol. 9, no. 8, pp. 506–515, September 2004.

[17] M. N. Kurchatova, A. S. Fomin, A. A. Shirokov, and N. A. Durnova, "Cytotoxicity of flavonoid-containing plant extracts toward the CHO cell line: A comparative study," Cell Tiss. Biol., vol. 16, pp. 80–85, February 2022.

[18] X. Hu, V. Laguerre, D. Packert, A. Nakasone, and L. Moscinski, "A simple and efficient method for preparing cell slides and staining without using cytocentrifuge and cytoclips," Int. J Cell Biol., vol. 2015, November 2015.

[19] J. Zhang, D. Chen, D. M. Han, Y. M. Chen, C. Dai, *et al*., "Tannic acid mediated induction of apoptosis in human glioma Hs683 cells," Oncol. Lett., vol. 15, no. 5, pp. 6845–6850, May 2018.

[20] T. P. N. Talorete, M. Bouaziz, S. Sayadi, and H. Isoda, "Influence of medium type and serum on MTT reduction by flavonoids in the absence of cells," Cytotechnology, vol. 52, no. 3, pp. 189–198, November 2006.

[21] A. Bahuguna, I. Khan, V. K. Bajpai, and S. C. Kang, "MTT assay to evaluate the cytotoxic potential of a drug," Bangladesh J Pharmacol., vol. 12. no. 2, pp. 115–118, April 2017.

[22] J. A. Plumb, R. Milroy, and S. B. Kaye, "Effect of the pH dependence of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide-formazan absorption on chemosensitivity determined by a novel tetrazolium-based assay," Cancer Res., vol. 49, no. 16, pp. 4435–4440, August 1989.

[23] P. Han, J. H. Kang, H. L. Li, S. X. Hu, H. H. Lian, *et al*., "Antiproliferation and apoptosis induced by tamoxifen in human bile duct carcinoma QBC939 cells via upregulated p53 expression," BBRS, vol. 385, no.2, pp. 251–256, July 2009.

[24] N. H. Haron, Z. Md. Toha, R. Abas, M. R. Hamdan, N. Azman, *et al*., "*In vitro* cytotoxic activity of *Clinacanthus nutans* leaf extracts against HeLa cells," Asian Pac. J Cancer Prev., vol. 20, no. 2, pp. 601–609, February 2019.

[25] R. Cao, H. Wallrabe, K. Siller, and S. R. Alam, "Single-cell redox states analyzed by fluorescence lifetime metrics and tryptophan FRET interaction with NAD(P)H," Cytometry A, vol. 95, no. 1, pp. 110–121, January 2019.

[26] W. Song, Y.-J. Ren, L.-L. Liu, Y.-Y. Zhao, Q.-F.- Li, *et al*., "Curcumin induced the cell death of immortalized human keratinocytes (HaCaT) through caspase-independent and caspase-dependent pathways," Food Funct., vol. 12, no. 18, pp. 8669–8680, September 2021.

[27] S. H. Z. Ariffin, W. W. Yeen, I. Z. Z. Abidin, R. M. A. Wahab, Z. Z. Ariffin, *et al*., "Cytotoxicity effect of degraded and undegraded kappa and iota carrageenan in human intestine and liver cell lines," BMC Complement. Altern. Med., vol. 17, no. 14, pp. 508–523, December 2014.

[28] E. Hollville, and S. J. Martin, "Measuring apoptosis by microscopy and flow cytometry," Curr. Protoc. Immunol., vol. 112, pp. 14.38.1–14.38.24, February 2016.

[29] C. D. Bortner, and J. A. Cidlowski, "Ions, the movement of water and the apoptotic volume decrease," Front. Cell Dev. Biol., vol. 25, no. 8: 611211. November 2020

[30] P. Wang, S. M. Henning, and D. Heber, "Limitations of MTT and MTS-based assays for measurement of antiproliferative activity of green tea polyphenols," PLos One, vol. 5, no. 4: e10202. April 2010

[31] H. Chasemi, A. Jamshidi, A. M. Ghatee, K. Mazhab-Jafari, M. Khorasani, *et. al*., "PPARγ activation by pioglitazone enhances the antiproliferative effects of doxorubicin on pro-monocytic THP-1 leukemia cells via inducing apoptosis and $G_2$/M cell cycle arrest," J Recept. Signal Transduct. Res., vol. 42, no. 5, pp. 429–438, October 2022.

[32] D. Karakas, F. Ari, and E. Ulukaya, "The MTT viability assay yields strikingly false-positive viabilities although the cells are killed by some plant extracts," Turk. J Biol., vol. 41, no. 6, pp. 919–925, December 2017.

[33] P. Pagliara, A. Scarano, A. Barca, S. Zuppone, T. Verri, *et al*., "*Ostreopsis cf. ovata* induces cytoskeletal disorganization, apoptosis, and gene expression disregulation on HeLa cells," J Appl. Phycology, vol. 27, no. 6, pp. 2321–2332, January 2015

[34] D. I. Hamdan, S. Salah, W. H. B. Hassan, M. Moris, H. M. A. Khalil, *et al*., "Anticancer and neuroprotective activities of ethyl acetate fractions from *Morus macroura* Miq. plant organs with ultraperformance liquid chromatography-electrospray ionization-tandem mass spectrometry profiling," ACS Omega vol. 7, no. 18, 16013–16027, April 2022.

[35] P. Rajendran, U. Maheshwari, A. Muthukrishnan, R. Muthuswamy, K. Anand, *et al*., "Myricetin: versatile plant based flavonoid for cancer treatment by inducing cell cycle arrest and ROS-reliant mitochondria-facilitated apoptosis in A549 lung cancer cells an *in silico* prediction," Mol. Cell Biochem., vol. 476, no. 1, pp. 57–68, January 2021.

[36] J. Che, T. Zhao, W. Liu, S. Chen, G. Yang, *et al*., "Neochlorogenic acid enhances the antitumor effects of pingyangmycin via regulating $TOP_2A$," Mol. Med. Rep., vol. 23, no. 2, pp. 158–166, February 2021.

[37] C.-D. Kim, J. D. Cha, S. Li, and I. H. Cha, "The mechanism of acacetin-induced apoptosis on oral squamous cell carcinoma," Arch. Oral. Biol., vol. 60, no 9, pp. 1283–1298, September 2015.

[38] M. Amigo-Benavent, S. Wang, R. Mateos, B. Sarria, and L. Bravo, "Antiproliferative and cytotoxic effects of green coffee and yerba mate extracts, their main hydroxycinnamic acids, methylxanthine and metabolites in different human cell lines," Food Chem. Toxicol., vol. 106(Pt A), 125–138, August 2017.

[39] N. R. Perron, and J. L. Brumaghim, "A review of the antioxidant mechanisms of polyphenol compounds related to iron binding," Cell Biochem. Biophys., vol. 53, no. 2, pp. 75–100, February 2009.
[40] A. A. Dayem, H. Y. Choi, G. M. Yang, K. Kim, S. K. Saha, *et al*., "The anti-cancer effect of polyphenols against breast cancer and cancer stem cells: Molecular Mechanisms," Nutrients, vol. 8, no. 9, pp. 581–618, September 2016.
[41] M. A. Baker, and A. Lawen, "Plasma membrane NADH-oxidoreductase system: A critical review of the structural and functional data," Antioxid. Redox. Signal., vol. 2, no. 2, pp. 197–212, Summer 2000.
[42] G. Loo, "Redox-sensitive mechanisms of phytochemical-mediated inhibition of cancer cell proliferation," J Nutr. Biochem., vol. 14, no. 2, pp. 64–73, February 2003.
[43] A. Das, D. Majumder, and C. Saha, "Correlation of binding efficacies of DNA to flavonoids and their induced cellular damage," J Photochem. Photobiol. B., vol. 170, pp. 256–262, May 2017.
[44] S. J. Hosseinimehr, V. Tolmachev, and B. Stenerlow, "$^{125}$I-labeled quercetin as a novel DNA-targeted radiotracer," Cancer Biother. Radiopharm., vol. 26, no. 4, pp. 469–475, August 2011.
[45] T. Walle, T. S. Vincent, and U. K. Walle, "Evidence of covalent binding of the dietary flavonoid quercetin to DNA and protein in human intestinal and hepatic cells," Biochem. Pharmacol., vol. 65, no. 10, pp. 1603–1610, May 2003.
[46] R. Solimani, "Quercetin and DNA in solution: Analysis of the dynamics of their interaction with a linear dichroism study," Int. J Biol. Macromol., vol. 18, no. 4, pp. 287–295, June 1996.
[47] X. Chen, Z. He, X. Wu, D. Mao, C. Feng, *et al*., "Comprehensive study of the interaction between *Puerariae Radix* flavonoids and DNA: From theoretical simulation to structural analysis to functional analysis," Spectrochim. Acta Part A Mol. Biomol. Spectrosc., vol. 231: 118109, April 2020.
[48] D. Atrahimovich, D. Avni, and S. Khatib, "Flavonoids-Macromolecules interactions in human diseases with focus on Alzheimer, atherosclerosis and cancer," Antioxidants (Basel), vol. 10, no. 3, pp. 423–438, March 2021.
[49] F. Virgili, and M. Marino, "Regulation of cellular signals from nutritional molecules: A specific role for phytochemicals, beyond antioxidant activity," Free Radic. Biol. Med., vol. 45, no. 9, pp. 1205–1216, November 2008.
[50] Z. Huang, F. Fang, J. Wang, and Ch.-W. Wong, "Structural activity relationship of flavonoids with estrogen-related receptor gamma," FEBS Lett., vol. 584, no. 1, pp. 22–26, January 2010.