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Calendula officinalis Extracts Protect against H_2O_2 Induced Chromosome Damage on HacaT Human Skin Cells

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Abstract

Background: *Calendula officinalis* extracts contain antioxidant compounds. The extracts tested in the current study protect against reactive oxygen species (ROS) induced cytotoxic activity towards human cells.

Objective: To determine the ability of *Calendula officinalis* extracts to protect against H_2O_2 induced chromosome damage on HaCaT human skin cells.

Methodology: The chromosome damage protection by four *Calendula officinalis* extracts was investigated in vitro using a dose-response (0.125, 0.5 and 1.0% (v/v)) on HaCaT human skin cells. Two water/ethanol based proprietary calendula extracts A and B and two proprietary aqueous calendula extracts C and D from Biodynamically grown plants were examined. Extracts were characterised using Folin-Ciocalteau and DPPH assays. Protection of extracts against chromosome damage was measured via micronuclei induction using the cytokinesis-blocked micronucleus assay. No genotoxicity was induced by the extracts themselves. Cells were also exposed to the Calendula extracts for 47 h before being exposed to an oxidative stress (300-μM hydrogen peroxide for 1 h).

Results: The oxidative stress induced micronuclei (MNi) at a frequency significantly higher ($P<0.05$; range=20-25 MNi/1000 binucleated cells, n=3) than the background frequency (media alone control; MNi range=7-13 MNi/1000 binucleated cells, n=3). When the cells were pre-treated with extracts before the oxidative stress, the level of protection against chromosomal damage by all Calendula extracts was significant ($P<0.05$). The frequency of MNi in the presence of extracts was reduced by an average increment of 20 MNi/1000 BN cells to 2-9 MNi/1000 BN cells at all doses tested.

Conclusion: The Calendula extracts protected against the chromosomal damage induced by oxidative stress in vitro. Such form of genetic damage has been linked with carcinogenesis.

Keywords: *Calendula officinalis*; Genotoxic protection; Chromosomal damage; Oxidative stress; CBMN (cytokinesis-block micronucleus) assay; Micronuclei (MNi)

Introduction

Alteration of genetic material can lead to cancer and can include chromosome breakage and loss, dicentric chromosomes and DNA damage, including mutations and gene amplification. The number of chromosome alteration is correlated within tumour process and malignant potential of the cancer [1]. DNA damage occurs from cellular sources (endogenous) and environmental agents (exogenous) [1]. Exogenous agents such as chemicals or radiation can interact with genetic material and induce genetic damage (genotoxicity), chronic toxicity and/or cell death (cytotoxicity) [2]. Exogenous agents can promote the formation of free radicals. Ultraviolet light (UV light) can lead to the formation of single oxygen (O) and other reactive oxygen species in the skin. Other atmospheric pollutants, such as ozone and nitrogen peroxide, lead to radical formation and may cause respiratory disease. Cigarette smoke also contains free radicals [3,4]. Oxidative stress arises from an imbalance between free radicals including reactive oxygen species (ROS), and antioxidant defences. ROS can damage cellular components including lipids, proteins and nucleic acids [5]. Oxidative damage to proteins or nucleic acids often increases the variety of damage products due to modifications in amino acids and nucleotides [6]. There are many conditions where oxidative damage is involved. Example includes the aging process, cancer, atherosclerosis, inflammatory conditions, viral infections, trauma, effects of cigarette smoke and interaction with environmental pollution [7-10]. An antioxidant is a substance which can inhibit or delay oxidative damage occurring and can therefore potentially protect against the damaging effects of ROS. Many studies have examined the chemical nature of fruits, vegetables, herbs and grains for their natural antioxidant activity [5,11] The majority of antioxidants isolated from higher plants are polyphenols with the main biological activity identified as anti-inflammatory, anti-carcinogenic, anti-bacterial and anti-viral [11-13]. The activity of different extracts of *Calendula officinalis* flower includes anti-tumoral, anti-inflammatory, anti-viral activity and anti-HIV properties [10,14-16]. *Calendula officinalis* extracts contain

antioxidant compounds and these include phenolic, Tocopherols and Carotenes [17]. Individual components of relevance to this study are quercetin, vitamin E, beta-Carotene and amino acids [18]. The extracts tested in the current study have been shown to protect human cells *in vitro* against ROS induced cytotoxic activity [17]. We tested four extracts of *Calendula officinalis* for protection against chromosome damage caused by free radicals using an *in vitro* human skin cell model.

Materials and Methods

Materials

Materials used were RPMI 1640 media and foetal bovine serum (FBS) were purchased from Gibco® Cell Culture. Cytochalasin B (Cyt-B) solution was purchased from Sigma™. Sodium dodecyl sulphate (SDS, approximately 99%), Phosphate buffered saline (PBS) was purchased from Sigma™. Hydrogen peroxide (H_2O_2) and Quercetin were purchased from Sigma™. Folin-ciocalteu reagent was purchased from Sigma™. Diff-Quik stains were purchased from Lab Aids (Australia). Media prepared for growth and treatment was RPMI medium supplemented with 10% FBS.

Characteristic	Extract A	Extract B	Extract C	Extract D
Density at 20°C	1.003 g/ml	1.002 g/ml	1.036 g/ml	0.973 g/ml
Total dry weight	8.80%	8.40%	13.97%	14.65%
Gallic acid equivalents (GAE)(mg GAE/g dry weight)	28	26	10.83	11.73

Table 1: Characteristics of different *Calendula officinalis* extracts. Shown are the results of density determination; total dry weight after freeze drying and Gallic acid aqueous (GAE) results performed by Folin-Ciocalteu assay.

Plant extracts

Plants extract preparation and characterization was as previous published [18]. Briefly, dried *Calendula officinalis* flowers were supplied by an industry partner (Jurlique International, Mount Barker, South Australia) from Biodynamically grown plants. Four different extracts were prepared independently. Extracts A and B were prepared as water/ethanol based proprietary calendula extracts and extracts C and D proprietary aqueous calendula extracts. When required, extracts were diluted in media to prepare the treatment solution. Total phenolic content; dry weight and density determination were used to characterise the plant extracts. The determination of density for all extracts was carried out at 20°C from the average of 10 independent measurements. Table 1 is presented data as mean ± SD, with errors calculated using the propagation of uncertainty equation (density=mass/volume). The percentage of total dry weights of all extracts (A,B,C or D) were determined after extracts samples were freeze dried at -56°C at 3.4 Pa for 24 h. The total dry solids were weighed once all volatiles were removed from samples.

Cell line

A human non-cancer keratinocytes cell line (HaCaT) was as a gift from the department of Haematology and Genetic Pathology-Flinders Medical Centre, school of medicine at Flinders University, Adelaide, South Australia. The cell line was maintained in RPMI-1640 media (RPMI-1640 powder reconstituted medium supplemented with L-glutamine 2 mM, penicillin 100U/ml, streptomycin 100 µg/ml and 10% FBS). Cells were seeded in tissue culture flasks at 5×105 cells/ml and incubated in a fully humidified atmosphere at 37°C with 5% CO_2. The cells were subcultured twice a week when they reached 60-80% confluence or greater.

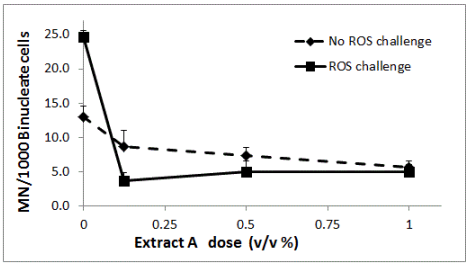

Figure 1: Dose-response effect of extract A on micronuclei frequency. HaCaT cells were plated as described in materials and methods and treated with calendula extract A for 48h. They were then treated for 1h without H_2O_2 (No ROS challenge) or with 1h 300 µM H_2O_2 (ROS challenge). The frequency of micronuclei (MN) per 1000 binucleated cells was determined using the cytokinesis Block micronucleus assay (as described in section 2.8). Data shown as mean ± SEM; n=3; 1% (v/v) A=0.88mg dry weight equiv/ml.

Cell treatment with Calendula extracts

Cells were treated *in vitro* for extract exposure as part of the genetic protection assay. Six wells of a 6-wells plate were seeded with 3×10^5 cells/well in a 2 ml volume and incubated for 19 h to allow the adherence of cells at 37°C in 5% CO_2. The media were aspirated and replaced with 2 ml of the treatment solution per well. The cells were treated for 48 h prior to CBMN assay analysis at doses derived from the MTT assay results for extracts A, B, C and D extracts (data not shown). Treatment solutions included A,B,C or D at 0, 0.125, 0.5 and 1 (v/v). The positive control compound used was Quercetin (16 µg/ml final concentration) with known antioxidant capacity. Media were used as a negative control.

Induction of oxidative stress by hydrogen peroxide (H_2O_2) to determine chromosome protection activity

Following incubation with or without extracts, oxidative stress was induced by H_2O_2 as previously published [18].

Folin-Ciocalteu assay

To prepare the calendula extracts, they were diluted 50-fold in 10 ml water. Gallic acid aqueous standards were prepared by adding 5.8, 12.8, 16.4, 22.8 and 27.8 mg in 250 ml of water. Then the Folin–Ciocalteu assay was carried out as previously described by [18,19]. For the assay, the mixtures were incubated 2 h at room temperature. Using a UV-Vis spectrophotometer the absorbance of each solution at 765 nm and 750 nm was measured. A calibration curve of gallic acid concentration versus absorbance was constructed from the Gallic acid aqueous standards. The gallic acid equivalents of the extracts was then determined in triplicate.

DPPH assay

The DPPH assay was performed to determine antioxidant activities of the extracts via radical scavenging ability using stable DPPH radical. A methanolic stock solution of Quercetin (1.6 mg/ml) was the positive control with dilutions in methanol (5, 4, 3, 2 and 1% (v/v)) used to determine the linear range of the DPPH assay. Calendula extracts were prepared and analysed as previously reported [18, 20]. The decrease in absorbance at 517 nm was determined with a Thermo Scientific Evolution Array UV-Visible spectrophotometer after 15 minutes for all samples.

Cytokinesis-block micronucleus (CBMN)

The CBMN assay was carried out as previously described [21] with the minor modifications was reported [22]. Briefly, after treatment Cyt-B was added at 4.5 µg/ml in media and the cultures were incubated at 37°C for 23 h. Cells were harvested by trypsinization and collected onto slides by centrifuging at 47 Xg (@6000 rpm) for 5 minutes using a cytospin. Slides were air-dried, fixed for 10 min in DiffQuick Fixative, and then stained with stain 1 (red DiffQuick Stain) and then Stain 2 (blue DiffQuick Stain). Slides were scored at a magnification of 250X or 40 X. Criteria for scoring micronuclei MNi, nucleoplasmic bridge (NPB) or nuclear buds (NBUDs) were as described [23]. The chromosomal damage induced by treatment, total number of MNI in BN cells were counted in total of 1000 BN.

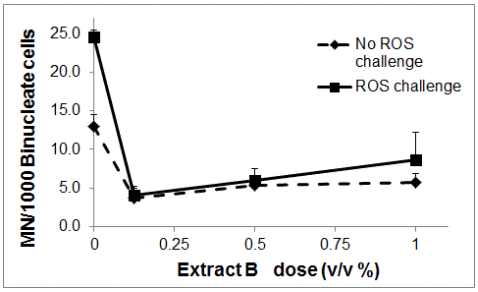

Figure 2: Dose-response effect of extract B on micronuclei frequency. HaCaT cells were plated as described in materials and methods and treated with calendula extract B for 48h. They were then treated for 1h without H_2O_2 (No ROS challenge) or with 1h 300 µM H_2O_2 (ROS challenge). The frequency of micronuclei (MN) per 1000 binucleated cells was determined using the cytokinesis Block micronucleus assay (as described in section 2.8). Data shown as mean ± SEM; n=3; 1% B=0.84mg dry weight equiv/ml.

Statistical analysis

Triplicate assays were performed for all experiments, and data is presented as the mean ± S.E.M. One way ANOVA (SPSS software, version 19.0) followed by Tukey's HSD post was used. T-test for independent samples was also used. Differences were considered statistically significant when the value of $P<0.05$.

Results

Characteristics of Calendula extracts

The physico-chemical properties of *Calendula officinalis* extract A, B, C and D were characterised (Table 1). The Folin-Ciocalteu assay was used to estimate polyphenol composition and is expressed using per dry weight determinations and gallic acid equivalents (Table 1). DPPH● assay characterization is also included (Table 3).

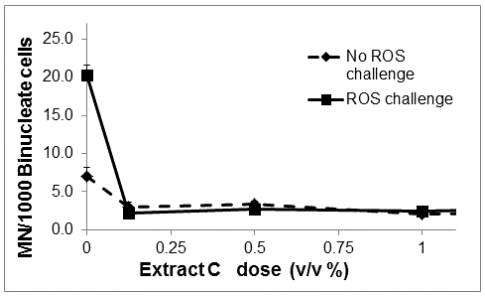

Figure 3: Dose-response effect of extract C on micronuclei frequency. HaCaT cells were plated as described in materials and methods and treated with calendula extract C for 48h. They were then treated for 1h without H_2O_2 (No ROS challenge) or with 1h 300 µM H_2O_2 (ROS challenge). The frequency of micronuclei (MN) per 1000 binucleated cells was determined using the cytokinesis Block micronucleus assay (as described in section 2.8). Data shown as mean ± SEM; n=3; 1% (v/v) C=1.4 mg dry weight equiv/ml.

Genotoxicity of Calendula plant extracts on Human Skin cells HaCaT

The *in vitro* genotoxicity and protective effects of Calendula plant extracts A, B, C and D on HaCaT Human Skin cells was performed after incubating cells with extracts for 48h. No genotoxicity was induced by the extracts themselves shown in (Figures 1-4). At no dose of any extract alone is the frequency of micronuclei greater than that of the zero dose control.

Protection induced by extracts against H_2O_2 induced oxidative stress on human skin cells

Oxidative stress induced micronuclei frequency by 300-µM hydrogen peroxide (H_2O_2) with no pre-treatment was significantly higher than the zero dose (untreated control) ($P<0.05$) in all experiments. Calendula extracts A,B,C and D induced protection against H_2O_2 induced micronuclei, as shown in (Figures 1-4). This was, at all doses, and statistically significant ($P<0.05$) for Human skin cells after treatment with each of the extracts for 48 h followed by 1h treatment with 300-µM H_2O_2. This is shown for all extracts in (Figs. 1-4) by the solid lines (ROS challenge series of experiments) where there is a significant difference ($P<0.05$) between cells in the absence of extract but with H_2O_2 (0 dose of extract) and all other doses of extracts in the presence of H_2O_2. The positive control of a known antioxidant, Quercetin significant protected against H_2O_2 induced micronuclei (Table 2, $P<0.05$). The protection level of all extracts was similar to the protection level of Quercetin against H_2O_2 induced oxidative stress genetic damage.

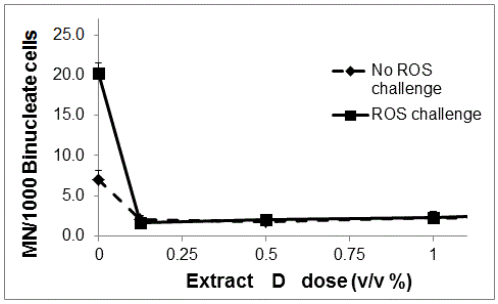

Figure 4: Dose-response effect of extract D on micronuclei frequency. HaCaT cells were plated as described in materials and methods and treated with calendula extract D for 48 h. They were then treated for 1h without H_2O_2 (No ROS challenge) or with 1h 300 µM H_2O_2 (ROS challenge). The frequency of micronuclei (MN) per 1000 binucleated cells was determined using the cytokinesis Block micronucleus assay (as described in section 2.8). Data shown as mean ± SEM; n=3; 1% (v/v) C= 1.47 mg dry weight equiv/ml.

Discussion

A previous study demonstrated protection by Calendula extracts against H_2O_2 induced cell killing [18]. Further work was required to cover the characterization of additional aspects of the calendula extracts protective action. The current study investigated the protection against chromosome damage by four calendula flower extracts on human skin cells (HaCaT) *in vitro*. Based on the cytotoxicity data for extracts A, B, C and D (data not shown) non-toxic doses were chosen for use in the CBMN assay. Firstly, the study showed the safety of calendula extracts as has been shown for medicinal plants [16,18,24,25]. No extracts were genotoxic by themselves at any dose tested on HaCaT skin cells. Total phenolics in extracts were determined by the Folin-Ciocalteu assay. The values for extracts A and B were 28 mg GAE/g dry weight and 26 mg GAE/g dry weights, and were 10.83 mg GAE/g dry weight for C and 11.73 mg GAE/g dry weights for D (Table 1). The levels of polyphenols obtained are equal to or greater than that obtained by others for extracts of calendula flower and other medicinal plants [19,26]. Consistently with this, the DPPH assay demonstrated that all extracts and the positive control quercetin, exhibited free radical inhibition at 2.5% dose ranging from 29-55% (Table 3). The genotoxicity protection of HaCaT skin cells *in vitro* against chromosome damage caused by ROS was explored by pre-treating cells with Calendula extracts for 48h then challenging with H_2O_2. Calendula flower extracts were negative in the Ames Salmonella/microsome mutagenicity assay and the extracts contained carotenes and flavonoids thus indicating high antioxidant activities [24]. Laser calendula extracts has shown significant inhibition of tumor growth of tumor cell proliferation when tested on human and murine tumor cell lines, therefore calendula flower extracts could be used in treating cancers [16]. Findings in the current study were consistent with previously observed antioxidant activity of calendula flower extracts, and the four extracts protected against chromosome damage from oxidative stress induced by H_2O_2 (known to induce free radicals including ROS). Genetic damage from oxidative stress induced by 300 µM H_2O_2 was significantly different from the background level of MNi in the negative control (un-treated control). The number of MNi/1000 BN in the un-treated control populations ranged from 7-13 MNi/1000 BN, n=3. Hydrogen peroxide, however, induced up to 24 MNi/1000 BN (range= 20-24 MNi), n=3. The four extracts offered protection against genetic damage induced by ROS with significant decreases in the number of MNi/1000 BN when HaCaT cells treated with extracts were challenged for 1h with H_2O_2 post-treatment. These results are similar to Quercetin the positive control for protection, which reduced genetic damage to only 8.7 MNi/1000 BN, as shown in Table 2. The bioactivity level of extracts appears high enough that there is no significant difference between all the treatment doses for each extract. Extracts A, B, C and D offered protection against MNi induction for all doses tested (Figures 1-4) related to DPPH and Folin-Ciocalteu results. The extracts were also characterized for Folin-Ciocalteu and consistently D extract was also lower than extracts A and B for this test as well. C extract varied in its performance in these two assays indicating it has a different composition of bioactives to the other extracts. This needs further investigation as the protection offered by the extracts did not fully reflect the chemical characterization results.

Pre-treatment	ROS challenge	[a]Experiment series 1	[b]Experiment series 2
Control	0	13.0 ± 1.5	7.0 ± 1.2
	300 µM	24.7 ± 0.9	20.3 ± 1.2
Quercetin	0	6.0 ± 0.6	4.3 ± 0.3
	300 µM	8.7 ± 1.2	9.3 ± 0.9
[a]Experiment series 1 was conducted to investigate Calendula extracts A and B. [b]Experiment series 2 was conducted for extracts C and D.			

Table 2: Effect of Quercetin pre-treatment and the media control on MN/1000 binucelated cells. Shown is the number of micronuclei/1000 bionucleated of control and quercetin after treat cells with or without H_2O_2. Values = mean ± SEM; n=3.

Increases in total number of MNi per 1000 BN cells indicates changes at the chromosome level, reflecting damage that is induced by H_2O_2 due to oxidative stress from free radicals. Free radical induced damage to DNA and chromosomes by a cellular redox imbalance from oxidative stress can result in cancer [27,28]. Micronuclei arise from either whole chromosome losses or acentric chromosome fragments. To investigate the specific type of chromosome damage responsible for the increases of MNi there is a need to use molecular techniques in combination with the CBMN assay e.g. probe for presence of centromeres in MNi using fluorescence probes [21]. Nucleoplasmic bridge (NPB) and Nucleoplasmic buds (NBUDs) were not observed after were cells exposed to hydrogen peroxide (H_2O_2). NPB can be due to dicentric chromosome and NBUDs can arise from gene amplification. The development of cancer is a result of the long term accumulation of mutations in human genes [29]. Also, some cancers are diagnosed with a genetic instability and of these, most are observed with instability at the chromosome level that can be chromosome breakage or whole chromosome losses [29]. Calendula extracts examined in this study offered protection against chromosomal damage induced by oxidative stress. Preventing induction of chromosomal damage using calendula extracts could reduce the risk of cancer. Interestingly, although not being used for protection, there is *Calendula officinalis* versus Trolamine in phase III randomized trial of *Calendula officianalis* for breast cancer [30].

Sample	Conc. (%)	Dry weight Equivalent (mg/ml)	Percentage (%) radical inhibition at 517 nm (mean ± S.E.M)
A	2.5	2.2	41.0 ± 2.0
B	2.5	2.1	44.4 ± 1.4
C	2.5	3.5	47.7 ± 9.1
D	2.5	3.8	28.7 ± 4.7
Quercetin	2.5	-	55.0 ± 1.6
Methanol	2.5	-	0.1 ± 0.1

Table 3: Free radical inhibition of extracts A, B, C and D, quercetin and methanol. Using DPPH assay Calendula extracts and quercetin (stock 1.61 mg/ml in methanol) were tested. Calendula extracts added neat to 2 ml DPPH solution. Each value represent the mean ± S.E.M; n = 3.

In conclusion, H_2O_2 damage can lead to oxidative stress and cause human cancer via chromosomal breakage or loss and DNA damage [21]. Calendula flower extracts protect HaCaT skin cells against an oxidative stress induced (by H_2O_2) chromosome damage by a decreased frequency of MNi. Further work on *calendula officinalis* extracts should include studying protection against UV light induced chromosome and mutation damage on human skin cells *in vitro* [31]. The extracts tested in this paper have potential applications as protectants due to antioxidants properties, including bio actives that act against oxidative stress induced damage to human cells.

Conflict of Interest Statement

The authors declare no conflict of interest

Acknowledgment

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References

1. Loeb KR, Loeb LA (2000) Significance of multiple mutations in cancer. Carcinogenesis 21: 379-385.
2. Würgler FE, Kramers PG (1992) Environmental effects of genotoxins (eco-genotoxicology). Mutagenesis 7: 321-327.
3. MURRAY CW, BOOTH AN, DEEDS F, JONES FT (1954) Absorption and metabolism of rutin and quercetin in the rabbit. J Am Pharm Assoc Am Pharm Assoc 43: 361-364.
4. Fusco D, Colloca G, Lo Monaco MR, Cesari M (2007) Effects of antioxidant supplementation on the aging process. Clin Interv Aging 2: 377-387.
5. Young IS, Woodside JV (2001) Antioxidants in health and disease. J Clin Pathol 54: 176-186.
6. de Zwart LL, Meerman JH, Commandeur JN, Vermeulen NP (1999) Biomarkers of free radical damage applications in experimental animals and in humans. Free Radic Biol Med 26: 202-226.
7. Hecht SS (1999) Tobacco smoke carcinogens and lung cancer. J Natl Cancer Inst 91: 1194-1210.
8. Ashok BT, Ali R (1999) The aging paradox: free radical theory of aging. Exp Gerontol 34: 293-303.
9. Rosenfeld ME (1998) Inflammation, lipids, and free radicals: lessons learned from the atherogenic process. Semin Reprod Endocrinol 16: 249-261.
10. Frankel EN (2007) Antioxidants in Food and Biology Facts and Fiction. The oily press, England.
11. Atoui AK, Mansouri A, Boskou G, Kefalas P (2005) Tea and herbal infusions: Their antioxidant activity and phenolic profile. Food Chemistry 89: 27-36.
12. Larson RA (1988) The antioxidants of higher plants. Phytochemistry 27: 969-978.
13. Shahidi F (2000) Antioxidants in food and food antioxidants. Nahrung 44: 158-163.
14. Katalinic V, Milos M, Kulisic T, Jukic M (2006) Screening of 70 medicinal plant extracts for antioxidant capacity and total phenols. Food Chemistry 94: 550-557.
15. Kalvatchev Z, Walder R, Garzaro D (1997) Anti-HIV activity of extracts from Calendula officinalis flowers. Biomed Pharmacother 51: 176-180.
16. Jiménez-Medina E, Garcia-Lora A, Paco L, Algarra I, Collado A, et al. (2006) A new extract of the plant Calendula officinalis produces a dual in vitro effect: cytotoxic anti-tumor activity and lymphocyte activation. BMC câncer 6: 119.
17. Re TA, Mooney D, Antignac E, Dufour E, Bark I, et al. (2009) Application of the threshold of toxicological concern approach for the safety evaluation of calendula flower (Calendula officinalis) petals and extracts used in cosmetic and personal care products. Food Chem Toxicol 47: 1246-1254.
18. Alnuqaydan AM, Lenehan CE, Hughes RR, Sanderson BJ (2015) Extracts from Calendula officinalis Offer in Vitro Protection Against H2O2 Induced Oxidative Stress Cell Killing of Human Skin Cells. Phytotherapy Research 29: 120-124.
19. Atanassova M, Georgieva S (2010) Comparative Polyphenol Composition and Antioxidant Capacity of the Bulgarian Plants (Dry Herbs). Electronic Journal of Environmental, Agricultural and Food Chemistry 9: 1514-1523.
20. Politeo O, Jukic M, Miloš M (2006) Chemical composition and antioxidant activity of essential oils of twelve spice plants. Croatica chemica acta 79: 545-552.
21. Fenech M (2007) Cytokinesis-block micronucleus cytome assay. Nat Protoc 2: 1084-1104.
22. Wang JJ, Sanderson BJS, Wang H (2007) Cyto- and genotoxicity of ultrafine TiO2 particles in cultured human lymphoblastoid cells. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 628: 99-106.
23. Fenech M (2000) The in vitro micronucleus technique. Mutat Res 455: 81-95.
24. Ramos A, Edreira A, Vizoso A, Betancourt J, López M, et al. (1998) Genotoxicity of an extract of Calendula officinalis L. J Ethnopharmacol 61: 49-55.
25. Re TA, Mooney D, Antignac E, Dufour E, Bark I, et al. (2009) Application of the threshold of toxicological concern approach for the safety evaluation of calendula flower (Calendula officinalis) petals and extracts used in cosmetic and personal care products. Food Chem Toxicol 47: 1246-1254.
26. Miliauskas G, Venskutonis PR, van Beek TA (2004) Screening of radical scavenging activity of some medicinal and aromatic plant extracts. Food Chemistry 85: 231-237.
27. Dizdaroglu M (1991) Chemical determination of free radical-induced damage to DNA. Free Radic Biol Med 10: 225-242.
28. Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M (2006) Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem Biol Interact 160: 1-40.
29. Lengauer C, Kinzler KW, Vogelstein B (1998) Genetic instabilities in human cancers. Nature 396: 643-649.
30. Pommier P, Gomez F, Sunyach MP, D'Hombres A, Carrie C, et al. (2004) Phase III Randomized Trial of Calendula Officinalis Compared With

Trolamine for the Prevention of Acute Dermatitis During Irradiation for Breast Cancer. Journal of Clinical Oncology 22: 1447-1453.

31. Fonseca YM, Catini CD, Vicentini FT, Nomizo A, Gerlach RF, et al. (2010) Protective effect of Calendula officinalis extract against UVB-induced oxidative stress in skin: evaluation of reduced glutathione levels and matrix metalloproteinase secretion. J Ethnopharmacol 127: 596-601.