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ORIGINAL PAPER

Bioactivity of Lemon Balm Kombucha

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Abstract There is inadequate published data referring to bioactivity of lemon balm tea and its Kombucha. The aim of this study, therefore, was to investigate antimicrobial, antiproliferative, genotoxic, and antigenotoxic potential of lemon balm tea and its Kombucha with consuming acidity. Antimicrobial activity was determined by agar-well diffusion method. Cell growth effects were determined in HeLa, MCF7, and HT-29 human tumor cell lines. Genotoxic and antigenotoxic effects were determined using chromosome aberration assay in Chinese hamster cell line CHO-K1. Differences between control and treated groups were evaluated using analysis of variance, at significance level of $p<0.05$. Kombucha from lemon balm tea (*Melissa officinalis* L.) exibited antimicrobial activity against prokaryotic microorganisms independently of their cell wall structure (both Gram-positive and Gram-negative bacteria), while there was no observed activity against eukaryots (yeasts and moulds). There was absence of genotoxic effects while antigenotoxic effects of lemon balm Kombucha and tea were confirmed on MMC-damaged CHO-K1 cells. For the explanation of cell growth effects that were not concentration dependent, concept of hormesis was used. Antiproliferative activity was lower compared with traditional Kombucha and *Satureja montana* L. Kombucha, with lemon balm tea showing higher activity than its Kombucha.

Keywords Antimicrobial activity · Cell growth activity · Chromosome aberration assay · Kombucha · *Melissa officinalis* L. · Tea

Abbreviations

ATCC	American type culture collection
CA	Chromosome aberration
CHO-K1	Chinese hamster ovary cell line
DET	Dye exclusion test
DMEM	Dulbecco's modified Eagle's medium
EDTA	Ethylenediaminetetraacetic acid
FCS	Fetal calf serum
HeLa	Human cervix cancer cell line
HT-29	Human colon cancer cell line
IC_{50}	Concentration that inhibits cell growth by 50%
MCF7	Human breast cancer cell line
MMC	Mitomycin C
PBS	Phosphate buffer solution
RPMI-1640	Roswell Park Memorial Institute medium
SRB	Sulforhodamine B
TCA	Trichloroacetic acid, TRIS-tris (hydroxymethyl)aminomethane

Introduction

Based on Blanc (1996), the consumption of Kombucha as a fermented beverage was practiced in 220 BC in Manchuria and spread to Russia where it is known as teakwas. The

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beverage was introduced into Germany during World War II and into France and France-dominated North Africa in 1950s (Blanc 1996). Kombucha made of black tea, green tea, decaffeinated black tea, and fruit teas (raspberry and blackberry) became popular in the United States, due to its refreshing and curative effects (Roussin 1996). In Balkan region, Kombucha has first appeared between World War I and World War II (Cvetkovic 2008). Kombucha has been claimed to be a prophylactic agent and beneficial to human health—as a diuretic in edemas, in arterosclerosis, in case of gout, sluggish bowels, for stones, etc. (Liu et al. 1996). Experience has also shown that Kombucha regulate the intestinal flora, strengthen and harmonize the metabolism, act as a natural antibiotic and help maintain acid-alkaline balance of the body (Sievers et al. 1995). Although there are numerous claims that drinking Kombucha is benefitial to health, they are not quite validated scientifically (Chu and Chen 2006).

Kombucha is traditionally prepared by fermenting sweetened (sucrose) black tea (*Camelia sinensis* L.). This medium is usually inoculated with cellulose pellicle formed during the previous cultivation, popularly known as a ″tea fungus″, and incubated statically under aerobic conditions for 7–10 days. This so-called tea fungus is actually a symbiosis of acetic acid bacteria (*Acetobacter xylinum, Acetobacter aceti*, and *Gluconobacter oxydans*) and yeasts (*Saccharomyces* sp., *Zygosaccharomyces* sp., *Torulopsis* sp., *Pichia* sp., and *Brettanomyces* sp.) (Greenwalt et al. 2000; Teoh et al. 2004). However, composition of "tea fungus" depends on geographic and climatic conditions as well as on local species of wild yeasts and bacteria that can explain differences in beverage components (alcohols, acidic acid, gluconic acid, lactic acid, etc.) (Mayser et al. 1995). Tea fungus culture used in this study contained at least five yeast strains (*Saccharomycodes ludwigii, Saccharomyces cerevisiae, Saccharomyces bisporus, Torulopsis* sp., and *Zygosaccharomyces* sp.) and two bacterial strains of the *Acetobacter* genera (Markov et al. 2001).

The yeasts ferment the sugar in the cultivation medium to ethanol, which is further oxidized by the acetic acid bacteria to acetic acid. The tea in the cultivation medium provides tea fungus with the necessary nitrogen compounds, of which especially important are purine derivatives (caffeine and theophylline), amply present in black tea (Hoffmann 1998). The final product is a sour, slightly carbonated, acidic beverage, comprised of sugars, organic acids, tea components, vitamins, and minerals, resembling cider. Many flavor compounds, including alcohols, aldehydes, ketones, esters, and amino acids have also been identified (Teoh et al. 2004).

Sweetened black tea has been the traditional and almost only recommended medium for preparing Kombucha. Malbasa et al. (2009) reviewed some attempts in applying atypical nutrients such as Coca-Cola, red wine, white wine, vinegar, extract of Echinacea, Mentha, etc. Studies of some alternative cultivation medium have shown that green tea (Greenwalt et al. 1998) and lemon balm tea (*Melissa officinalis* L.) (Velicanski 2008) have more stimulating effect on the Kombucha fermentation than black tea, yielding the fermentation product in a shorter time frame that was in the case of green tea explained by its higher caffeine content. Lemon balm is a well-known aromatic herb used to give fragrance to the different food and beverage products. In the USA, Department of Agriculture (USDA) refers to *M. officinalis* L. loosely as "common balm" (http://plants.usda.gov/java/profile?symbol=MEOF2). Lemon balm has also been used as a medicinal plant for treatment of headaches, gastrointestinal disorders, nervousness, and rheumatism (Dastmalchi et al. 2008; Stangler Herodez et al. 2003). *M. officinalis* L. essential oil is a well-known antibacterial and antifungal agent, and it is also responsible for the mild depressive and spasmolytic properties of the plant (Mimica-Dukic et al. 2004).

There is inadequate published data referring to bioactivity of lemon balm tea and its Kombucha. The aim of this study, therefore, was to investigate antimicrobial, antiproliferative, genotoxic and antigenotoxic potential of lemon balm tea and its Kombucha with consuming acidity.

Materials and Methods

Plant Material

Aerial parts of the cultivated flowering plant *M. officinalis* L. were collected in July 2009 in Vojvodina, Serbia. Voucher specimens (number 2184) were confirmed and deposited at the Herbarium of the Department of Biology and Ecology, Faculty of Natural Science, University of Novi Sad, Novi Sad, Serbia.

Cultural Conditions of the Tea Fungus

Substrate for Kombucha fermentation was prepared by adding 70 g/l of commercial sucrose to tap water. After boiling of which, 5 g/l of dry crushed leaves of lemon balm were added. The tea leaves were steeped for 15 min and removed by filtration. After cooling to 30 °C, the inoculum (left over Kombucha from previous process) was added in an amount of 10% (*v*/*v*). Prepared Kombucha (0.33 l) was poured into flasks (Ø=8 cm, capacity 0.72 l) and incubated under aerobic conditions at 28 °C. The incubation period was terminated when optimal consuming acidity of 3.5–4.5 g/l was achieved (Cvetkovic et al. 2008).

Chemical Analyses

The pH value of fermented liquid samples was determined by electronic pH-meter (HI 9321, Woonsocket, USA). Titratable acidity of fermented beverages was determined by potentiometric titration with NaOH, c=0.1 mol/l, after the removal of CO_2 (Office Internationale de la vinge et du vin 1990).

Samples Used in Antimicrobial, Cell Growth, and Chromosome Aberration Assays

For the determination of antimicrobial activity Kombucha beverage (titrable acidity=4.56 g/l), acetic acid solution at the same concentration as in fermented tea (4.56 g/l), decoction of unfermented *M. officinalis* L. tea (dry weight, 5 g/l), neutralized Kombucha (prepared by neutralizing Kombucha beverage with 0.1 mol/l NaOH) and heat-denaturated Kombucha (treated at 100 °C for 10 min) were used. For the analysis of chromosome aberration (CA) frequency and cell growth effects consuming concentrations and their serial dilutions in 0.9% NaCl were used. Dry weights of *M. officinalis* L. tea and *M. officinalis* L. Kombucha were m=7.3 and m=47.5 g/l, respectively. Achieved final concentrations were 0.045, 0.18, 0.73 and 0.295, 1.185, 4.75 μg/ml, respectively, for CA assay and in the range of 1.95–500 μg/ml for cell growth assay. Samples were filtered through a 0.22-μm microfilters to remove cells and obtain sterility.

Test Microorganisms

Microorganisms used in antimicrobial tests were: Gram-negative bacteria—*Pseudomonas aeruginosa* (ATCC 27853), *Proteus mirabilis* (ATCC 35659), *Escherichia coli* (ATCC 25922), *Salmonella enteritidis* (ATCC 13076), *Erwinia carotovora* (NCPPB 595); Gram-positive bacteria—*Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 10876), *Sarcina lutea* (ATCC 9341); yeasts—*S. cerevisiae* (112, Hefebank, Weihenstephan, Germany), *Candida pseudotropicalis* (clinical isolate), *Rhodotorula* sp. (natural isolate) and moulds—*Aspergillus niger* (ATCC 16404), *Penicillium aurantiogriseum*, and *Aspergillus flavus* (natural isolates).

Experimental Scheme

Experimental scheme of current study with key instrumentation data and test criteria is given in Fig. 1.

Antimicrobial Test

Antimicrobial activity of test strains was determined by the agar-well diffusion method. Test strains of bacteria were grown on Müeller–Hinton agar slants and that of yeasts and moulds grown on Sabouraud dextrose agar slants, respectively, for 24 h at 37 or 25 °C and checked for purity (Mayo 1998; Sreeramulu et al. 2000). Cells were then suspended in sterile 0.9% NaCl solution. Suspensions for inoculation (1 ml; 1×10^7 cells/ml) were homogenized with melted (45 °C) Müeller–Hinton or Sabouraud dextrose agar (19 ml) and poured into Petri dishes. Wells (Ø=9 mm) were made with a sterile metal tube by a vacuum pump. Samples (100 μL) were then transferred into the wells. Plates were than incubated at 37 °C for 24 h (bacteria) and 25 °C for 48–72 h (yeasts and moulds) when the diameter of inhibition zone was measured.

Grow and Culture of the Cell Lines

For the estimation of cell growth effects, human tumor cell lines HeLa (cervix epitheloid carcinoma), MCF7 (breast adenocarcinoma), and HT-29 (colon adenocarcinoma) were used. In chromosome aberration assay and dye exclusion test Chinese hamster cell line CHO-K1 (ovary, epithelial) was used. Cell lines were grown in DMEM (PAA Laboratories GmbH, Pashing, Austria) with 4.5% glucose (HeLa, MCF7, and HT-29) and RPMI 1640 medium (PAA Laboratories GmbH, Pashing, Austria) with L-glutamine (CHO-K1), supplemented with 10% heat inactivated fetal calf serum (FCS; NIVNS, Serbia), 100 IU/ml of penicillin and 100 μg/ml of streptomycin (Galenika, Belgrade, Serbia). All investigated cell lines grow attached to the surface. They were cultured in 25 cm^2 flasks (Corning, New York, USA) at 37 °C in atmosphere of 5% CO_2 and 100% humidity, sub-cultured twice a week and a single cell suspension was obtained using 0.1% trypsin (Serva, UK) with 0.04% EDTA.

Sulforhodamine B Assay

Cell lines were harvested and plated into 96-well microtiter plates (Sarstedt, Newton, USA) at seeding density of 3×10^3 cells per well, in a volume of 180 μl, and pre-incubated in complete medium supplemented with 5% FCS, at 37 °C for 24 h. Serial dilutions of tea, Kombucha and solvent were added (20 μl/well) to achieve required final concentrations and control. Microplates were then incubated at 37 °C for an additional 48 h. Cell growth was evaluated by the colorimetric sulforhodamine B (SRB) assay according to Skehan et al. (1990). Cells were fixed with 50% TCA (1 h, +4 °C), washed with distilled water (Wellwash 4, Labsystems; Helsinki, Finland) and stained with 0.4% SRB (30 min, room temperature). The plates were then washed with 1% acetic acid to remove unbound dye. Protein-bound dye was extracted with 10-mM TRIS base. Absorbance (A) was measured on a microplate

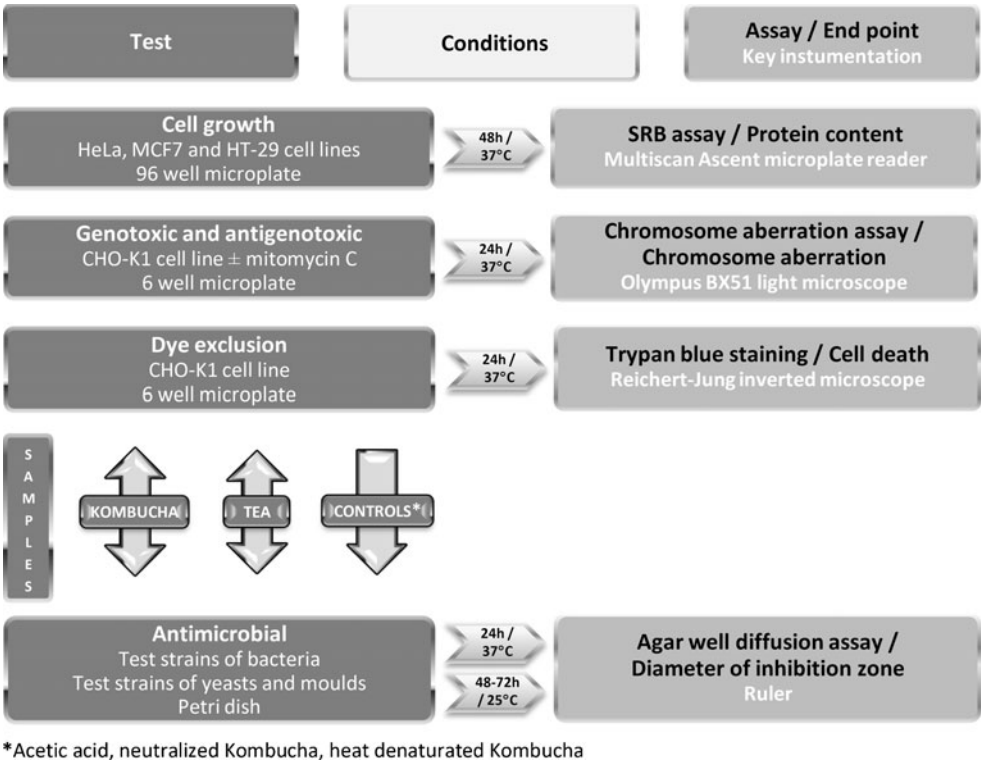

Fig. 1 Experimental scheme used to investigate bioactivity of *Melissa officinalis* L. tea and its Kombucha

reader (Multiscan Ascent, Labsystems; Helsinki, Finland) at 540/620 nm.

Chromosome Aberration Assay and Dye Exclusion Test

Cells were seeded in 6-well plates (Corning, New York, USA), in 5 ml of medium with 10% FCS, at seeding density of 25×10^5 cells per well and exposed to test samples for 24 h. Mitomycin C (MMC; Bristol-Myers Squibb, USA) was diluted in PBS to achieve final concentration of 0.1 μg/ml, defined in previous pilot experiment as adequate for accurate scoring (data not shown).

The chromosome aberration (CA) assay was performed according to Verma and Babu (1995). Immediately after cell seeding, substanties were added for 24 h. At the end of the treatment, cells were washed with complete fresh medium and 0.1 μg/ml of colcemid solution was added 1.5 h before harvesting. The cells were than treated with hypotonic solution (0.56% KCl) for 25 min at 37 C in fully humidified atmosphere with 5% CO_2 and fixed three times with methanol/glacial acetic acid (3:1, *v*/*v*) for 15 min. The slides were stained with 2% Giemsa and scored for structural chromosome aberrations, using light microscopy (Olympus BX51) and 3.2 mega pixel digital camera (Olympus CAMEDIA C3040) attached to the computer.

Trypan blue dye exclusion test (DET) was performed according to Yoshida et al. (2008).

Statistical Analysis

Results of antimicrobial activity were expressed as mean ± SD of three independent experiments. Dye exclusion test was performed in two independent experiments, in triplicate. Cell survival and percent of cytotoxicity were estimated according to the formulas: percent of viable cells=(number of viable cells/total cell number)×100; percent of cytotoxicity=[1−(number of viable cells/total cell number)]×100. Results of cell growth activity were expressed as mean ± SD of three independent experiments, performed in quadruplicate. Effect on cell growth was expressed as a percent of the control, and calculated as: (At/Ac)×100 (%), where At is the absorbance of the test sample and Ac is the absorbance of the control. Differences between control and treated groups were evaluated using one-way analysis of variance, at significance level of $p<0.05$ (Microsoft Office Excel 2003 software). CA frequency was expressed as mean ± SD of three independent experiments each scored on minimum of 100 metaphase cells per point. The CA was determined only in the cells containing 19–23 (modal number±2) chromosomes. Structural CA were categorized as chromatid and chromosome types, according to Savage (1975). Differences between control and treated groups were evaluated using one-way analysis of variance with Fisher's least significant difference using Statistica software, Version 8 (StatSoft Inc, Tulsa, USA), at significance level of $p<0.05$.

Results and Discussion

The battery of multi-endpoint bioassays used that are based on whole cell response of bacteria, yeast and moulds, mammalian and human cell lines is powerful indicator of metabolic, biochemical, and genetic alterations that arise under the influence of evaluated compounds.

Antimicrobial Activity

After 3 days of cultivation in sweetened lemon balm tea titratable acidity in its Kombucha was 4.56±0.03 g/l and pH=2.89±0.05. Lemon balm Kombucha and control samples of acetic acid solution and heat-denaturated Kombucha had expressive antimicrobial activity against Gram-negative bacteria (*S. enteritidis*, *E. coli*, *P. aeruginosa*, *P. mirabilis*, *E. carotovora)* and Gram-positive bacteria (*S. aureus* and *B. cereus)* (Table 1). Tested samples did not express any antimicrobial activity against *S. lutea* (Gram-positive bacteria) or yeasts *S. cerevisiae*, *C. pseudotropicalis*, and *Rhodotorula* sp., and moulds *A. niger, A. flavus*, and *P. aurantiogriseum* (Table 1). Of all tested samples acetic acid solution had the highest antimicrobial activity. Neutralized Kombucha was active only against *E. coli* (zone of biostatic activity was 30 mm). Unfermented tea did not show any antimicrobial activity against tested microorganisms.

Lemon balm Kombucha had similar activity as acetic acid solution against both Gram-positive and Gram-negative bacteria. Acording to this results, it can be inferred that acetic acid is main antimicrobial agent in Kombucha beverage. Antimicrobial activity of acetic acid and other weak organic acids is a result of cytoplasmic acidification that inhibits cell growth (Ludovico et al. 2003). Sreeramulu et al. (2000) and Steinkraus et al. (1996) also marked acetic acid as main antimicrobial agent in black tea Kombucha. In their studies Kombucha beverages had much higher (3–5 times) bactericidal effect against *E. coli, S. aureus*, and *B. cereus* than lemon balm Kombucha, probably due to higher content of acetic acid in beverages. Also, lemon balm Kombucha showed higher activity toward tested bacteria then Kombucha beverages from black and winter savory (*Satureja montana* L.) tea (Cetojevic Simin et al. 2008) (with titratable acidity 3.55 and 3.94 g/l, respectively).

Neutralized lemon balm Kombucha had minimal inhibitory activity toward *E. coli* that attributes to the presence of antimicrobial components in Kombucha other than acetic acid. Sreeramulu et al. concluded that other antimicrobial components are ethanol, proteins or tannins originally present in tea or their derivatives as well as microbial metabolites produced by bacteria or yeasts (Sreeramulu et al. 2001). There was no significant difference between antimicrobial activity of Kombucha samples before and after heating, that means antimicrobial components are thermostable.

There are no published studies about antimicrobial activity of lemon balm decoct (with drinkable levels of tea). In this study, unfermented lemon balm tea (dry weight, 5 g/l) did not show any antimicrobial activity, probably due to low concentration of active components. The unfermented black and green tea also showed no antimicrobial properties against most of the test organisms even at 70 g/l dry tea, except for *S. aureus* that was minimally inhibited when the dry weight of tea reached 35 g/l and higher (Hoffmann 1998). Ertürk (2006) determined minimal inhibitory concentration of ethanolic extract of lemon balm leaves and twigs against bacteria and moulds and obtained result were 10–25 g/l, depending on tested microorganism.

Lemon balm Kombucha and acetic acid solution did not inhibit the growth of yeasts and moulds (eukaryotic microorganisms) probably due to their acidophilic/acidotolerant properties that makes them more resistant to organic acids such as acetic acid that is main antimicrobial agent in Kombucha. Because of that, potential contamination

Table 1 Antimicrobial activity of lemon balm Kombucha and controls (acetic acid and heat-denaturated Kombucha)

Microorganism	Kombucha TA=4.56 g/L		Acetic acid c=4.56 g/L		Heat-denaturated Kombucha	
	A	B	A	B	A	B
Salmonella enteritidis	13.85±0.54	28.12±1.2	17.23±0.36	n.d.	15.25±0.32	28.67±0.89
Escherichia coli	13.67±1.54	30±0.0	16.67±0.58	n.d.	14.4±0.89	30±0.0
Proteus mirabilis	15±1.0	n.d.	17.75±0.96	n.d.	17±0.82	n.d.
Pseudomonas aeruginosa	14.4±0.89	n.d.	17±0.71	n.d.	16±0.00	n.d.
Erwinia carotovora	17.83±1.18	n.d.	22.8±0.84	n.d.	21.6±0.89	n.d.
Staphylococcus aureus	16±1.22	n.d.	16.8±2.17	n.d.	15.8±1.64	n.d.
Bacillus cereus	14.33±1.54	n.d.	15±1.73	n.d.	14.25±1.7	n.d.

Diameter of inhibition zone (mm). Results are expressed as mean ± SD of three independent experiments

A microbicidal activity, *B* microbiostatic activity, *nd* not detected, *TA* titratable acidity, *c* concentration

with moulds and yeasts from air exists in case of growing Kombucha at home. Greenwalt et al. (2000) also reported the absence of activity of black tea Kombucha (titratable acidity 7.2 g/l) toward pathogenic yeast *Candida albicans*.

Chromosome Aberration Frequency and Cell Viability

In both undamaged and MMC-damaged CHO–K1 cells 24 h treatment with lemon balm tea and its Kombucha resulted in concentration-dependent decrease of CA frequency, compared with control values (Fig. 2). Decrease of CA frequency in undamaged cells was statistically significant ($p<0.05$) at tea concentrations higher than 0.18 μg/ml (Fig. 2a) and at Kombucha concentrations higher than 0.295 μg/ml (Fig. 2b). Decrease of CA frequency in MMC-damaged cells was highly significant ($p<0.001$) for all examined concentrations of both lemon balm tea and its Kombucha (Fig. 2). The majority of aberrations found (Fig. 3) in both undamaged and MMC-damaged CHO-K1 cells were of chromosomal type (Tables 2, 3) that indicates influence on the cell cycle in pre-replication period.

After a 24-h incubation period with lemon balm tea and Kombucha, IC_{50} was not detected in any applied concentration. There was concentration-dependent proliferation in all samples determined by DET (data not shown).

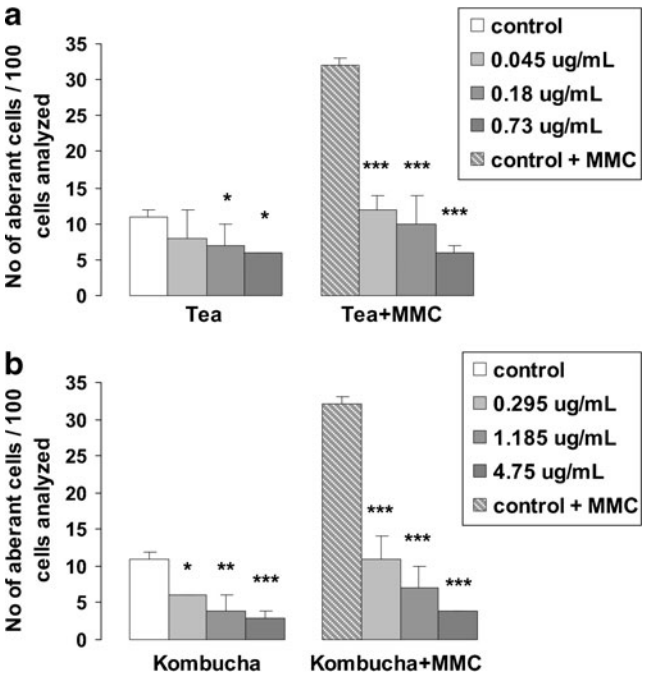

Fig. 2 Induction of chromosome aberrations by *Melissa officinalis* L. **a** Tea and **b** Kombucha in CHO-K1 cells (*$p<0.05$; **$p<0.005$; *** $p<0.001$. One-way analysis of variance with Fisher's least significant difference, compared with control). *MMC* mitomycin C. Results are expressed as mean ± SD of three independent experiments each scored on minimum of 100 metaphase cells per point

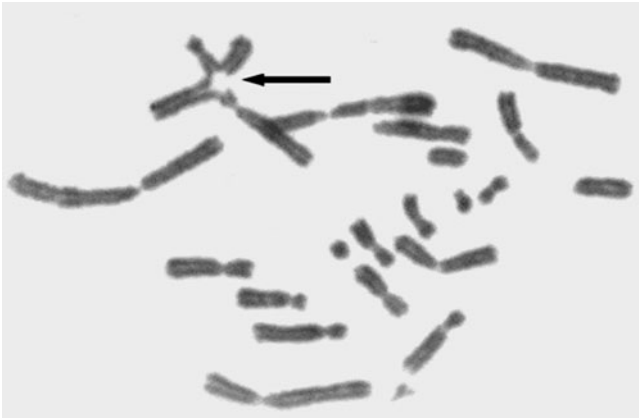

Fig. 3 Aberrant metaphase in CHO-K1 cell line. *Arrow* indicates chromatide breakage

Data about genotoxic or antigenotoxic potential of Kombucha are still insufficient. Obtained results showed that lemon balm Kombucha and tea did not induce CA but decreased CA frequency in both undamaged and MMC-damaged CHO-K1 cells, probably due to their antioxidative activity. MMC is alkylating agent that induces intrastrand cross-linking of DNA and generation of oxygen free radicals (Pritsos and Sartorelli 1986). As a consequence a broad range of genotoxic effects and chromosomal alterations results (Lorge et al. 2006) such as chromosomal aberrations (Snustad and Simmons 2001). *M. officinalis* L. possess antioxidant activity that corresponds to the content of phenolic compounds. It has been shown that lemon balm extracts are good free radical scavengers that exhibit significant protective effect on hydrogen peroxide induced toxicity (López et al. 2009). Study of antioxidant activity confirmed that Kombucha exhibited increased time-dependent antioxidant properties. During fermentation, small molecules with higher antioxidant activities are released due to depolymerization of the arubigins and this is offered as explanation of increased total phenol content found in Kombucha (Chu and Chen 2006). Also, oral administration of Kombucha in rats during chronic treatment within 45 days induced significant activation of antioxidant defense system and declared this traditional beverage as a strong natural antioxidant (Yogesh et al. 2003). Results of this study are in accordance with previous findings where Kombucha from *S. montana* L. reduced micronuclei frequency on MMC-treated and MMC-untreated human peripheral blood lymphocytes. In this experiment, sister chromatide exchange frequency was higher compared with control values that indicated intensive reparation of primary DNA damage after the treatment with *S. montana* L. Kombucha (Mrdjanovic et al. 2007). Pereira et al. (2005) evaluated genotoxic effect of rosmarinic acid in brain tissue of rats using comet assay and also found no DNA damage.

Table 2 Chromosome aberrations (%) detected in MMC-damaged and undamaged CHO-K1 cell line after the treatment with *Melissa officinalis* L. Kombucha

Sample/ Treatment	Concentration (μg/mL)	Chromatid			Chromosome					
		Gap	Break	Total% of aberrations	Gap	Break	Dic	Ring	Ac	Total % of aberrations
Control	0.0	3.2	1.4	4.6	3.2	1.0	0.0	0.0	2.2	6.4
Kombucha	0.29	1.0	0.5	1.5	0.0	3.0	0.0	0.0	1.5	4.5
	1.18	2.0	0.0	2.0	0.0	1.0	0.0	0.0	1.0	2.0
	4.75	1.0	0.0	1.0	0.0	1.0	0.0	0.0	1.0	2.0
Control+MMC	0.0+0.1	6.2	4.1	10.3	11.3	4.1	2.0	0.0	4.3	21.7
Kombucha+MMC	0.29+0.1	2.1	1.2	3.3	2.0	5.2	0.5	0.0	0.0	7.7
	1.18+0.1	1.0	0.0	1.0	4.6	1.4	0.0	0.0	0.0	6.0
	4.75+0.1	2.0	0.0	2.0	0.0	1.0	0.0	0.0	1.0	2.0

CA frequency was expressed as mean of three independent experiments each scored on minimum of 100 metaphase cells per point
MMC mitomycin C, *Dic* dicentric chromosome, *Ac* acentric fragment

Antiproliferative Activity

Number of cells, of attached Cell lines, seeded at the beginning of the experiment is dependent on time in culture, volume of the medium, size of attaching surface, cell size, doubling time, plating efficiency, and, at least, on concentration of serum in the medium. Because of possible growth stimulation (proliferation) that could be induced by the investigated substance it is necessary to calculate the exact number of cells to be seeded in order to produce sub-confluent monolayer of attached cells at the end of the experiment. In our experiments, under conditions given in Material and methods section, HeLa, MCF7 and HT-29 cell line were seeded at 3,000 to 4,000 cells per well of 96-well microplate, as even 5,000 cells per well seeded can result in overgrowth of cells in *some treatments* and exhaustion of growth medium at the end of 24+48 h=72 h experimental scheme. This can, by itself, influence cell growth, and interfere with experimental results, i.e., it can "push" cell growth into the plateau phase in *these treatments* and lead to false *general conclusions*.

As already mentioned, total time in culture in our experimental scheme is 72 h. After the initial seeding of the cells they are pre-incubated for 24 h to allow them to attach and enter into exponential growth phase prior to the addition of examined substances. The lag phase for the cultured cells can be approximated to 24 h (Freshney 2005). After pre-incubation, substances to be investigated are added for additional 48 h, which spans through the exponential phase.

In this research all experimental treatments were evaluated compared with untreated controls (i.e. controls have been

Table 3 Chromosome aberrations (%) detected in MMC-damaged and undamaged CHO-K1 cell line after the treatment with *Melissa officinalis* L. tea

Sample/ Treatment	Concentration [μg/mL]	Chromatid			Chromosome					
		Gap	Break	Total% of aberrations	Gap	Break	Dic	Ring	Ac	Total% of aberrations
Control	0.0	3.2	1.4	4.6	3.2	1.0	0.0	0.0	2.2	6.4
Tea	0.04	1.0	0.5	1.5	1.5	1.0	0.5	0.0	3.5	6.5
	0.18	1.0	0.0	1.0	0.5	0.5	0.0	0.0	4.0	6.0
	0.73	2.0	0.5	2.5	2.0	0.0	1.0	0.0	0.5	3.5
Control+MMC	0.0+0.1	6.2	4.1	10.3	11.3	4.1	2.0	0.0	4.3	21.7
Tea+MMC	0.04+0.1	3.0	1.0	4.0	2.5	1.0	1.0	0.0	3.5	8.0
	0.18+0.1	0.3	2.7	3.3	3.0	1.0	0.0	1.7	1.0	6.7
	0.73+0.1	2.0	0.0	2.0	0.5	2.0	0.0	0.0	1.5	4.0

CA frequency was expressed as mean of three independent experiments each scored on minimum of 100 metaphase cells per point
MMC mitomycin C, *Dic* dicentric, *Ac* acentric fragment

treated with solvent). For the assessment of antitumor activity, along with cell lines derived from tumor tissues, the use of non-tumor cell line(s) as well as the use of panel of positive and negative controls is invaluable in the evaluation of obtained results. In our laboratory, we are finishing the new protocol that will include these valuable controls.

M. officinalis L. tea and its Kombucha affected cell growth depending on cell line, but none affected cell growth by 50% inhibition (Fig. 4). All observed cell growth effects showed typical hormetic, biphasic response that is characterized with cell growth stimulation at lower concentrations that arises in cell growth inhibition at higher concentrations. The most prominent effect on cell growth and also difference between the activity of tea and Kombucha was observed in HeLa cell line. Kombucha and tea induced stimulation of HeLa cell line growth in the lower concentration range (1.95–30 μg/ml). Tea exhibited the most significant inhibition of HeLa cell line growth ($p<0.01$), but only at concentrations higher than 100 μg/ml (IC_{20} was reached at 500 μg/ml) (Fig. 4a). Kombucha also stimulated MCF7 cells in the lowest concentration range (under 10 μg/ml), but inhibited their growth at highest concentration (500μg/ml) (Fig. 4b). Neither Kombucha nor tea effected the growth of HT-29 cell line in the investigated concentration range (Fig. 4c).

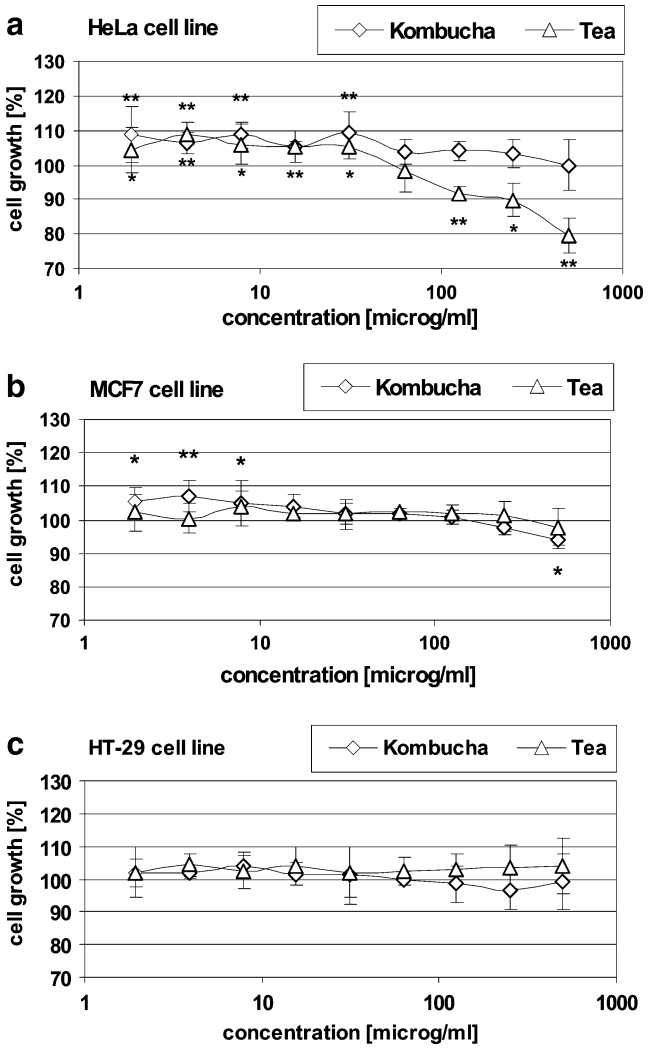

Fig. 4 Cell growth activity of lemon balm tea and its Kombucha in **a** HeLa, **b** MCF7, and **c** HT-29 cell line. *$p<0.05$; **$p<0.01$. One-way analysis of variance, compared with control. Results are expressed as mean ± SD of three independent experiments, performed in quadruplicate

Based on IC_{20} value lemon balm Kombucha showed lower activity toward HeLa cell line compared with black tea Kombucha and *S. montana* Kombucha (Cetojevic Simin et al. 2008). Compared with this two Kombuchas, lemon balm Kombucha showed similar activity ($p<0.05$) toward MCF7 cell line, but had no activity against HT-29 cells. Only in HeLa cell line there was a marked difference between the activity of lemon balm tea and Kombucha, with lemon balm tea showing higher antiproliferative activity. In previous research *M. officinalis* L. chloroform extract, among other examined lemon balm extracts, exhibited strongest antiproliferative effect in HeLa and MCF-7 cell lines with IC_{50} values of 0.09 mg/ml and 0.10 mg/ml, respectively (Canadanovic-Brunet et al. 2008).

It is reasonable to propose that these active substances contained in chloroform extract of lemon balm are responsible for antiproliferative effects in HeLa and MCF-7 cell line. For the explanation of cell growth effects of both lemon balm Kombucha and tea towards HeLa and MCF7 cell line that were no concentration-dependent well-known concept of hormesis may be used. In pharmacology such dose responses have been studied with the aid of synthetic agonists and antagonists of receptors witch mediate hormetic biphasic effects (low dose stimulation and high dose inhibition) (Calabrese and Baldwin 2001a; Calabrese and Baldwin 2001b). A single agonist with differential binding (i.e., high and low receptor affinities) affecting two opposite acting receptors will induce hormetic-like biphasic dose responses in numerous biological systems as has been shown for dozens of receptor systems (Calabrese 2005). It is believed that phenolics can exert their effects on the different signaling pathways such as mitogen-activated protein kinases, activator protein-1,or nuclear factor-κB either separately or sequentially, as well as possibly interacting between/among these pathways, which can offer complementary and overlapping mechanism of action (Yang and Liu 2009). It is hypothesized that the additive and synergistic effects of phytochemicals in fruits and vegetables are responsible for their potent antioxidative and anticancer activities and that the benefits of this diet are attributed to the complex mixture of phytochemicals present in whole foods (Liu 2004).

Conclusions

Kombucha from lemon balm tea (*M. officinalis* L.) showed significant antimicrobial activity against Gram-positive and Gram-negative bacteria. Acetic acid was marked as main antimicrobial agent. Growth of eukaryotic cells (yeasts and moulds) was not inhibited by lemon balm Kombucha. Cell growth, genotoxic, and antigenotoxic effects were evaluated based on differences between control and treated groups, at significance level of $p<0.05$. There was absence of genotoxic effects while antigenotoxic effects of lemon balm tea and its Kombucha were confirmed on MMC-damaged CHO-K1 cells by decreased chromosome aberrations frequency. For the explanation of cell growth effects that were not concentration dependent, concept of hormesis was used. Antiproliferative activity was lower compared with traditional Kombucha and *S. montana* L. Kombucha, with lemon balm tea showing higher activity than its Kombucha.

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