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ORIGINAL ARTICLE

Antiproliferative and antimicrobial activity of traditional Kombucha and *Satureja montana* L. Kombucha

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Summary

Purpose: *To carry out a preliminary investigation of the biological activity of Kombucha beverages from Camellia sinensis L. (black tea) and Satureja montana L. (winter savory tea), that have consuming acidity.*

Materials and methods: *Cell growth effect was measured by sulforhodamine B colorimetric assay on HeLa (cervix epithelioid carcinoma), HT-29 (colon adenocarcinoma), and MCF-7 (breast adenocarcinoma). Antimicrobial activity to bacteria, yeasts and moulds was determined by agar-well diffusion method.*

Results: *Consuming Kombuchas had the most expressive antimicrobial activity against all investigated bacteria, except Sarcina lutea, while unfermented tea samples had no activity. Traditional Kombucha showed higher activity against Staphylococcus aureus and Escherichia coli than acetic acid, while both neutralized Kombuchas had bacteriostatic activity on Salmonella enteritidis.*

Examined Kombuchas did not stimulate cell proliferation of the investigated cell lines. Antiproliferative activity of winter savory tea Kombucha was comparable to that of traditional Kombucha made from black tea. Furthermore, in HeLa cell line Satureja montana L. Kombucha induced cell growth inhibition by 20% (IC_{20}) at lower concentration compared to the activity of water extract of Satureja montana L. obtained in our previous research.

Conclusion: *Presence of more active antiproliferative component(s) in Satureja montana L. Kombucha compared to Satureja montana L. water extract and antimicrobial component(s) other than acetic acid in both Kombuchas is suggested.*

Key words: antiproliferative, antimicrobial, *Camellia sinensis* L, *Satureja Montana* L, Kombucha

Introduction

Kombucha is a traditional fermented beverage with a history of several thousand years in the East and yet it is quite popular today in the West. The beverage has been claimed to be a prophylactic agent beneficial to human health - as a diuretic, in edemas, in arteriosclerosis, in case of gout, sluggish bowels, for stones, etc. [1,2]. Experience has also shown that Kombucha beverage can regulate the intestinal flora, strengthen cells, harmonize metabolism, function as a natural antibiotic, and help maintain pH, i.e. the body's acid-alkaline balance [3]. Current strong and increasing interest in the consumption of the product derives from its purported therapeutic benefits, which range from weight loss to curing cancer and AIDS [4]. However, many of these effects have not been proven scientifically [5].

Kombucha is prepared by fermenting sweetened black tea with tea fungus. The tea fungus is a symbiosis of acetic acid bacteria (*Acetobacter aceti, Acetobacter pasteurianus, Gluconobacter oxidans*) [5] and yeasts (*Saccharomyces* spp., *Zygosaccharomyces* spp., *Torulopsis* spp., *Pichia* spp., *Brettanomyces* spp.) [5-7]. The final product is a sour, slightly sparkling, acidic beverage.

Analyses of the fermented liquid have revealed

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the presence of acetic, lactic and gluconic acids as major chemical compounds. Other components are alcohols, aldehydes, ketones, esters and amino acids [4].

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 [1]. Because of that, sweetened black tea (*Camelia sinensis* L.) has been the traditional and almost only recommended medium for preparing Kombucha.

Studies of some alternative cultivation median have shown that green tea has more stimulating effects on the Kombucha fermentation than black tea, yielding the fermentation product in a shorter time frame [8]. The stimulating effect of green tea on Kombucha culture was explained by a higher caffeine content compared to black tea [9]. Sweetened tea of *Echinacea purpurea* L. can also be used for Kombucha fermentation and the obtained beverage has outstanding antioxidant properties [10]. It is known that *Echinacea* spp., herbal medicines and dietary supplements are traditionally used as immunostimulants in the treatment of inflammatory and viral diseases. Also, Kombucha can successfully be obtained from peppermint tea [11]. Using lemon balm tea (*Melissa officinalis* L.) as an alternative medium Kombucha beverage is obtained in a shorter time compared to black tea [12].

Previous studies showed that Kombucha can be successfully produced from winter savory tea (*Satureja montana* L.), although the process lasts longer than in the case of black tea [13]. Winter savory tea has antiseptic, aromatic, carminative, digestive, expectorant and stomach effects, and because of that it is chosen as an alternative medium for Kombucha fermentation.

There are no published studies referring to the antiproliferative and antimicrobial activity of Kombucha beverage with consuming acidity. The aim of this paper was to investigate the biological activity of traditional Kombuchas obtained from *Camellia sinensis* L. tea and *Satureja montana* L. tea, that have consuming acidity.

Materials and methods

Cultural conditions of the tea fungus

Substrate for Kombucha fermentation was prepared by adding 70 g/L of commercial sucrose in tap water, and after boiling 5 g/L of dry crushed leaves of black (*Camellia sinensis* L.) or winter savory tea (*Satureja montana* L.) was prepared in the same way. The tea leaves were steeped for 15 min and removed by filtration. After cooling to about 30° C, the inoculum (Kombucha beverage from previous process) was added in amount of 10% (v/v). Then the 0.33 L of the prepared medium was poured into small flasks (Ø=8 cm, capacity 0.72 L) and incubated under aerobic conditions at 28° C. The incubation period was terminated when the Kombucha beverages achieved optimal consuming acidity 3.5-4.5 g/L of acids [14].

Samples for determination of antiproliferative activity

The dry weight of Kombucha beverages were m=65.8 mg/mL (black tea Kombucha) and m=70.8 mg/mL (winter savory tea Kombucha). For analysis of the antiproliferative effects serial dilutions in 0.9% NaCl of Kombucha beverages were prepared to achieve the required working concentrations (0.0195-10 mg/mL). Samples were filtered through a sterile microfilter (0.22 μm) to remove cells.

Cell lines

The 3 human tumor cell lines used in the study were HeLa (cervix epithelioid carcinoma), MCF-7 (breast adenocarcinoma) and HT-29 (colon adenocarcinoma). The cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, BRL, UK) with 4.5% glucose supplemented with 10% heat inactivated fetal calf serum (FCS; NIVNS, Serbia), 100 IU/mL of penicillin and 100 μg/mL of streptomycin (Galenika, Serbia). The cells were sub-cultured twice a week and a single cell suspension was obtained using 0.5% trypsin (Serva, UK). All cell lines were cultured in 25 cm^2 flasks (Corning, New York, USA) at 37° C in atmosphere of 5% CO_2 and 100% humidity. Exponentially growing cells were used throughout the assay. A treatment period of 2 days was selected since the control cells were still in the exponential phase at that time.

Sulforhodamine B (SRB) assay

Cells were harvested and plated into 96-well microtiter plates (Corning, New York, USA) at seeding density of 3×10^3 cells per well, in a volume of 180 μL, and preincubated in complete medium supplemented with 5% FCS, at 37° C for 24 h. Serial dilutions of Kombuchas were added to all wells (20 μL/well), except control, to achieve the required final concentrations (1.95-1000 μg/mL). Microplates were then incubated at 37° C for an additional 48 h.

Cell growth was evaluated by the colorimetric SRB assay according to Skehan et al. [15]. Cells were fixed (50% trichloroacetic acid [TCA], 50 μL/well, 1 h, +4° C), washed 4 times with distilled water (Wellwash

4, Labsystems, Helsinki, Finland) and stained (0.4% SRB, $C_{27}H_{29}N_2O_7S_2Na$, 100 μL/well, 30 min, at room temperature). The plates were then washed 4 times with 1% acetic acid to remove unbound dye. Protein-bound dye was extracted with 10 mM TRIS base (200 μL/well). Absorbance (A) was measured on a microplate reader (Multiscan Ascent, Labsystems, Helsinki, Finland) at 540/620 nm. The effect on cell growth was expressed as a percent of the control, and calculated as:

(At/Ac) × 100 [%]

At - absorbance of the test sample

Ac - absorbance of the control

Chemical analyses

The pH value of the fermented liquid samples was determined by electronic pH-meter (HI 9321, Woonsocket, USA). The total acidity of the fermented beverage samples was determined by potentiometric titration with NaOH, c = 0.1 mol/L, after the removal of CO_2 [16].

Samples for determination of antimicrobial activity

1. Kombucha beverage (for black tea Kombucha total acidity was 3.55 g/L and for winter savory tea Kombucha 3.94 g/L).
2. Acetic acid solution at the same concentration as in fermented tea.
3. Unfermented tea - decoct (dry weight 5 g/L).
4. Neutralized Kombucha (prepared by neutralizing Kombucha beverage with 0.1 mol/L NaOH).

Samples were filtered through a sterile microfilter (0.22 μm) to remove cells.

Test microorganisms

Gram negative bacteria: *Pseudomonas aeruginosa* (ATCC 27853), *Proteus mirabilis* (ATCC 35659), *Escherichia coli* (ATCC 25922); Gram positive bacteria: *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 10876), *Sarcina lutea* (ATCC 9341); yeasts: *Saccharomyces cerevisiae* (112, Hefebank Weihenstephan), *Candida pseudotropicalis* (clinical isolate), *Rhodotorula* spp. (natural isolate); and moulds: *Penicillium aurantiogriseum*, *Aspergillus niger* and *Aspergillus flavus* (all natural isolates) were used as test microorganisms.

Antimicrobial activity

Antimicrobial activity was determined by the agar-well diffusion method. The strains were grown on Mueller-Hinton (bacteria) or Sabouraud Dextrose (yeasts and moulds) slants 24 h at 37° C or 25° C, respectively, and checked for purity. After incubation, the cells were washed from the surface of agar and suspended in sterile physiological solution. The number of cells in 1 ml of suspension for inoculation, measured by Mc Farland nephelometer, was 1×10^7 cfu/mL. The 1 ml of the suspensions was homogenized with 19 mL of melted (45° C) Mueller-Hinton or Sabouraud dextrose agar and poured into Petri dishes. Wells of 9 mm in diameter were made with a sterile metal tube by means of a vacuum pump. Sterile samples (100 μL) were then transferred into the wells of agar plates inoculated with test microorganisms. Plates were incubated at 37° C (bacteria) or 25° C (yeasts and moulds) for 24 h (bacteria) or 48 h (yeasts and moulds) when the diameter of halo zone was measured.

Statistical analysis

Antiproliferative activity data were expressed as mean (SD) of 3 experiments carried out in quadruplicate. Significant differences between values were determined using two-tailed Student's t- test. The significance level was 95% ($p\leq 0.05$) or 99% or ($p\leq 0.01$).

The evaluation of antimicrobial activities of the samples was carried out in 3 repetitions and results were expressed as mean (SD).

Results

Antiproliferative activity

Kombucha in black or winter savory tea effected cell growth depending on cell line, but none of them affected cell growth by 50% inhibition (Figures 1-3).

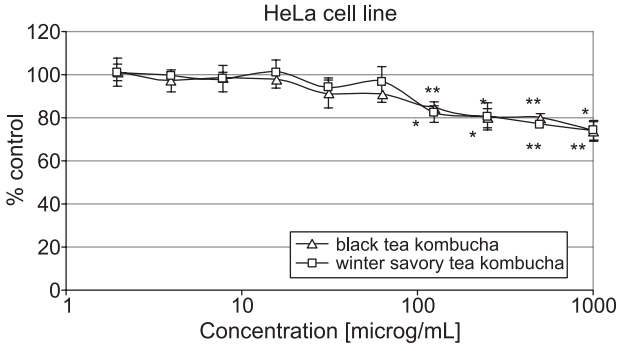

Figure 1. Antiproliferative activity of black tea and winter savory tea Kombuchas in HeLa cell line. Data are the mean ± SD of 3 experiments, performed in quadruplicate. (*$p\leq 0.05$, **$p\leq 0.01$; Student's t-test, significantly different from the control).

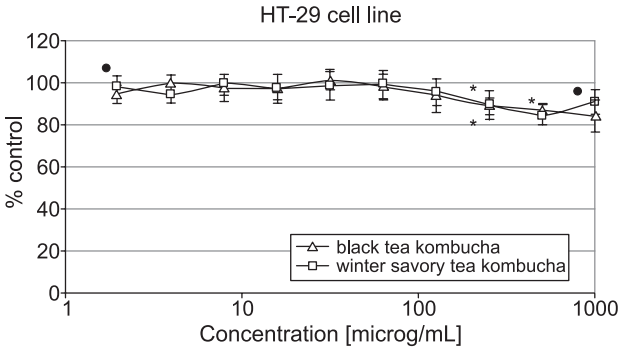

Figure 2. Antiproliferative activity of black tea and winter savory tea Kombuchas in HT-29 cell line. Data are the mean ± SD of 3 experiments, performed in quadruplicate (*p≤ 0.05; Student's t-test, significantly different from the control; •p≤0.05, Student's t-test, significantly different between Kombuchas).

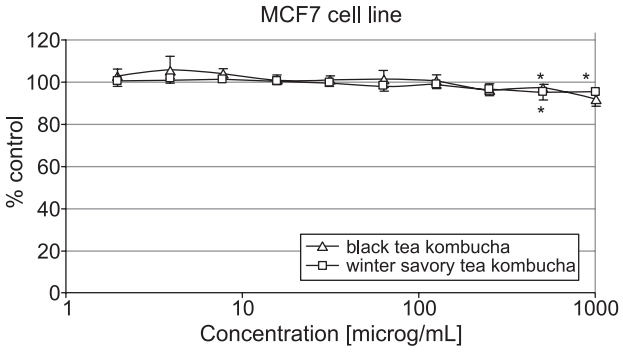

Figure 3. Antiproliferative activity of black tea and winter savory tea Kombuchas in MCF7 cell line. Data are the mean ± SD of 3 experiments, performed in quadruplicate (*p≤ 0.05; Student's t-test, significantly different from the control).

In HeLa cells IC_{20} value for both Kombuchas was achieved at concentration ≈ 250 μg/mL (Figure 1). Both Kombuchas inhibited the growth of HT-29 and MCF-7 cells by 15% and 10%, respectively, but only at their highest concentrations (Figures 2, 3).

No differences were observed in the activities of the 2 different Kombuchas within the same cell line compared to control. Differences between the Kombuchas were of statistical significance ($p \leq 0.05$) only in HT-29 cells (at 3.91 and 1000 μg/mL concentrations: black tea Kombucha showed slightly higher antiproliferative activity at those concentrations (Figure 2).

Antimicrobial activity

Results of antimicrobial activities of Kombucha beverages from black tea and *Satureja montana* L. tea are shown in Tables 1 and 2, respectively. They show that Kombucha obtained from black or *Satureja montana* L. winter savory tea and acetic acid solution had the most expressed antimicrobial activity. Kombucha and acetic acid solutions had almost the same bactericidal or bacteriostatic activity against Gram negative bacteria *Salmonella enteritidis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, and Gram positive *Staphylococcus aureus* and *Bacillus cereus* (sporogenic bacteria). None of the samples expressed antimicrobial activity against *Sarcina lutea* (Gram positive bacterium).

Kombucha and acetic acid solution showed marginal activity against *Aspergillus flavus* and *Penicillium aurantiogriseum* which are organisms with eucaryotic cell type. However, growth of other tested eucaryotes (yeasts: *Saccharomyces cerevisiae*, *Candida pseudotropicalis*, *Rhodotorula* spp. and mould *Aspergillus niger*) were not inhibited by any sample applied.

Neutralized Kombucha prepared from black tea expressed bactericidal activity only toward *Bacillus cereus* and bacteriostatic activity against *Salmonella enteritidis* and *Proteus mirabilis*. Kombucha from *Satureja montana* L. tea showed only bacteriostatic activity toward *Salmonella enteritidis*. Unfermented tea, prepared as decoct of 5 g/L dry tea (drinkable levels of tea), showed no antimicrobial properties against test organisms.

Discussion

The growth inhibition activity of *Camellia sinensis* L. and *Satureja montana* L. Kombuchas was evaluated *in vitro* in a panel of 3 histologically different human cancer cell lines: HeLa (cervix epithelioid carcinoma), MCF-7 (breast adenocarcinoma) and HT-29 (colon adenocarcinoma). Antiproliferative effects were observed at a 1.95-1000 μg/mL range of mass concentrations and were within consuming concentrations of Kombucha beverages. The results presented herein were obtained by assessing cellular viability with the SRB colorimetric assay, with SRB dye binding to protein basic amino acid residues and providing a sensitive index of cellular protein content [15,17].

No differences were observed in the activities of the 2 different Kombuchas within the same cell line. None of the Kombuchas attained cell growth by 50%, i.e. none reached IC_{50} value, but both induced inhibition of HeLa cell line with IC_{20} values at concentration of 250 μg/mL. In our previous investigation HeLa cell line was also the most susceptible to water extract of *Satureja montana* L. (IC_{20} and IC_{50} values were achieved at 400 μg/mL and 840 μg/ml, respectively)

Table 1. Antimicrobial activity of traditional Kombucha (from black tea) (diameter of halo $zone_{mean}$ (SD) [mm])

Microorganism	*Kombucha TA=3.55 g/L*		*Acetic acid c=3.55 g/L*		*Neutralized Kombucha*	
	A	*B*	*A*	*B*	*A*	*B*
Salmonella enteritidis	12.33 (0.6)	29.0 (1.73)	13.0 (0.58)	ND	ND	24.67 (0.6)
Escherichia coli	13.67 (0.6)	ND	13.0 (0.50)	ND	ND	ND
Proteus mirabilis	ND	15.67 (0.6)	ND	17.33 (0.6)	±	20.0 (0.00)
Pseudomonas aeruginosa	12.0 (0.0)	ND	12.0 (0.0)	ND	ND	ND
Staphylococcus aureus	12.33 (0.6)	ND	ND	14.0 (0.00)	ND	ND
Bacillus cereus	9.33 (1.53)	9.33 (0.71)	10.33 (1.5)	10.67 (0.6)	9.33 (1.35)	ND
Penicillium aurantiogriseum	±	ND	±	ND	ND	ND

A: microbiocidal activity, B: microbiostatic activity, ND: not detected, ±: boundary of antimicrobial activity (without growth inside and on brim of wells, zone about 9 mm), TA: titratable acidity, c: concentration, SD: standard deviation

Table 2. Antimicrobial activity of winter savory tea Kombucha (diameter of halo $zone_{mean}$ (SD) [mm])

Microorganism	*Kombucha TA=3.94 g/L*		*Acetic acid C=3.94 g/L*		*Neutralized Kombucha*
	A	*B*	*A*	*B*	*B*
Salmonella enteritidis	13.33 (0.6)	30.0 (1.53)	14.0 (0.58)	ND	21.33 (0.58)
Escherichia coli	14.0 (0.0)	ND	14.67 (0.58)	ND	ND
Proteus mirabilis	ND	18.0 (0.0)	ND	18.0 (0.0)	ND
Pseudomonas Aeruginosa	12.67 (0.6)	ND	13.33 (0.47)	ND	ND
Staphylococcus aureus	ND	15.33 (0.58)	ND	14.67 (0.6)	ND
Bacillus cereus	9.33 (0.58)	11.0 (0.0)	9.33 (0.58)	11.33 (0.6)	ND
Penicillium aurantiogriseum	±	ND	±	ND	ND
Aspergillus flavus	±	ND	±	ND	ND

For abbreviations see footnote of Table 1

[18]. This could suggest the presence of more active antiproliferative components in *Satureja montana* L. Kombucha compared to *Satureja montana* L. water extract. Water extracts of *Satureja montana* L. exhibited antiproliferative effect to HeLa and HT-29 cells at higher concentrations, but at lower concentrations they induced cell proliferation. MCF-7 cells responded by growth stimulation to water extracts at whole concentration range [18], probably due to the presence of phytoestrogens or growth-stimulating factors. It is known that genistein, a natural isoflavone phytoestrogen, stimulates the growth of estrogen-dependent MCF-7 cells at low concentrations [19,20]. However, the examined Kombuchas did not stimulate cell proliferation.

The lack of differences in the antiproliferative activity of 2 Kombuchas may suggest that the active component is acetic acid, but the different response of histologically diverse cell lines may be due to in-

trinsic, protective properties of the examined cells. Some authors suggest that cellular mechanisms which control RON gene expression may be dysfunctional in colon and breast carcinoma cells [21], leading to their elevated protection against the examined Kombuchas. RON is a member of the MET proto-oncogene family and is highly expressed in HT-29 cell line but not in normal colon epithelial cells [22], and many epithelial tumor cells [23]. Interestingly, a high expression pattern of RON is also observed in human primary breast carcinoma tissues and cell lines [24].

Kombucha beverages obtained from *Camellia sinensis* L. (black tea) and *Satureja montana* L. (winter savory tea) and appropriate acetic acid solution had the most expressed antimicrobial activity against all investigated bacteria, except *Sarcina lutea*. There were no significant differences in antimicrobial activities of the 2 different beverages. As can be seen from results, acetic acid is the major antimicrobial agent in the tested Kombuchas. It is well known that weak organic acids, such as acetic and benzoic acid, have antimicrobial activity [25]. Undissociated part of the organic acid molecule gets into the cell and the result of dissociation is cytoplasmic acidification that inhibits cell growth. However, traditional Kombuchas showed higher activity against *Staphylococcus aureus* and *Escherichia coli* than acetic acid, while both neutralized the Kombuchas' exhibited bacteriostatic activity on *Salmonella enteritidis*. This could imply the existence of an antimicrobial component(s), other than acetic acid, responsible for the antimicrobial activity.

Sreramulu et al. [26] tested the antimicrobial activity of Kombucha from black tea (with 8.5 g/L acetic acid content) against pathogenic bacteria. They also marked acetic acid as major antimicrobial agent. Similar results were published by Steinkraus et al. [27] who investigated *in vitro* the antimicrobial activity of black tea Kombucha with 10.5 g/L acetic acid. In that paper [27] Kombucha showed much higher (3-5 times) bactericidal effects against *Escherichia coli, Staphylococcus aureus* and *Bacillus cereus* than samples tested in this study (Tables 1 and 2), probably because of high content of acetic acid.

In our study unfermented black tea and winter savory tea did not show any antimicrobial activity against test microorganisms. Antimicrobial activity was found in extracts and concentrates of black and winter savory tea by Toda et al. [28] and Pepeljnjak et al. [29]. Greenwalt et al. [8] showed that unfermented tea (dry tea concentration 4.4 g/L) did not have antimicrobial activity against test microorganisms.

Generally, yeasts and moulds are acidophilic/acidotolerant organisms and that fact can explain the resistance of the tested strains to acetic acid and Kombucha beverages. In the work of Greenwalt et al. [8] *Candida albicans,* a common human pathogen, was not inhibited by any test solutions (Kombucha, unfermented tea and neutralized beverage) except the tested commercial vinegar (50 g/L acetic acid). Because of that, a potential danger from contamination with moulds and yeasts from air exists in the case of growing Kombucha at home.

The examined Kombucha beverages did not stimulate cell proliferation of the investigated cancer cell lines. The antiproliferative activity of the winter savory tea Kombucha was comparable to that of the traditional Kombucha made from black tea. Furthermore, in HeLa cell line *Satureja montana* L. Kombucha induced IC_{20} at lower concentrations compared to the activity of water extract of *Satureja montana* L. obtained in our previous research [18].

We speculate that more active antiproliferative component(s) in *Satureja montana* L. Kombucha compared to *Satureja montana* L. water extract and antimicrobial component(s) other than acetic acid in both Kombuchas may be present.

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