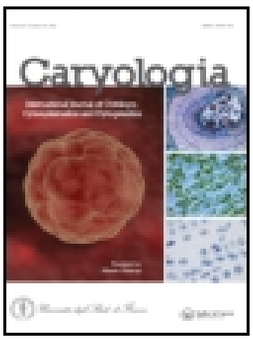

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Mitotic activity of a new phytotherapeutic product with a trade name of "Ganomix"

Mieczysław Kuraś[a], Krzysztof Gulewicz[b], Olga Bemowska-Kałabun[a] and Małgorzata Wierzbicka[a]

[a]Faculty of Biology, University of Warsaw, Warsaw, Poland; [b]Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznan, Poland

ABSTRACT

Water extracts of *Uncaria tomentosa* (Vilcacora) bark and *Ganoderma lucidum* (Reishi) spores that are rich in biologically active compounds, are increasingly widely used in prevention and treatment of many serious medical conditions. They are considered an important treatment option, complementary to phytotherapeutics and synthetic medicines. A new formulation with a trade name of Ganomix was developed based on these two components. The aim of this study was to determine mitotic activity of Ganomix and to compare the activity of Ganomix with the activity of its individual components. The biological activity was assessed using the following plant tests: *Allium* test (Levan test) and Phytotoxkit biotest. In addition, the chemical composition of selected components was analyzed. Water extracts of Vilcacora bark and Reishi spores, as well as their combination in Ganomix, exhibited varying biological activities. The Vilcacora bark extracts had inhibitory effects at the great majority of concentrations tested, while Reishi spore extracts were mainly stimulatory at the same range of concentrations. However, Ganomix extract showed an intermediate effect compared with Vilcacora bark and Reishi spore extracts. The promising results of these investigations give hope for the effective use of Ganomix in prevention and therapy of different sicknesses.

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KEYWORDS
Ganomix; *Uncaria tomentosa*; *Ganoderma lucidum*; mitotic activity

1. Introduction

Close association between conventional medicine and traditional medicine based on medicinal plants may be justified in order to achieve the best available results in the prevention and therapy of civilization diseases (Taraphdar et al. 2001; Surh 2003; Cordain et al. 2005; Beliveau and Gingras 2006). Of the herbal remedies used widely in Poland, *Uncaria tomentosa* (Willd. ex Schult.) DC. (Vilcacora), *Ganoderma lucidum* (Reishi), and Ganomix, which is a mixture of the two compounds (1:1 ratio) produced by Centrum Ziołolecznictwa Wilcaccora, are most important. Ganomix has a Good Manufacturing Practice (GMP) certificate issued by the Chief Sanitary Inspectorate in Poland.

Vilcacora (*Uncaria tomentosa* (Willd. ex Schult.) DC.) is one of the most popular Peruvian medicinal plants, and products obtained from its bark, leafs or roots have been used in traditional medicine for thousands of years. The broad-spectrum effects of Vilcacora are explained by the presence of indole and oxindole alkaloids that can be divided into two groups: pentacyclic oxindole alkaloids (POAs) and tetracyclic oxindole alkaloids (TOAs). POAs have been proven to be responsible for nonspecific and cellular immune response. In addition, they demonstrated cytostatic activity against neoplastic cells. TOAs lower blood cholesterol levels and reduce platelet aggregation, and have an effect on the central and peripheral nervous systems. Moreover, Vilcacora contains a range of other compounds, such as polyphenols, sterols, quinic acid glycosides, triterpenes and their derivatives, that work synergistically with alkaloids. These compounds have anti-inflammatory and antiviral properties, protect blood vessels and remove reactive oxygen species which are known to induce DNA damage in cells (Sheng et al. 1998; Wurm et al. 1998; Keplinger et al. 1999; Falkiewicz and Łukasiak 2001; Sandoval et al. 2002; Kuraś et al. 2009).

Reishi mushroom (*Ganoderma lucidum*) is an important source of nutrients and therapeutic agents. Spores, which are rich in proteins, amino acids, polypeptides, polysaccharides, terpenes, vitamins and organic germanium, constitute the most pharmacologically active components of Reishi mushroom. Reishi contains more than 100 antioxidants. Polysaccharides and triterpenes are pharmacologically important, as they increase interferon production (thus increasing immunity), destroy free radicals, promote antigen-producing B-cell generation, increase the number of macrophages that help white blood cells fight infections, as well as enhance synthesis of DNA, RNA and proteins in the liver, bone marrow and blood. Lanostane-type triterpenes, the so-called ganoderic acids, decrease blood cholesterol levels, and

CONTACT Mieczysław Kuraś kuras@biol.uw.edu.pl

reduce blood vessel constriction through the increase in angiotensin production. They also have analgesic and detoxification activities, decrease blood viscosity, protect the liver and kill cancer cells. These multiple ingredients of Reishi help to control the whole body's function. Moreover, Reishi mushroom relieves harmful effects of chemotherapy and radiation therapy (Wu et al. 2001; Bao et al. 2002; Chen et al. 2002; Gao et al. 2003; Zhang et al. 2003; Boh et al. 2007; Kao et al. 2014).

Vilcacora and Reishi mushroom products are increasingly widely used in prevention and treatment of many diseases, including various malignancies, and are considered important adjunctive treatments to synthetic drug therapy. They are both components of dietary supplements. Ganomix is a mixture of Vilcacora bark and water extracts of Reishi mushroom spores, and is produced and marketed exclusively in Poland.

The aim of this study was to determine the biological activity of Ganomix and to compare the mitotic activity of Ganomix with the activity of its individual components.

2. Materials and methods

2.1. The origin of the biological material

Vilcacora bark is provided by the Instituto Peruano de Investigacion Fitoterapica Andina DROGUERIA IMPIFA (Lima, Peru), and Reishi mushroom spores are provided by the Fujian Xianzhilou Nutra-Industry Co. Ltd. (Fuzhou, China). Ganomix is a combination of these components and is produced by the Centrum Ziołolecznictwa „*Wilcaccora*" in Łomianki near Warsaw (Poland).

2.2. Chemical analysis

Qualitative and quantitative analyses of alkaloids contained in Vilcacora bark and water extract of Vilcacora bark were performed using methodology described by Sturm and Stuppner (1992) and Pilarski et al. (2010). The profile of polyphenolic compounds in this component has been reported by Bors et al. (2011). The water extract of *Uncaria tomentosa* was obtained in the following manner: 10 g of ground bark was extracted with 50 ml of distilled water (6 h, 400°C). Next, the sample was centrifuged at 4500 rpm and the extract decanted.

Qualitative and quantitative analyses of Reishi extract involved total protein, fat, fatty acids, sugars, alkaloids, macro- and microelements. The Kjeldahl method, the Bertrand method and the method described by Daniewski et al. (2002) were used to determine the content of total protein, carbohydrates and fat/fatty acids, respectively. Macro- and microelement contents of samples were also determined: three independent weighted portions of the sample (approximately 0.1 g) were mineralized with concentrated nitric acid using the Mars 5 Microwave System (CEM Corporation, USA) (mineralization parameters: Stage 1; maximum wattage: 1200 W; power: 50%; ramp time: 25 minutes; pressure control: 160 PSI; maximum temperature: (210˚C); hold time: 25 min). Solutions were filled up to 10 ml after mineralization. Then concentrations of individual elements were measured using the ICP-OES VISTA-MPX (Varian, USA) optical emission spectrometer and ICP-MS (Varian, USA) mass spectrometer.

2.3. Ecotoxicological analyses

Mitotic and biological activities of Vilcacora, Reishi mushroom and Ganomix water extracts were analyzed using the *Allium* test (Levan 1938; Wierzbicka 1987) and Phytotoxkit biotest (Phytotoxkit 2016 Standard operational procedure). Three experimental variants were used: water extract of ground Vilcacora bark, water extract of Reishi mushroom spores and mixture of Vilcacora and Reishi extracts (Ganomix). The extracts of Vilcacora, Reishi and Ganomix were prepared in the following manner: 2 g of preparation was placed in an Erlenmeyer flask and shaken with 100 ml distilled water for 6 h in the thermostat at 37°C. After this time the samples were centrifuged at 8000 rpm for 20 min in a centrifuge (Heraeus Biofuge Primo R, Thermo Scientific). Supernatants were separated and transferred into three properly labeled beakers and filled up with distilled water to 500 ml. The initial solutions (100%) were used to obtain further dilutions (*Allium* test: 50, 25, 12.5, 6.25, and 3.125%; Phytotoxkit test: 50, 25 and 12.5%).

2.3.1. Allium test

The effects of three test extracts on mitotic activity namely quantity of dividing cells (mitotic and phase indices) were examined. Mitotic and phase indices were calculated using the Lopez-Saez and Fernandez-Gomez method (1965). Material evaluated in the *Allium* test was fixed after 6, 24, 48 and 72 h. Five onions were used in each variant; 2-mm long fragments of *A. cepa* root tips were cut off after the above-specified incubation periods. Then, the root tips were macerated and stained with acetic orcein (2% orcein in 45% acetic acid) at room temperature for 24 h. After this time, mashed specimens of *Allium cepa* root tips were prepared (of one root tip each). The specimens were examined under light microscope using a "bright field" illumination technique. Thirty cells were counted under a microscope.

2.3.2. Phytotoxkit biotest

Lepidium sativum L. was used as a sensitive bioindicator in this test. The Phytotoxkit test was performed using test plates lined with cellulose wadding soaked in 30 ml of test substance or distilled water (control). Ten seeds were put in one row on a filter paper and placed on each plate. Covered test plates stacked vertically on racks were then incubated in darkness at 23°C for four days.

Table 1. The average percentage content of alkaloids in Vilcacora bark and Vilcacora bark water extract.

Alkaloids	Bark (%)	Bark water extract (%)
Uncarin F + Speciophyllin	10.11	15.99
Mitraphyllin	21.77	24.97
Rhynchophyllin	0.56	0.27
Unidentified	13.85	13.89
Unidentified	15.43	14.13
Isomitraphyllin	8.02	8.54
Pteropodin	20.71	17.08
Isopteropodin	9.55	5.13
Total	100.00	100.00
% content per dry mass of the bark	0.75	1.19

The test plates were scanned with a computer scanner every 24 h. The length of plant roots was measured using Image Tool software (Image Tool for Windows, version 3.00, Copyright 1995-2002, The University of Texas Health Science Center, San Antonio). The root tolerance index (RTI) was calculated based on the results of measurements [(A – B)/A]·100, where: A = length of roots grown on the control soil; B = length of roots grown on the test soil (Phytotoxkit 2016). The test procedure was repeated four times. Statistical analyses were performed using STATISTICA software (StatSoft, Inc. 2014. STATISTICA, version 12. www.statsoft.com). The Kruskal–Wallis non-parametric test was used to compare multiple independent samples. The significance α level was 0.05.

3. Results

3.1. Analysis of chemical composition

The Vilcacora bark used in Ganomix contained 0.75% alkaloids (Table 1). The proportion of alkaloids contained in the bark was highest for mitraphyllin (21.77%) and pteropodin (20.71%), and lowest for rhynchophyllin (0.56%). Two unidentified alkaloids were also detected which represent 29.28% of all alkaloids in the bark. The water extract of Vilcacora bark contains the highest proportion of the alkaloids mitraphyllin (24.97%), pteropodin (17.08%), and uncarin F with speciophyllin (15.99%). The extract contained 28.02% unidentified alkaloids. The content of alkaloids in dry extract was 1.19% (Table 1). Bors et al. (2011) found considerable amounts of polyphenolic compounds such as procyanidin B2, (–)-epicatechin, procyanidin C1, caffeic acid derivatives, chlorogenic acid, quercetin derivatives, kaempferol derivatives and proanthocyanidin polymers, in the water extract of Vilcacora bark. These compounds constituted about 10.5% of the dry extract.

It was found that Reishi spores used in Ganomix contain significant amounts of protein, fat, macro- and microelements (Table 2). The contents of protein and free peptides, fats, alkaloids and sugars in Reishi spores were 17.5, 25.42, 1.37, and 1.87%, respectively. Furthermore, the spores contained fatty acids, including oleinic acid, linoleic acid and palmitic acid. Germanium was also detected in the spores (Table 2). In addition to these, Reishi contains a wide variety of bioactive molecules, such as terpenoids, steroids, phenols, nucleotides and their derivatives, glycoproteins, and polysaccharides. Reishi mushroom proteins contain all the essential amino acids and are especially rich in lysine and leucine. The low content of fat and high proportion of polyunsaturated fatty acids to total fatty acids are considered significant contributors to the health value of these mushrooms (Chang and Buswell 1996; Borchers et al. 1999; Sanodiya et al. 2009).

3.2. Analysis of chemical composition

3.2.1. Allium test

An elevated mitotic index (MI) value (i.e. the ratio of the number of cells actively dividing to the total number of cells) in the *Allium* test indicates increased mitotic activity, while decreased or very low MI indicates partial or total inhibition of biological activity. Regular mitotic cell divisions characteristic of normal cells in the *Allium* test (MI about 10%) were observed in control cells. Water extracts of Vilcacora bark (at all concentrations tested) demonstrated concentration-proportional inhibitory effect during the 0–24 h incubation period. The inhibitory activity of water extracts was greatest at concentrations of 100% and 50%, and weakest at a concentration of 3.125%. Test results suggest that Vilcacora bark extracts inhibit cell divisions at nearly all concentrations tested (Figure 1a). However, Reishi spore extracts had a stimulatory effect on mitotic cell activity. After 72 h of incubation with water extracts, the MI was higher compared to the control value at five concentrations tested, and lower just by 50% only at the highest concentration (100%). The effects of Reishi extracts were exactly opposite to those of Vilcacora extracts at the same concentrations (Figure 1b). Ganomix extracts exhibited almost intermediate effect compared to the two others (Figure 1c). The results suggest that inhibiting effects of Vilcacora and stimulatory effects of Reishi on cell division are complementary to each other, so the effect of Ganomix would be intermediate. Various concentrations of extracts offer a wide range of possibilities as they can be matched according to immunomodulatory needs. Therefore, different therapeutic effects could be achieved with different combinations of Ganomix components.

Changes in the mitotic phase index (a percentage of cells in particular mitotic phases) were observed together with changes in MI during incubation with water extracts (Figures 1d, e, f). Significant increase in the percentage of prophase cells with increased proportion of "cc" forms was clearly evident after a 6-h incubation with Vilcacora extracts. This process still progressed after 24 h of incubation, but was visibly inhibited at 48 and 72 h time points (Figure 1d). Significant increase in the prophase index (up to 85%) with corresponding decrease in the number of cells in other phases of

Table 2. Chemical content analysis of Reishi mushroom (spore) extract.

Moisture (%)								7.8
Protein (including free amino acids: lysine, histidine, arginine, proline, leucine, valine, asparagine, tyrosine) and free peptides (%)								17.5
Fat (%)								25.42
Fatty acids: percent of total fat content (%)								
Hexadecanoic (palmitic) acid		Stearic acid		Oleic acid		Linoleic acid		Linolenic acid
15.256		3.131		63.613		15.919		2.081
Eicosenoic acid		Erucid acid		Palmitoleic acid		Behenic (docosanoic) acid		
0.000		0.000		0.000		0.000		
Alkaloids (% content per dry mass)								1.37
Sugars (%)								1.87
Macro- and microelements (mg kg^{-1})								
Al	Ca	Cr	Cu	Fe	Li	Mg	Mn	Na	Ni
130	680	0.50	15	110	<0.05	225	3.0	30	0.15
Sc	Sr	Ti	Zn	Cd	Co	Pb	Se	V	Ge
0.04	4.0	7.5	19	0.20	0.06	0.10	0.04	0.30	0.013
Macro- and microelements (%)								
K	P	S						
0.13	0.16	0.10						

mitotic divisions was also observed after 6 h of incubation with Reishi spore extracts. Changes were also found in numerous chromosomes (cc); however, their number was smaller compared to cells treated with Vilcacora extracts after the same time (Figures 1d, e). Test results demonstrated significant differences in the effect on cell activity between Vilcacora and Reishi extracts, with notably increased activity of cells treated with Reishi extracts. These results suggest stimulatory effects of the extracts. When cells were incubated with Ganomix extracts, the Phase MI values were intermediate relative to the previous two: the prophase index was decreased while metaphase and anaphase indexes, as well as the number of changed "cc" nuclei, were increased (Figures 1d, e, f). However, the prophase index increased again after 24, 48, and 72 hours of incubation. This index reflects stimulation of metabolic activity that could also be translated into stimulation of an immune response (immunostimulation).

3.2.2. Phytotoxkit biotest

The growth rate of *L. sativum* roots treated with Vilcacora extracts was reduced compared to the control during the entire test period. During the first three days, statistical significant differences in the root growth rate compared to control were observed for the highest concentrations of Vilcacora bark extracts (Figure 2a). The RTI was reduced compared to the control for all concentrations of Vilcacora extracts during the entire incubation period, indicating their inhibitory effects. The inhibitory effects of Vilcacora extracts were directly proportional to concentration (Figure 2d).

The growth rate of *L. sativum* roots incubated with Reishi spore extracts was increased compared to control on the first day of incubation, and decreased for the remaining time. Statistically significant differences in growth rates of roots treated with the highest concentrations of Reishi extracts compared to the control were observed between the second and fourth day of the test (Figure 2b). The RTI for Reishi extracts was increased (indicating stimulatory effects) compared to the control on the first day of the test and reduced (indicating inhibitory effects) for the remaining time. The effects of Reishi extracts were directly proportional to concentration (Figure 2e).

The growth rate of *L. sativum* roots treated with Ganomix extracts was reduced compared to the control during the entire test period. Statistical significant differences in the root growth rate compared to the control were observed for the highest concentrations of Ganomix extracts during the entire test period (Figure 2c). The RTI was reduced compared to the control for all concentrations of Ganomix extracts during the entire incubation period, indicating their inhibitory effects. Initially, the effects of Ganomix extracts were stronger than those of Vilcacora and Reishi extracts, but tended to diminish and became intermediate later on (Figures 2d, e, f).

4. Discussion

As shown above, *Uncaria tomentosa* and *Ganoderma lucidum* preparations are rich source of biological active compounds and nutrients. The following question arises: does a mixture of both have influence on biological activity and nutrition value? Combination of Vilcacora and Reishi extracts in one formulation resulted in accumulation of chemical compounds shared by both components with the addition of compounds contained in individual components of the mixture, and may lead to significant enhancement of their health effects. In the present study we focused attention on the effect of such a combination on the mitotic activity of a new preparation (Ganomix).

The choice of the method, supported on plant tests for mitotic activity studies, was not accidental. The cognitive

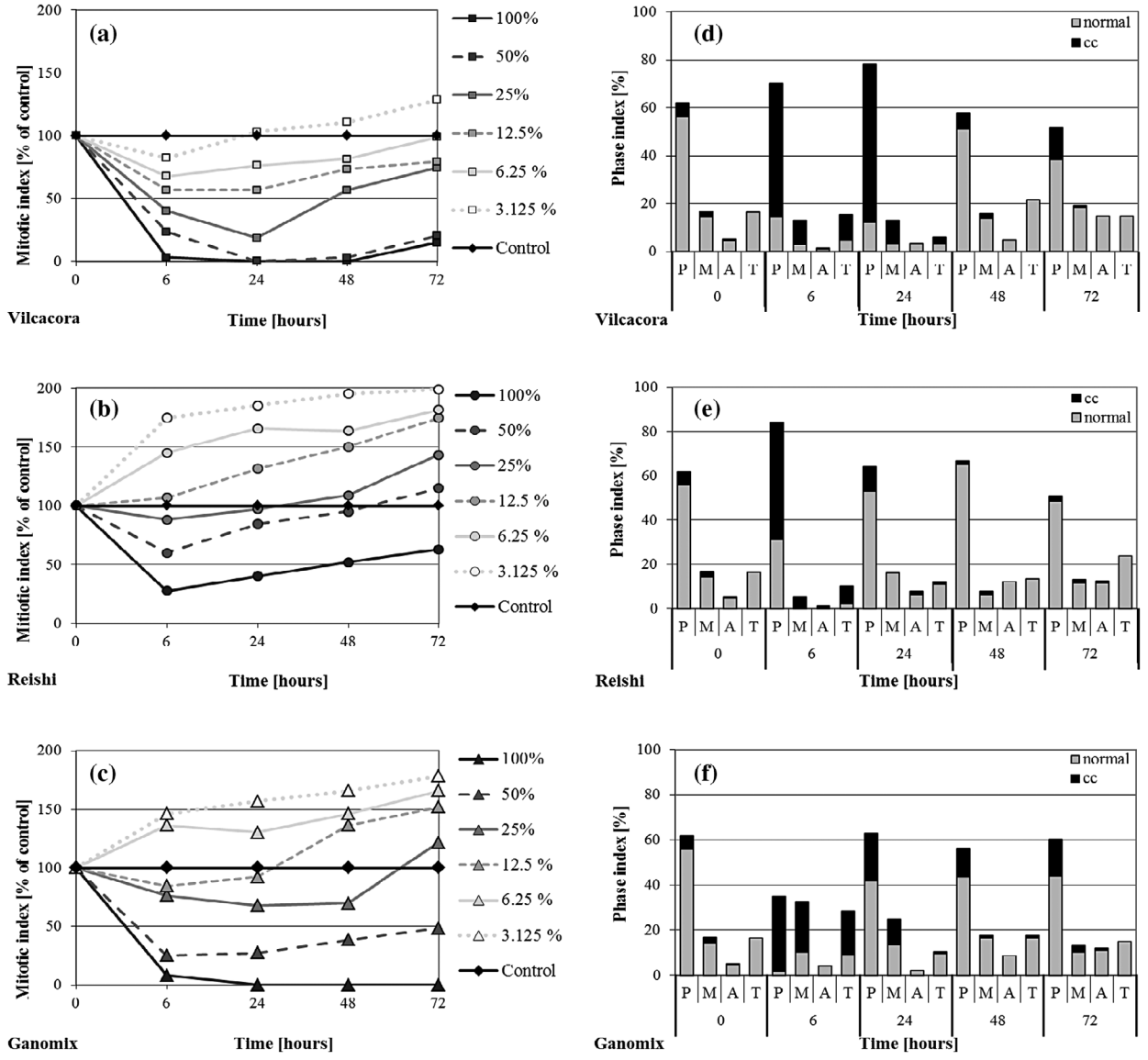

Figure 1. (a, b, c) Mitotic index of *Allium cepa* root tip cells in the *Allium* test; (d, e, f) phase index of *Allium cepa* root tip cells in the *Allium* test. The test was performed on subsequent concentrations of: (a, d) Vilcacora extract; (b, e) Reishi extract; (c, f) Ganomix, and distilled water as a control solution. Legend: P: prophase; M: metaphase; A: anaphase; T: telophase; normal: phases of normal mitotic cycle; cc: phases of abnormal mitotic cycle.

value of plant tests for determining mitotic activity is unquestionable, especially at early stages of research. They are used in studies on the effects of drugs and other chemicals on the human body, despite evident phylogenetic and physiological differences between plant and animal cells. Numerous comparative studies have been conducted using plant, animal and human tests to evaluate the activity of different compounds, and their results are largely consistent (Nilan 1978; Grant 1994; Kuraś et al. 2006,;2009; Abratowska et al. 2014; Wierzbicka et al. 2015). The *Allium* test (Levan 1938) is based on the observation of chromosome divisions in root meristematic cells of *Allium cepa* L. This test is used to investigate the effects of various factors on mitotic activity, life cycle, aberration induction, structure and ultrastructure of cells, cellular respiration and many other living processes (Kuraś et al. 2006, 2009) including, but not limited to, identification of cells exhibiting neoplastic transformation. This test is a very good bioassay that is comparable with many tests performed on *in vitro* animal/cancer cell cultures (Grant 1994). To verify the results obtained in the *Allium* test, the Phytotoxkit microbiotest was also performed. The Phytotoxkit biotest measures the growth rate of plant roots exposed to test substance for several days. Growth rates are then compared with those of control plants. The assessment of root growth inhibition is useful to determine the activity of chemical compounds (ISO 11269-1 1993; Phytotoxkit 2016; Wierzbicka et al. 2015). The Phytotoxkit biotest confirmed results obtained from the *Allium* test, despite the fact that they measured biological activity in different ways.

Studies have shown that the mitotic activity of investigated extracts was varied. The effects of Vilcacora bark extracts were inhibitory at the great majority of

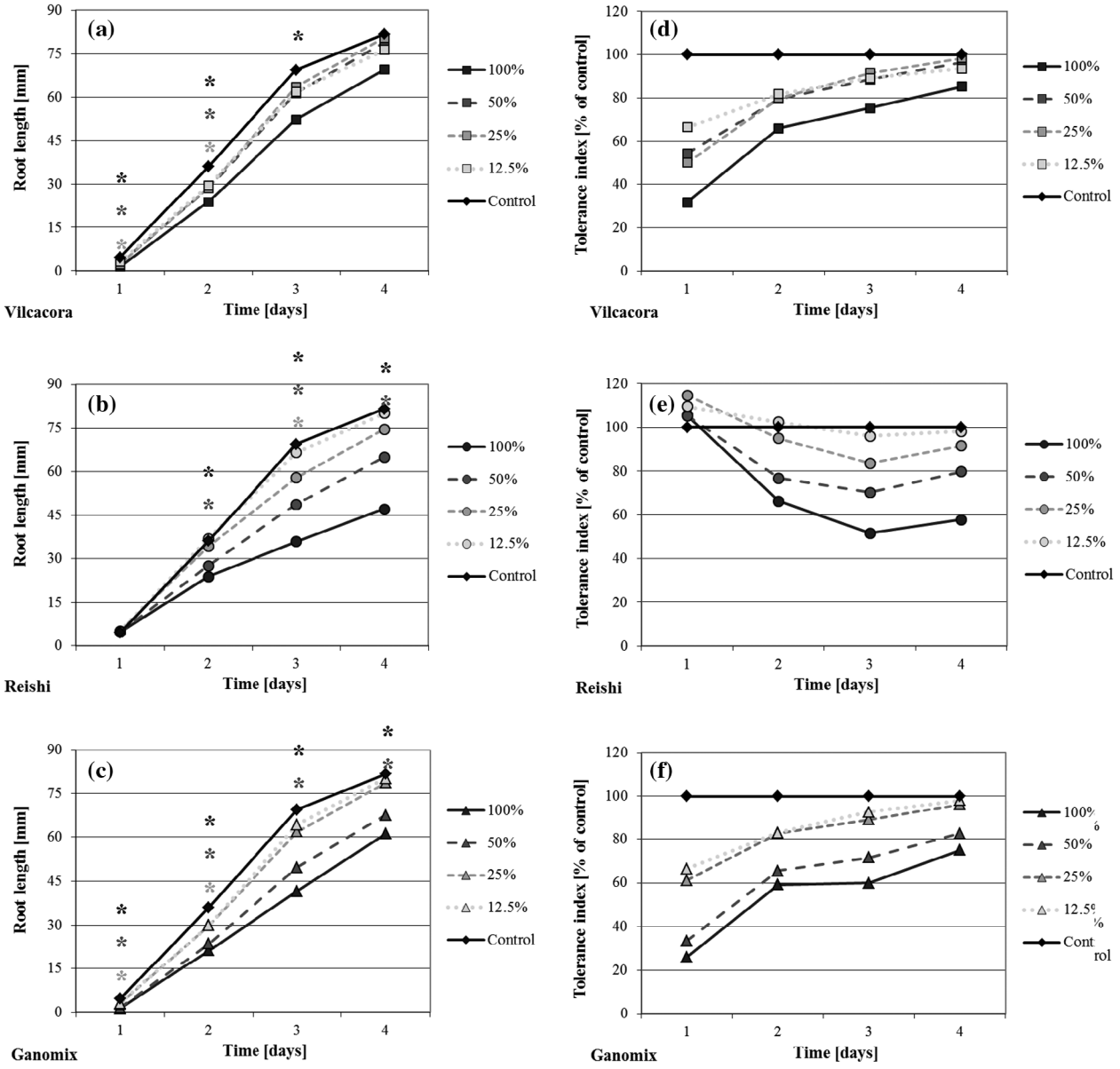

Figure 2. (a, b, c) The root growth rate of *L. sativum*; (d, e, f) the root tolerance index (RTI) for *L. sativum*. The test was performed on subsequent concentrations of: (a, d) Vilcacora extract; (b, e) Reishi extract; (c, f) Ganomix, and distilled water as a control solution. Legend: * indicates statistically significant differences in root growth rates (a, b and c) compared to control plants; colors indicate the results obtained with different concentrations. Statistical analyses were performed using the STATISTICA software. The Kruskal–Wallis non-parametric test was used to compare multiple independent samples. The significance α level was 0.05.

concentrations tested, while those of Reishi spore extracts were mainly stimulatory at the same range of concentrations. Ganomix extract showed an intermediate effect compared with Vilcacora bark and Reishi spore extracts. The impact of Reishi and Vilcacora on mitotic and other biological activity of cells was also shown by other researchers (Chen et al. 2002; Gao et al. 2003; Kuraś et al. 2006, 2009).

Another study (Kuraś et al. 2006) showed that *Uncaria tomentosa* extracts affect the organization of chromatin, which may be related to a disturbed balance of the quantity of histones or other proteins responsible for controlling the proper structure of nuclear chromatin. The strongly shortened and thickened chromosomes were observed in prophases and metaphases (cc divisions) (Kuraś et al. 2006). Kuraś et al. (2009) in alkaloid-rich and alkaloid-free fractions (different in chemical composition) obtained from *U. tomentosa* bark observed condensation and contraction of chromosomes (more in alkaloid-rich) with retardation and/or inhibition of mitoses and changed mitotic phases. The results described in these works (Kuraś et al. 2006, 2009) overlap with those obtained in our work for water extracts of Vilcacora bark.

Chen et al. (2002) showed that a mixture of *Ganoderma lucidum* and its spores (MLGLGS) has inhibitory action on tumor cells at high concentration and high dosage. Also, preclinical studies have established that the *G. lucidum* polysaccharide (GLPS) fractions have potent antitumor activity, which has been associated with the

immunostimulating effects of GLPS (Gao et al. 2003). Gao et al. (2003) showed that polysaccharides fractions extracted from *G. lucidum* (called Ganopoly®) enhanced the immune responses in patients with advanced-stage cancer. The *Allium* test in our research showed that Reishi spore extracts had a stimulatory effect on mitotic cell activity.

These studies indicate that the combination of Reishi and Vilcacora in preparation Ganomix allows for the averaged effect on the mitotic activity of *A. cepa* cells.

Based on the results of tests and reports on the chemical composition and health benefits of Vilcacora and Reishi extracts, combination of the two products in Ganomix would enhance the individual health properties of each component. The above-mentioned effects of Vilcacora and Reishi mushroom products are closely related to their chemical composition. Each product contains a wide variety of ingredients, and some of them are shared by both components of Ganomix. Combination of Vilcacora and Reishi extracts in one formulation resulted in accumulation of chemical compounds shared by both components with the addition of compounds contained in individual components of the mixture, and may lead to significant enhancement of their health effects. Apart from many alkaloids and triterpenes, the new Ganomix formulation contains a group of polysaccharide and triglyceride compounds which increase the extent of biological activity of the product.

5. Conclusions

The effects of Vilcacora bark extracts containing significant amounts of alkaloids were inhibitory (at the great majority of concentrations tested), and the effects of Reishi spore extracts were stimulatory (at the same range of concentrations). However, the Ganomix extract showed an intermediate effect compared with Vilcacora bark and Reishi spore extracts. These results give hope for the effective use of Ganomix in medicine.

Disclosure statement

No potential conflict of interest was reported by the authors.

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