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ORIGINAL RESEARCH PAPER

Metabolic activity of tree saps of different origin towards cultured human cells in the light of grade correspondence analysis and multiple regression modeling

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Abstract
Tree saps are nourishing biological media commonly used for beverage and syrup production. Although the nutritional aspect of tree saps is widely acknowledged, the exact relationship between the sap composition, origin, and effect on the metabolic rate of human cells is still elusive. Thus, we collected saps from seven different tree species and conducted composition-activity analysis. Saps from trees of Betulaceae, but not from Salicaceae, Sapindaceae, nor Juglandaceae families, were increasing the metabolic rate of HepG2 cells, as measured using tetrazolium-based assay. Content of glucose, fructose, sucrose, chlorides, nitrates, sulphates, fumarates, malates, and succinates in sap samples varied across different tree species. Grade correspondence analysis clustered trees based on the saps' chemical footprint indicating its usability in chemotaxonomy. Multiple regression modeling showed that glucose and fumarate present in saps from silver birch (*Betula pendula* Roth.), black alder (*Alnus glutinosa* Gaertn.), and European hornbeam (*Carpinus betulus* L.) are positively affecting the metabolic activity of HepG2 cells.

Keywords
tree saps; hepatocytes; data mining; natural beverages

Introduction

Collection of tree saps has been practiced for centuries across all continents of the Northern Hemisphere. More than 33 species have been exploited as a source of sap for nutritional, medicinal, and cosmetic applications, with the birches (*Betula* sp.) and maples (*Acer* sp.) being the most commonly used [1,2]. Ethnobotanical reports indicate that alder (*Alnus glutinosa*), hornbeam (*Carpinus betulus*), willow (*Salix alba*), and walnut (*Juglans regia*) were also tapped in various regions of Europe and North America [1–3].

Practitioners of traditional medicine were employing raw tree saps in the treatment of pneumonia, the managements of scurvy, and to facilitate the healing of skin lesions [2,4]. Numerous studies report that tree saps and sap-based syrups of various origin were used in folk medicine for the treatment of liver-related disorders and to generally improve health. In Denmark, birch sap was recommended against hepatitis [5]. Birch sap tapped in Russia was used to dissolve gallstones [6]. Revitalizing application of tree saps was common in Poland, Lithuania, and Romania [2]. Fresh sap obtained from maples and birches were occasionally allowed to ferment into an alcoholic beverage [1]. Current uses of tree saps involve almost exclusively beverage and syrup production from different species of birches and maples.

Tree saps are complex biological matrices [7,8]. Initial studies on the chemical composition of the saps were focused on the quantification of the components affecting syrup manufacturing process, i.e., sugars and free amino acids [9–11]. More recently, efforts were made to quantify the components of tree saps that mediate health-beneficial properties including minerals, vitamins, and phenolic compounds [7,12,13] and to verify tree saps applications known from folk medicine [14–18]. Majority of this studies were focused only on the assessment of the sap content of birches and maples, tree species producing saps the most commonly utilized in food industry. Thus, there is a limited number of reports discussing variabilities in sap composition across wide range of tree species.

To address the importance of the nutritional and energetic properties of the saps in vitro, we tapped trees of seven different species and investigated whether supplementation of standard culture medium with raw tree saps affects the metabolic activity of human HepG2 liver cells. In parallel, to analyze the intra- and interspecies variability in sap composition we quantified the concentration of main sap constituents using HPLC. Then, the grade correspondence analysis (GCA) was used to visualize and study the relationships between different sap constituents across investigated species. This approach enabled the clusterization of tree species producing saps of similar composition and identification of the saps components displaying similar pattern of prevalence. Multiple regression model formulated to describe the effects of saps components on the HepG2 metabolism revealed that glucose and fumaric acid are the key components of the saps that drive the activity of the saps in HepG2 cells.

Material and methods

Chemicals

Standards of carbohydrates (fructose, glucose, and sucrose) and organic acids (fumaric, malic, and succinic) were supplied by Sigma Aldrich (USA). Mixture of anions (fluorides, chlorides, nitrates, nitrites, sulphates, bromides, and phosphates) used as a reference was from Thermo Scientific (USA). Acetonitrile gradient grade, ammonium acetate, and ethanol were from Merck (Germany). Water was deionized and purified by Ultrapure Millipore Direct-Q 3UV-R (Merck).

Trees

The total number of 31 trees of seven different species were enrolled into this study, including silver birch (BP, *Betula pendula* Roth.; $n = 4$), black alder (AG, *Alnus glutinosa* Gaertn.; $n = 5$), European hornbeam (CB, *Carpinus betulus* L.; $n = 4$), white willow (SA, *Salix alba* L.; $n = 4$), boxelder (AN, *Acer negundo* L.; $n = 5$), Norway maple (AP, *Acer platanoides* L.; $n = 5$), and black walnut (JN, *Juglans nigra* L.; $n = 4$). The trees were located at the Institute of Applied Biotechnology and Basic Sciences of the University of Rzeszów (Werynia, Subcarpathian Province, Poland, 50°15′22″ N, 21°48′29″ E; Fig. 1).

Fig. 1 Approximate location of the trees selected for tapping. The map represents the area surrounding the Institute of Applied Biotechnology and Basic Sciences, University of Rzeszów (Werynia, Subcarpathian Province, Poland).

Saps collection

Sap samples were collected between February 26 and March 16, 2014. Tapping was carried out by employing a protocol based on previously published approach targeted to minimize the risk of microbial contamination [19]. In brief, a piece of bark was removed from a tree with a sterile chisel on the south side of a trunk, 50 cm above the ground level. The exposed spot was treated with 70% ethanol for 30 s. Subsequently, holes (10 mm diameter, 4–6 cm deep, 30° angle) were drilled using hand drill. The sap was allowed to flow freely for 5 min to clean the cut from any remaining wood shavings. Samples were collected into 15-mL conical tubes. The wound was sealed with Koro-Derma pruning ointment (Bros, Poland). The saps were stored at −20°C until further analysis.

Cell culture

Human hepatocellular carcinoma HepG2 cells (ATCC: HB-8065) were maintained in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin, and 0.1 mg/mL streptomycin (all from Life Technologies, USA). The cells were cultured in humidified atmosphere of 95% air and 5% CO_2 at 37°C.

MTS metabolic assay

The HepG2 cells were seeded out in a 96-well plate at 1 × 104 cells per well and allowed to attach overnight. Then, the cells were treated with 22 µm filtered tree saps (5%, 20%, or 50%) or vehicle (H_2O) in serum-free media (100 µL/well of total volume). After 72 h of incubation the CellTiter MTS reagent (Promega, Germany) was added (20 µL/well). Three hours later the absorbance was recorded at 490 nm using ELx800 plate reader (BioTek Instruments, USA).

Quantification of saccharides

HPLC Varian system controlled by Varian Workstation software version 6.9.1 with evaporative light scattering detector Varian Evaporative Light Scattering Detector was used. Chromatographic separation was performed on Cosmosil Sugar-D 4.6 × 250 mm column (Nacalai Tesque, Inc., Japan) with use mixture of acetonitrile and water (80:20, v/v) at 1 mL/min flow rate. The temperature of thermostat and autosampler was 35°C and 4°C, respectively. Detector parameters were as follow: flow rate of gas of 1.2 dm^3/min, nebulizer and evaporator temperature of 80°C.

Quantification of inorganic anions

Dionex ICS 1000 ion chromatograph with conductometric detector, controlled by Chromeleon version 6.8 software was used. Chromatographic separation was carried out on IonPack AS 14A analytical column (Thermo Scientific) at flow rate of mobile phase 1 mL/min and at 30°C.

Quantification of organic anions

VWR Hitachi Chromaster 600 chromatograph (Merck) with Diode Array Detector and EZChrom Elite software was used. Separation was performed at 25°C, on ZIC-HILIC 4.6 × 150 mm column (Merck) using acetonitrile and water solution of ammonium acetate at concentration 40 mM (77:23, v/v) as a mobile phase; the flow rate was 1 mL/min. Quantification was performed at 210 nm. The samples were evaporated to dryness and dissolved in mobile phase.

Statistical analysis

Evaluation of the differences in cellular metabolic activity elicited by culture media supplementation with tree saps collected from different species versus vehicle-treated cells was carried out by one-way analysis of variance (ANOVA) and Dunnett's test using GraphPad Prism v5.03 (GraphPad Software, USA). Comparison between the levels of sap components in different tree species was carried out using one-way ANOVA followed by Tukey's test with correction for unequal sample sizes using Statistica v. 12.0 (StatSoft, USA). Plots were generated using GraphPad Prism. Probability $p < 0.05$ was considered significant throughout the study.

The grade correspondence analysis (GCA) was performed with GradeStat 3.0.1 software (Institute of Computer Science, Polish Academy of Sciences, Poland). GradeStat was also used to generate Spearman correlation matrix.

Multiple regression model [20,21] was created using SAS statistical software system package v 9.4 (SAS, USA). First, variables (saps components) characterized by high Spearman correlation ($|r| > 0.8$) were excluded from the further analysis to avoid multicollinearity. Next, the best subset of predictors (saps components) was selected based on the Bayesian information criterion (BIC), the adjusted R^2, and the Akaike information criterion (AIC). Obtained multiple regression model was then validated based on Shapiro–Wilk normality test and heteroskedasticity test of White.

Results

Tree saps modulate the metabolic activity of cultured cells

To assess the capability of tree saps to affect cellular metabolic activity the human HepG2 liver cell model was employed. At concentration as low as 5% the saps from BP and AG were able to slightly, but significantly, stimulate the metabolism of HepG2 cells (Fig. 2a). At higher dose (20%) the saps obtained from BP, AG and CB boosted the

metabolic rate of HepG2 cell more than two-fold compared to control cells (Fig. 2b). However, when the cells were treated with media containing 50% of the saps significant cytotoxic effect was observed for AN and JN saps whereas saps from BP, AG, CB, SA and AP displayed no significant effect (Fig. 2c).

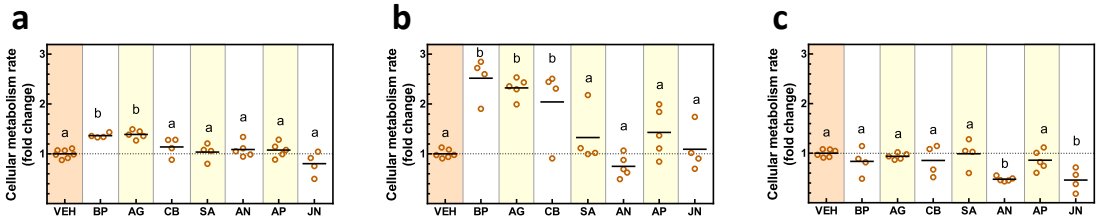

Fig. 2 Effects of tree saps on metabolic activity of HepG2 cells. HepG2 cells were treated with vehicle (H_2O) or tree saps for 72 h. Saps were used at the concentration of 5% (**a**), 20% (**b**), and 50% (**c**) in serum-free medium. MTS assay was performed and metabolism rates relative to vehicle-treated cells were plotted. The effect of the saps on HepG2 metabolism rate vs. control was statistically assessed using one-way ANOVA followed by Dunnett's post hoc test. Different letters indicate significant differences at $p < 0.05$. VEH – vehicle; BP – *Betula pendula* Roth.; AG – *Alnus glutinosa* Gaertn.; CB – *Carpinus betulus* L.; SA – *Salix alba* L.; AN – *Acer negundo* L.; AP – *Acer platanoides* L.; JN – *Juglans nigra* L.

Content of saccharides, organic and inorganic anions varies between saps of different origin

Metabolic rate of sap-treated HepG2 cells varied greatly not only across tested species, but also between individual trees within a species (Fig. 1). Since tree saps are complex biological matrices abundant in components that can differently modulate the metabolism of cultured cells [16,22], we decided to investigate the composition of the collected saps in order to identify key components that were responsible for the observed differences in HepG2 metabolism rate.

All investigated members of Betulaceae produced saps containing varying levels of glucose and fructose with no detectable amounts of sucrose (Fig. 3). Oppositely, saps from SA and JN as well as AN and AP were positive for sucrose (concentrations ranging from 10 to 80 mM). No fructose nor glucose were detected in saps collected from SA, JN, AN, and AP (apart from single sample of JN origin).

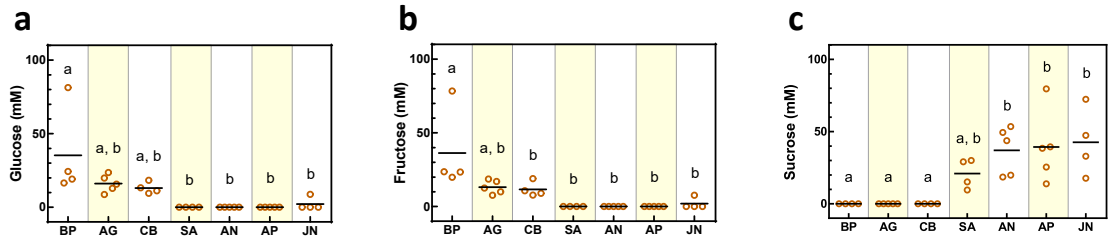

Fig. 3 Concentration of (**a**) glucose, (**b**) fructose, and (**c**) sucrose was measured in tree saps obtained from *Betula pendula* Roth. (BP), *Alnus glutinosa* Gaertn. (AG), *Carpinus betulus* L. (CB), *Salix alba* L. (SA), *Acer negundo* L. (AN), *Acer platanoides* L . (AP), and *Juglans nigra* L. (JN). Differences in mean sap content of glucose, fructose, and sucrose between different tree species were statistically evaluated using one-way ANOVA and Tukey's post hoc test. Different letters indicate significant differences at $p < 0.05$.

Chlorides were detected in almost all investigated samples (29 of 31) with the highest levels in the saps of CB and AP origin and with the lowest levels in BP and SA saps (Fig. 4a). Elevated concentrations of nitrates were present in selected sap samples from AN and AP. However, no significant differences were observed between mean nitrates concentrations across all investigated species (Fig. 4b). Sulphates concentration in SA saps was significantly higher than in saps obtained from all other species (Fig. 4c).

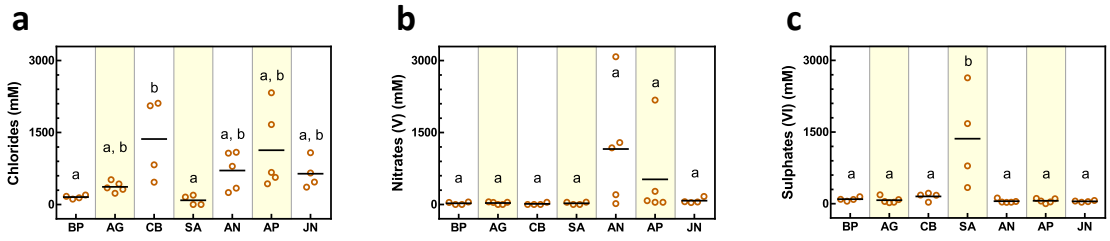

Fig. 4 Differences in the concentration of selected inorganic anions across saps collected from seven tree species. Content of (**a**) chlorides, (**b**) nitrates, and (**c**) sulphates was assessed in tree saps obtained from *Betula pendula* Roth. (BP), *Alnus glutinosa* Gaertn. (AG), *Carpinus betulus* L. (CB), *Salix alba* L. (SA), *Acer negundo* L. (AN), *Acer platanoides* L . (AP), and *Juglans nigra* L. (JN). One-way ANOVA followed by Tukey's post hoc test was used to statistically evaluate the differences in the content of chlorides, nitrates, and sulphates across saps obtained from the investigated tree species. Different letters indicate significant differences at $p < 0.05$.

Fumaric acid was not detected in AN saps nor in most of SA saps (Fig. 5a). Saps isolated from CB and AG were characterized by moderate mean fumaric acid concentrations (from 1 to 3 μM), while saps from BP, AP, and JN contained, on average, more than 4 μM of this organic acid (Fig. 5a). Malic acid was detected in the saps from all investigated species, apart from all AN and two SA trees (Fig. 5b). The highest malic acid content was observed in BP (10.0 ±1.6 μM). Succinic acid was present in the saps from selected CB and SA individuals and was undetected in the saps of BP, AG, AN, AP, and JN origin (Fig. 5c).

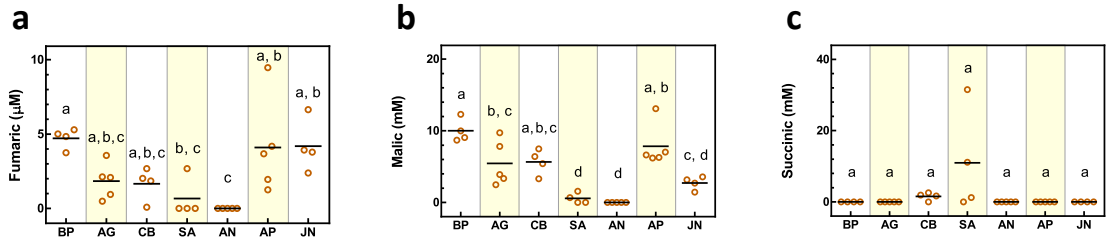

Fig. 5 Concentration of fumaric, malic and succinic acids in tree saps. Concentration of (a) fumaric acid, (b) malic acid, and (c) succinic acid was measured in tree saps obtained from *Betula pendula* Roth. (BP), *Alnus glutinosa* Gaertn. (AG), *Carpinus betulus* L. (CB), *Salix alba* L. (SA), *Acer negundo* L. (AN), *Acer platanoides* L . (AP), and *Juglans nigra* L. (JN). Statistical evaluation of interspecies differences in the content of fumaric, malic, and succinic was carried out using one-way ANOVA and Tukey's post hoc test. Different letters indicate significant differences at $p < 0.05$.

In order to get more insight into the interspecies variability in sap composition and to identify the tree species that produce the most similar saps, we utilized GCA approach. The overrepresentation map (Fig. 6a) clustered together the trees producing the most similar saps in regard to their chemical content. SA trees were at the top of the map. AN, AP, and JN were tethered together at the center of the map, whereas CB, AG, and BP were localized at the bottom of the map. Component-wise succinic acid was overrepresented in the same set of sap samples as sulphates. Similarly, nitrates, sucrose, and chlorides were often present together. The last set of components that were similarly represented in the investigated saps was consisted of fumaric acid, malic acid, glucose, and fructose. In conclusion, succinic acid and sulphates were overrepresented in the saps from SA trees (upper left corner of the map); nitrates, sucrose, and chlorides were overrepresented in the saps from AN, AP, and JN; fumaric acid, malic acid, glucose, and fructose were overrepresented in the saps from CB, AG, and BP. Noteworthy, trees from the same taxonomic family were clustered together, i.e., their saps were the most similar to each other. Similar picture of saps composition was delivered by Spearman's rank correlation map (Fig. 6b).

Glucose content correlated positively with fructose levels ($r = 0.99$) in all investigated samples, i.e., the higher glucose content, the higher fructose levels. Conversely, sucrose concentration was negatively correlated with glucose content in respective tree sap

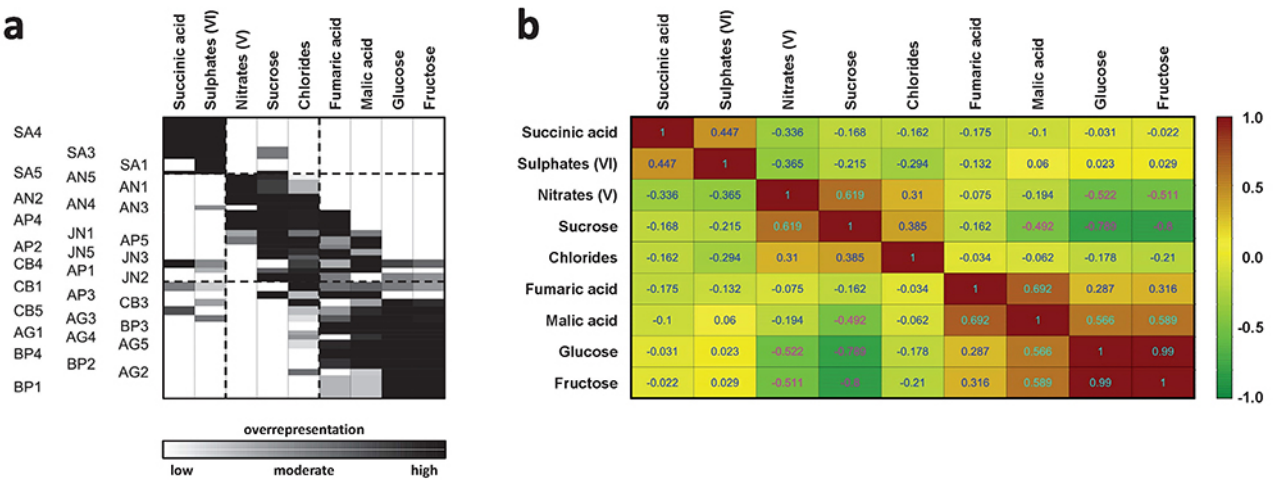

Fig. 6 Global differences in the content of tree saps obtained from *Betula pendula* Roth. (BP), *Alnus glutinosa* Gaertn. (AG), *Carpinus betulus* L. (CB), *Salix alba* L. (SA), *Acer negundo* L. (AN), *Acer platanoides* L . (AP), and *Juglans nigra* L. (JN). **a** Grade correspondence analysis (GCA) was conducted and visualized as an overrepresentation map. The components that are highly overrepresented in the saps obtained from given trees are clustered together and marked in dark gray or black. The most underrepresented tree sap components in a given subset of trees are in white, and are also located next to each other on the map. Dotted lines separate the clusters of the most similar sets of individual trees and respective saps components. **b** Spearman's rank correlation coefficient matrix was created to identify saps components that vary together across investigated saps. The redder a particular field is, the more positive is the correlation between respective components. Greenfields designate negative correlation.

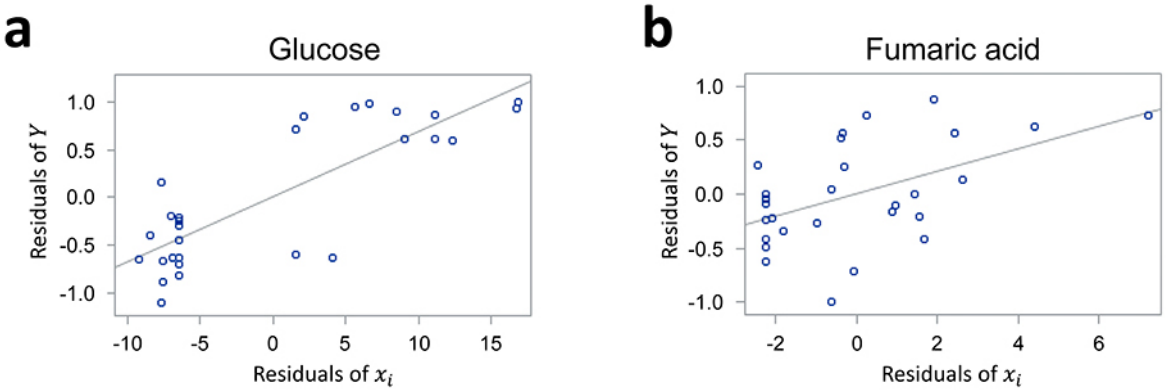

Fig. 7 Glucose and fumaric acid as key sap components driving the metabolism boost of HepG2 cells. Partial regression plot between metabolic rate of HepG2 cells versus glucose (**a**) and fumaric acid (**b**). Partial regression plot is a plot where: on x axis we have residuals of x_i (chosen predictor; glucose or fumaric acid content) against all remaining predictors and on y axis we have residuals of Y (meatabolic rate of HepG2 cells) regressed on all predictors with x_i omitted. The idea of this plot is that we look for influence of x_i on Y when linear influence of all other variables has been accounted for.

samples ($r = 0.789$). In general, saps containing more monosaccharides were lower in sucrose and vice versa.

Glucose and fumaric acid drive the metabolic activity of tree saps

Due to qualitative and quantitative differences in saps' composition we decided to construct a mathematical model that could decipher the effects of individual components of the tested saps on the metabolism of HepG2 cells. Created multiple regression model revealed that glucose ($p < 0.0001$) and fumaric acid ($p < 0.006$) were the key sap components that were positively affecting the metabolic activity of HepG2 cells (Fig. 7). This model was able to explain about 70% ($R^2 = 0.7206$, adjusted $R^2 = 0.6982$) of the observed variability in HepG2 metabolic rate elicited by tested saps, meaning that the remaining 30% of the effect depended on the components that were not assessed in this study.

Discussion

We decided to employ the HepG2 cell line to study the effects of raw tree saps on the metabolic rate of cultured cells. HepG2 cells produce liver-specific proteins and maintain a variety of metabolic functions characteristic for liver cells, thus making them a good model for in vitro metabolic studies [23–25]. MTS assay based on the reduction of tetrazolium salt by cytoplasmic and mitochondrial dehydrogenase enzymes was utilized to monitor the metabolic activity of the cultured cells [26].

In our metabolic assay we observed that saps from BP, AG, and CB were the strongest inducers of HepG2 metabolism, with approximately two-fold increase over the control cells when used at 20% concentration. This boost, however, was strongly suppressed when the saps were used at higher doses (50%). Moreover, significant reduction in metabolic rate was observed in cells treated with 50% sap from AN and JN, indicating possible cytotoxic effects of highly-concentrated saps. Observed drop in the metabolic rate of cultured HepG2 cells at high doses of tree saps may also be explained by the dilution of culture media and consequent restriction on growth factors and nutrients.

Chemical composition analysis of the tree saps delivered the information on the inter- and intraspecies variability in saccharides as well as inorganic and organic anions. For instance, tree species could be divided into the group producing saps rich in sucrose (SA, AN, AP, and JN) and the group producing saps containing glucose and fructose (BP, AG, and CB). In significance, all investigated trees were growing in the same park area. Moreover, the trees belonging to the same species were located no more than 15 m apart. Despite these facts, concentrations of some key components varied up to over 100-fold between the saps collected from different individuals of the same species. This clearly illustrates that even that major differences in the content of key saps components can be observed not only between species, but also between individual trees of the same species. Thus, we hypothesized that intra- and interspecies variability in the sap composition may affect the biological activity of the saps.

To visualize the variability in saps composition, we generated the overrepresentation map based on GCA analysis [27]. The main concept of GCA is to order the variables/objects (saps components/individual trees) in such a way that neighboring variables (saps components) are more similar than those further apart. Concomitantly, neighboring objects (individual trees) are also more similar than those further apart. Obtained overrepresentation map showed which variable–object pairs (component–tree pairs) are over- or under-represented. This approach enabled us to clearly demonstrate that intraspecies variability in tree sap composition was lower than interspecies variability as trees of the same species were grouped together across the map. Moreover, the observed pattern stays in line with the taxonomical classification of the tree species investigated in this study, as the species belonging to the same taxonomical family were always clustered together on the map. This indicates that the GCA could be used in chemotaxonomic studies.

In order to determine which tree saps components have a major effect on the observed changes in the HepG2 metabolic activity we utilized a multiple regression modeling. Multiple regression does, however, assume that dose–effect relationships are described by linear function and cannot predict the effect of unknown factors. Nevertheless, with this simple mathematical tool we were able to identify key sap components affecting the metabolic activity of HepG2 cells, namely glucose and fumaric acid. Glucose and fructose, primary sources of carbon in cell culture, directly affect the regulation of multiple metabolic pathways. Our in vitro MTS assay demonstrated that tree saps rich in these monosaccharaides facilitated the metabolism of HepG2 cells, whereas sucrose-contacting saps were deprived of this activity. Interestingly, glucose alone, but not sucrose, is able to induce growth of HepG2 cells [28]. Since glucose levels were highly correlated with fructose content across all studied saps, one may expect that fructose also positively affects the growth of HepG2 cells. In fact, it has been previously demonstrated that HepG2 cells are able to utilize fructose as energy source [28].

Fumarate is an intermediate in the tricarboxylic acid cycle (the Krebs cycle). It is produced during the oxidation of succinate, reaction catalyzed by succinate dehydrogenase. This reaction generates 1 mole of $FADH_2$, an equivalent of 1.5 mole of ATP, from 1 mole of fumarate. It has been previously demonstrated that, apart from energy generation, fumarate protects liver cells from cytotoxicity, enhances DNA synthesis, and

stimulates the proliferation of hepatocytes [29]. Accordingly, our study demonstrates that the concentration of fumarate in tree saps correlates positively with the metabolic activity of cultured human HepG2 cells of liver origin.

Recent in vivo study in rats indicated that maple syrup may improve liver function through downregulation of genes involved in ammonia formation [30]. Yoo and colleagues demonstrated that sap obtained from *Acer pictum* Thunb. (*Acer okamotoanum*) boosts alcohol metabolism in rats and protects against ethanol-induced apoptosis in hepatocytes [31]. In this study, we showed that raw tree saps from *Betula pendula* Roth., *Alnus glutinosa* Gaertn., and *Carpinus betulus* L. can stimulate metabolic activity in HepG2 model of human hepatocytes in vitro. These observations support the revitalizing and detoxifying properties of tree saps known from folk medicine.

In conclusion, we identified key tree sap components that modulate the metabolism of cultured HepG2 cells in vitro, namely glucose and fumaric acid. Consequently, saps from silver birch, black alder, and European hornbeam that are rich in glucose and fumarate are the most nutritional for cultured HepG2 cells. Furthermore, we showed that GCA enables the identification of phytochemicals displaying similar pattern of prevalence across different species. Therefore, GCA is potentially suitable data mining tool for studying the interspecies variability, especially in the context of chemotaxonomy. Finally, our data corroborate the traditional opinion of nourishing and liver-protecting properties of tree saps on a cellular level.

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