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Beyond the Traditional Applications of Raspberry *(Rubus idaeus)* Leaf: An *in vitro*, *in vivo* and *in silico* Study

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Abstract: The consumption of raspberry (*Rubus idaeus L.*) leaves has a long tradition, especially as a "general pregnancy tea", although the scientific data are insufficient and contradictory. Phytochemical comparation of extracts from cultivated and wild raspberry leaves, *in silico* prediction of their biological activities and acute toxicity followed by *in vitro* antiradical activity and effects on the viability/proliferation of HeLa cells and isolated rat uterus were performed. Leaves from cultured (v. Polka) and wild individuals were extracted with distilled water, 70% v/v ethanol or 70% v/v methanol. All samples exhibited high polyphenol content and antiradical activity, with the 70% v/v ethanol extract of wild *R. idaeus* showing the strongest free radical scavenging ability. *In silico* analyzes predicted that raspberry leaves possess numerous compounds with anti-inflammatory, apoptosis-agonistic, antinociceptive and NO signaling-related activities. Potentially toxic levels of the tested compounds could not be achieved with regular tea drinking. The tested extracts have no noticeable effects on the viability/proliferation of HeLa cells. The effects on spontaneous contraction of the isolated rat uterus were modest. Although safety is not a concern, further studies are needed to justify or deny the efficacy of raspberry leaf tea in folk medicine for healthy pregnancy and easy delivery.

Keywords: *R. idaeus* L.; leaves extracts; phytochemical comparation; *in silico* prediction; HeLa cell viability/proliferation, uterus contraction.

1. Introduction

The genus *Rubus*, one of the largest in the Rosaceae family, comprises more than 700 species divided into 12 subgenera, where only a few of which are cultivated. Modern raspberry cultivars are derived from the European red raspberry (*Rubus idaeus* L.), North American red raspberry (*Rubus strigosus* Michx.), the black raspberry (*Rubus occidentalis* L.) and the purple raspberries (*Rubus neglectus* Peck) that are hybrids between red and black raspberries [1]. The known number of

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raspberry varieties is consistently increasing, with several hundred varieties under cultivation. The wild *R. idaeus* thrives throughout the Balkans at elevations above 1000 m above sea level, primarily on the borders of beech forests, but it can also be found in vineyards, large orchards, hedges, pastures, abandoned meadows, and other poorly managed environments [2].

The biological potential of raspberry fruit and its benefits to human health have been examined extensively due to the widespread use of this fruit in human diet [1]. Also, the medicinal applicability of raspberry leaves was recognized long ago and has been used in traditional medicine of different cultures for the treatment of numerous disorders. In the past centuries, red raspberry leaves have been most consumed as a "general pregnancy tea" and uterotonic. Today's pregnant women's knowledge of the use of raspberry leaf during pregnancy appears to be steadily increasing; country-specific awareness varies between 7% and 56% [3]. The Traditional Balkan and Southeast European medicine list the leaves of both wild and cultivated raspberry as having potential benefits for reducing labor pains, preventing miscarriage, and relieving morning sickness during pregnancy [4]. The possible benefits of raspberry leaves tea also include inducing labor, preventing late pregnancy, reducing discomfort in the pre-labor phase and improving cervical ripening. In addition to facilitate the course of pregnancy and childbirth, raspberry leaves are known for their various other pharmacological properties, including diuretic, antidiabetic, and anti-diarrheal effects, and also for treatment of respiratory and circulatory diseases [5-8]. The Committee on Herbal Medicinal Products at the European Medicines Agency (HMPC/EMA) recommends the use of *Rubus idaeus folium* in the form of tea or dry water extract for symptomatic relief of minor spasm associated with menstrual periods, symptomatic treatment of mild inflammation in the mouth or throat and mild diarrhoea [9]. Modern applications of raspberry leaves include its use as an additive to drinks, nutritional supplements, preparations of functional herbal teas, and chocolate, in order to enhance their nutritive and flavour-forming properties [10].

Having in mind that plant extracts consist of large number of pharmacologically active substances, it is very demanding to examine in detail the pharmacological activity of each of them and consequently to gain insight into the potential areas of their application. Consequently, new methods are needed to uncover the biological activities of natural products components, including their interactions with established biological targets and their related pharmacotherapeutic effects. One of the relatively new approaches for pharmacological screening of plant compounds is use of *in silico* prediction methods. The computer program PASS (Prediction of Biological Activity for Substances) is one of the software developed for prediction of biological activity spectrum [11], while the computer program GUSAR was developed for prediction of acute toxicity potential [12] of chemical compounds. These programs provide theoretical evaluation of compound's overall biological potential, as well as acute toxicity potential which can serve as a basis for further researches.

Although the consumption of both wild growing and cultivated raspberry leaves has a long tradition, the available research data are still insufficient and sometimes contradictory, particularly regarding the difference between phytochemical characteristics of cultivated and wild growing raspberry, their potential pharmacological activities, mechanisms of action and possible side effects [13-16]. Thus, the aim of the present study was to conduct the phytochemical comparation of cultivated and wild growing *R. idaeus* leaves extracts and using *in silico* predictions to get a potential insight into their biological activities and acute toxicity. Following these steps, an *in vitro* radical scavenging activity and the effect on cell viability and proliferation will be studied. Finally, the effect of cultivated and wild *R. idaeus* leaf extracts will be examined on an isolated rat uterus model.

2. Materials and Methods

2.1. Preparation of Plant Extract

The developed leaves of cultivated (v. Polka) and wild individuals of the plant species *Rubus idaeus* were collected near the coast of the Vlasina Lake (42°44’19.0” N 22°19’24.0” E) in July of 2017.The localities from which the wild and cultivated raspberries were harvested were at approximately the same altitude, sun exposure, about 300 meters apart, and the cultivated raspberries were not additionally irrigated. The plant material was dried in a dark, ventilated place, and then

crushed to the consistency of coarse powder (*Pulvis grossus*) before extraction. Wild growing (MM) and cultivated (GM) raspberry leaf extracts (drug/solvent ratio was 1/10) were obtained by maceration [17] with distilled water (MM H_2O, GM H_2O), 70% v/v ethanol (MM 70EtOH, GM 70EtOH) or 70% v/v methanol (MM 70MOH, GM 70MOH), respectively. All prepared extracts were evaporated under reduced pressure (using a rotary vacuum evaporator), at a temperature of up to 45˚C, until dryness. Dry extracts (after determining the yields) were transferred and stored in well-closed glass containers in a refrigerator at 4 ˚C until analysis.

2.2. Phytochemical Analysis

2.2.1. Determination of Total Phenolic Content

The total phenolic content of the wild and cultivated *R. idaeus* leaves extracts were determined using the Folin-Ciocalteu method [18]. The absorbance was measured spectrophotometrically at 725 nm after standing test tubes for 40 minutes to develop a blue color, comparing it to a blank that contained the extraction solvent instead of the sample. The total phenolic content of the investigated extracts was calculated using a catechin calibration curve (range 1-5 µg/mL) and expressed as mg catechin equivalents (CE) per gram of extract.

2.2.2. Determination of Total Tannin Content

The total tannin content of the wild and cultivated *R. idaeus* leaves extracts were determined using the same Folin-Ciocalteu method [18]. After adding polyvinylpolypyrrolidone, all phenolic chemicals are present in the supernatant, except tannins. The tannin content of investigated extracts are also expressed as mg of catechin equivalents (CE) per gram of extract, and obtained from the difference between total phenolic content and non-tannic polyphenols.

2.2.3. Determination of Total Flavonoid Content

The total flavonoid content of the wild and cultivated *R. idaeus* leaves extracts were estimated according to Lamaison and Carnat (1990) [19]. The absorbance was measured spectrophotometrically at 430 nm after standing test tubes for 10 minutes to develop a yellow color from chelates of flavonoids and $AlCl_3$. The total flavonoid content of investigated extracts were calculated and expressed as mg rutin equivalents (Ru) per gram of extract using a rutin calibration curve (range 1-5 µg/mL).

2.3. In silico Analysis

The PASS software, developed for prediction of biological activity of chemical compounds, algorithm operates on the concept of the "biological activity spectrum," which is an intrinsic characteristic of a compound that indicates its range of biological activities resulting from interactions with different biological entities. This spectrum offers a theoretical evaluation of the compound's overall biological potential which can serve as a basis for further research. PASS online 9.1 software (http://www.way2drug.com/PASSOnline/index.php) accessed through Way2Drug predictive service was used to perform *in silico* analysis of the officially raspberry leaves compounds listed in Assessment report on *Rubus idaeus* L., folium [9]. The obtained data included Pa (potential activity) and Pi (potential inactivity) values, with the criteria of Pa>Pi. A Pa value greater than 0.7 indicates high potential biological activity of the compound, value between 0.5 and 0.7 indicates medium bioactivity, while a value less than 0.5 indicates relatively low bioactivity [20]. GUSAR software (https://www.way2drug.com/GUSAR/acutoxpredict.html) was accessed through the same service, and was used for prediction of acute oral toxicity potential of raspberry leaves compounds according to calculated LD_{50}.values. As input both programs used MOL files of the raspberry leaves' compounds. Obtained results of *in silico* prediction of pharmacological activity and acute rat toxicity were used as a basis for further analyses in this work.

2.4. Determination of Antiradical Activity

The antiradical activity of the wild and cultivated *R. idaeus* leaves extracts were determined using the DPPH (1,1-diphenyl-2 picrylhydrazyl) free radical scavenging assay [21,22]. The absorbance was measured on an ELISA microplate reader at 540 nm after 30 minutes in the dark. The percentage of DPPH free radical inhibition was calculated using the formula:

$$\% \text{ DPPH} = (Ac - As) / Ac \times 100$$

where Ac is the absorbance of the control, and As is the absorbance of the sample. Results are expressed as IC_{50} value, extract concentration capable of neutralizing 50% of free DPPH radicals, in µg/ml.

2.5. Determination of Cell Viability and Proliferation

Determination of cell viability and proliferation was performed in the Laboratories of Scientific Research Center for Biomedicine, Faculty of Medicine, University of Niš, Niš, Serbia. HeLa S3 cells (human cervical adenocarcinoma cell line) cells were grown in DMEM (Dulbecco's Modified Eagle's Minimal Essential Medium, Gibco™, Thermo Fisher Scientific, USA) supplemented with L-glutamine, penicillin-streptomycin and 10% fetal bovine serum. All experiments with cells were performed in a vertical sterile chamber (Klimaoprema d.o.o., Croatia). Viability and proliferation of HeLa cells was estimated after exposure to the wild and cultivated *R. idaeus* leaves extracts [23-25]. Dry extracts were dissolved in supplemented DMEM and the following test concentrations were prepared: 2mg/mL, 0.2 mg/mL, 0.02 mg/mL and 0.002 mg/mL. All extracts were sterilized by filtration through a 20 µm filter. The effective concentrations of the extracts were twice as low due to volume dilution in supplemented DMEM.

For cell viability estimation 10^5 cells in supplemented DMEM were seeded in individual wells (96-well cell culture plates). After 24h tested extracts or supplemented DMEM (control) was added to the cells. The cells were incubated for further 24h in humified atmosphere, with 5% CO_2 at 37°C (Innova CO-48 New Brunswick Scientific CO2 Incubator, Artisan Technology Group, USA). For cell proliferation assay 10^4 cells in supplemented DMEM were seeded in individual wells and after 24h different concentrations of tested extracts or supplemented DMEM (control) were added to the wells. The cells were incubated for further 72h. In each experimental setting every concentration of extract was tested in triplicate.

At the end of the incubation period the incubation medium was removed, the cells were washed with buffered salina and 20 µl of MTT [3-(4,5-dimethylthiazol-2)-2,5 diphenyltetrazolium bromide] was added. After 4 hours of incubation at 37°C, the resulting formazan crystals were dissolved by the addition of isopropanol. Spectrophotometric measurement of MTT reduction was performed at an optical density of 540 nm, using a multi-channel spectrophotometer Multiscan Ascent No354, Thermo Labsystems, Finland) [26].

The obtained results were interpreted based on the viability and proliferation of HeLa cells obtained from the three repeated tests. The viability/proliferation of each group of treated cells was determined by the formula:

$$\% \text{ cell viability/proliferation} = \text{average absorbance/average absorbance of the control} \times 100$$

Cell morphology was evaluated under an inverted microscope (Observer Z1, Carl Zeiss, Germany). According to their morphology, cells were classified into adherent and non-adherent phenotypes, as well as those with and without filopodial extensions. The potentially toxic effect of the extracts on the cells was assessed through the evaluation of the change in their morphology in the presence of extracts, compared to that in the control group of cells. Cells detached from the substrate were considered dead.

2.6. Determination of the Extract Effect on Rat Uterine Contractions

2.6.1. Animals and Housing

For this study adult female Wistar rats (200-250 g) housed under standard laboratory conditions in the Vivarium of the Medical Faculty, University of Niš, were used. All experiments were conducted at the Scientific Research Center for Biomedicine and the Institute of Physiology, Faculty of Medicine, University of Niš, Niš, Serbia. According to the declaration of Helsinki and European Community guidelines for the ethical handling of laboratory animals (EU Directive of 2010; 2010/63/EU), all experimental procedures with animals were conducted and also approved by the Animal Ethics Board of the Republic of Serbia (No. 323-07-06862/2016 05/2).

2.6.2. The Uterus Isolation and Preparation

On the morning of the experiment, the animals were sacrificed after overnight fasting. The uterus strips were washed with Krebs solutions and then suspended in a tissue bath maintained at 37°C, containing Krebs solutions. Tissue was tied to the bottom of the bath, while the other end of the isolated uterus preparation was attached to an isotonic force transducer (TSZ-04-E, Experimetria Ltd., Budapest, Hungary). For recording and analyzing uterus responses, a SPEL Advanced ISOSYS Data Acquisition System was used (Experimetria Ltd.). Before the beginning of the experiment, the uterus preparations were suspended under 1 g of pressure and acclimatized for 45 minutes.

2.6.3. Effects of the Wild and Cultivated R. idaeus Leaves Extracts on Spontaneous Uterus Contractions

The wild and cultivated *R. idaeus* leaves extracts solutions were introduced to a tissue bath to examine their influence on spontaneous uterus contractions after the stabilization period. Increasing concentrations of each sample (0.01–1mg/mL) were successively added to the tissue bath. Inhibition of uterus contractility was expressed as a percentage of the basal tone compared with baseline values as described previously [27].

2.7. Statistical Analysis

Statistical analyses were performed using the IBM SPSS Statistics 20 software package. The hypothesis of equality of means was tested with one-way ANOVA followed by Tukey's post hoc test. Pearson's linear correlation coefficient was used for the correlation analysis. Probability values (p) of less than 0.05 were considered statistically significant.

3. Results and Discussion

3.1. Comparing Wild and Cultivated Varieties Raspberry Leaf Extracts: Extraction Methods and Environmental Factors

Traditional medicine is primarily based on the application of the specific parts of the plants in the form of various extracts to prevent or treat various conditions. Given that the preparation and implementation of the so-called traditional remedies were very often transferred through generations orally, there is a high probability that the original way of preparation and indications for their use have changed over time. Therefore, from a scientific point of view, it is very important to evaluate the preparation of herbal medicines, their pharmacological potential, as well as to justify their use in traditional medicine.

Although the preparation of herbal medicines is thought to be simple, the extraction process itself is greatly affected by the solvent used, its polarity and pH, as well as the temperature at which extraction is carried out [28]. In this work, we used water as a the most commonly used solvent in traditional medicine, which is also a very polar solvent and increases the swelling of the plant material,

then diluted ethanol, which is the most commonly used in pharmacy for the preparation of extracts and tinctures, and diluted methanol, which extracts the most of the active plant constituents. All solvents were used cold to reduce the possibility of decomposing the active principles.

To avoid potential influence of environmental factors on raspberry leaves composition, wild and cultivated raspberries were collected at the same vegetative phase, approximately at the same altitude, sun exposure, about 300 meters apart, and the cultivated raspberries were not additionally irrigated or fed. The results presented in Table 1 showed that the highest yield of extracts was achieved by using water as an extraction agent, and that was with cultivated raspberry leaves (25.5%) compared to the yield of wild raspberry leaves (23.12%). Also, with the application of either solvent, the extracts obtained from the leaves of cultivated raspberries have a higher yield (Table 1). Given that the influence of changing factors of the external environment is reduced to a minimum by the targeted selection of areas for collection of plants material, higher yields of extracts obtained from the leaves of cultivated raspberries in comparison to wild raspberries for all used solvents could be attributed to the internal characteristics of the cultivated raspberry of v. Polka.

3.2. Comparing Phytochemical Composition of Wild and Cultivated Varieties Raspberry Leaf Extracts

Considerable amounts of phenolic compounds were found in all tested samples, ranging from 56.34 ± 2.59 mg CE/g (MM 70EtOH) to 145.77 ± 3.83 mg CE/g (GM H2O) for the total phenolic content, 31.57 ± 3.04 mg CE/g (MM 70EtOH) to 129.81 ± 6.34 mg CE/g (GM 70MOH) for the total tannin content and 50.38 ± 0.3 mg Ru/g (MM H2O) to 96.31 ± 1.44 mg Ru/g (GM 70EtOH) for the total flavonoid content. The water extract of wild and cultivated raspberry leaves showed the highest content of phenolic compounds. Water extract of wild raspberry leaves had the highest tannin content, while in the extracts obtained from cultivated raspberries, the highest tannin content was found in the 70% methanolic extract and the tannin fraction represent more than a half of the total polyphenolic compounds of all cultivated raspberry leaves extracts. In addition, water extracts of wild and cultivated raspberry leaves had the highest flavonoid content. Previous studies, as well as present phytochemical results showed that raspberry leaves extracts contain significant amounts of phenolic compounds, which could be useful in their quality estimation [1,6,10,29,30]. While the high contents of both total phenolic compounds and total flavonoids in water extracts gives justification for use of raspberry tea in folk and traditional medicine.

Table 1. Phytochemical composition and radical scavenging activity of raspberry leaf extracts

Type of extract	Yields of extracts (%)	Total phenolic content (mg CE/g)	Total tannin content (mg CE/g)	Total non-tannin content (mg CE/g)	Total flavonoid content (mg RuE/g)	Radical scavenging activity (IC_{50} µg/ml)
MM 70MOH	18.04	72.78 ± 5.32^{b}	34.86 ± 0.75^{a}	37.92 ± 4.57^{c}	50.38 ± 0.3^{b}	9.50 ± 0.71^{b}
MM 70EtOH	16.04	56.34 ± 2.59^{a}	31.57 ± 3.04^{a}	24.77 ± 0.45^{b}	56.2 ± 0.80^{c}	12.48 ± 0.65^{a}
MM H2O	23.12	138.38 ± 2.01^{d}	59.39 ± 2.43^{b}	78.99 ± 0.42^{d}	58.96 ± 0.24^{a}	166.03 ± 4.77^{d}
GM 70MOH	23.18	145.07 ± 7.09^{d}	129.81 ± 6.34^{e}	15.26 ± 0.75^{a}	75.71 ± 0.64^{d}	18.02 ± 0.47^{d}
GM 70EtOH	17.84	105.87 ± 2.39^{c}	77.35 ± 2.09^{c}	28.52 ± 0.3^{b}	76.84 ± 0.35^{e}	24.22 ± 0.82^{c}
GM H2O	25.5	145.77 ± 3.83^{d}	109.62 ± 1.28^{d}	36.15 ± 2.55^{c}	96.31 ± 1.44^{c}	150.07 ± 3.51^{d}

MM 70MOH: extract of wild *R. idaeus* leaves obtained with 70% v/v methanol (hydromethanolic extract)
MM 70EtOH: extract of wild *R. idaeus* leaves obtained with 70% v/v ethanol (hydroethanolic extract)
MM H2O: extract of wild *R. idaeus* leaves obtained with distilled water (water extract)
GM 70MOH: extract of cultivated *R. idaeus* leaves obtained with 70% methanol (hydromethanolic extract)
GM 70EtOH: extract of cultivated *R. idaeus* leaves obtained with 70% ethanol (hydroethanolic extract)
GM H2O: extract of cultivated *R. idaeus* leaves obtained with distilled water (water extract)
Data are presented as mean ± SD and further compared using One-Way ANOVA followed by Tukey's *post hoc* test.

Based on the obtained results it should be noted that the content of total phenolic, tannin and flavonoids in extracts of cultivated raspberry leaves obtained by maceration with different solvents was significantly higher than in the same type of extracts of wild raspberry leaves ($p < 0.05$). Studies have shown that the *Rubus* plant family genotype and other cultural parameters, such as environmental conditions, can influence the total amount of phenolic compounds and thus their antioxidant activity

[31]. In the present work the influence of environmental factors was reduced to a minimum, thus the observed differences in the total amount of phenolic compounds could be attributed to the internal characteristics of different raspberry varieties.

3.3. Comparing Antiradical Activity of Wild and Cultivated Varieties Raspberry Leaf Extracts

Phenolic compounds are the most important type of phytochemicals regarding their potential medical use. As effective antioxidants, polyphenols act as chelators of metal ions that catalyze oxidation reactions, thus preventing reactions triggered by a single active oxygen atom. Also, they serve as reducing agents, by blocking free radicals and inhibiting the activity of enzymes such as lipoxygenases [10,29]. The results of the antiradical activity of different extract obtained from leaves of wild and cultivated *R. idaeus* DPPH (2,2-diphenyl-1-picrylhydrazyl) are presented in Table 1. The hydro methanolic leaf extracts of wild raspberry showed the most pronounced antiradical activity with an IC_{50} value of 9.5 ± 0.71 μg/ml, while the water leaf extract of wild plants showed the lowest antioxidant potential activity with an IC_{50} of 166.03 ± 4.77 μg/ml. Additionally, there is a statistically significant difference ($p<0.05$) in the radical scavenging potential when the activity of water extract of wild and cultivated raspberry leaf. It was demonstrated that wild raspberry extracts exhibited higher antioxidant activity compared to cultivated varieties [32], which is consistent with the results of the present study. In a study of Costea et al. (2016) [29] 50% ethanol extract of raspberry leaves had low DPPH scavenging capacity, compared to the standard chlorogenic acid, with EC_{50} values being above 0.0227 mg/mL. This value is close to results for both our hydroetanolic extracts MM 70EtOH and GM 70EtOH: 0.0125 and 0.0242 mg/mL, respectively (Table 1).

3.4. In Silico Analysis of R. idaeus leaves compounds

Considering chemical structure of tested compounds, it was possible to predict biological activities of 45 out of 49 compounds present in raspberry leaf extract. Analyzing potential activity spectra predicted by PASS for one constituent showed 3367 different biological activities for Pa>Pi, 1284 different activities for Pa>0.5 and 768 activities for Pa>0.7. The data obtained by *in silico* prediction are large and complex (Available in Supporting information), and considering traditional use of raspberry leaves, further evaluation was focused on pharmacological activities related to the women's reproductive health for the Pa>0.5 (Table 2). One other group of possibly important activities that can influence smooth muscle contraction and labor course is related to nitric oxide (NO), such as nitric oxide antagonist, nitric oxide donor, nitric oxide scavenger, nitric-oxide synthase inhibitor and nitric-oxide synthase stimulant activity. *In silico* results predicted that all tested compounds of raspberry leaves have certain impact on NO level although with possibility Pa>Pi. Among them the most prominent nitric oxide scavenger activity (Pa>0.5) was predicted only for cycloartenol, while nitric oxide antagonist activity was predicted for hyperoside, kaempferol, kaempferol 3-O-*β*-D-galactopyranoside, nerol, quercetin, quercetin 3-O-*β*-D galactopyranoside, quercetin 3-O-*β*-D glucopyranoside, and squalene for Pa>0.5, and for kaempferol-3-O-α-L-arabinopyranoside, rutin, *α*-Amyrin, and *β*-Amyrin for Pa>0.7.

Assessment Report (EMA/HMPC/44209/2012 Corr.1) states that raspberry leaves tea is often recommended to be taken to facilitate and stimulate labor and to shorten its duration [9], but the conclusion of Committee on Herbal Medicinal Products of the European Medicinal Agency is that scientific documentation of these effects is questionable [33]. Thus, *R. idaeus folium* is approved as traditional herbal medicinal product for the symptomatic relief of minor spasm associated with menstrual periods, symptomatic treatment of mild inflammation in the mouth or throat and symptomatic treatment of mild diarrhea. Although at first glance it seems that predicted pharmacological effects of raspberry leaves compounds in terms of antiinflammatory (35/45), apoptosis agonists (33/45), and antinociceptive activity (22/45) (Table 2) are not related to female reproductive health, in depth analysis led to a different conclusion. Mechanism of labor, its beginning, course and the very end have not yet been fully clarified, but there are a lot of evidences that inflammation and apoptosis, in the cells of the cervix, are very important for initiation of labor, and that pain that accompanies childbirth [34].

Table 2. Predicted pharmacological activities of raspberry leaves compounds related to the women's general health, course of pregnancy and childbirth for Pa>0.5

Pharmacological activity	No. of compounds	The number with which the compounds are labeled in the table legend
Antiinflammatory	35	1, 3, 7, 9, 10, 12, 14, 15, 16, 18, 19, 20, 21, 22, 23, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45.
Apoptosis agonist	33	1, 2, 3, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 18, 19, 20, 21, 22, 23, 28, 29, 30, 31, 32, 33, 34, 36, 38, 40, 41, 43, 44, 45.
Antinociceptive	22	1, 2, 3, 12, 13, 16, 17, 18, 22, 24, 25, 26, 27, 30, 31, 35, 36, 37, 39, 41, 42, 43.
Antioxidant	21	1, 7, 9, 10, 14, 15, 18, 19, 20, 21, 23, 25, 29, 30, 31, 32, 33, 38, 40, 44, 45.
Anticarcinogenic	19	1, 5, 6, 9, 10, 11, 15, 18, 19, 20, 21, 23, 25, 29, 30, 31, 32, 38, 40.
Immunosuppressant	15	1, 10, 12, 18, 20, 21, 23, 25, 28, 30, 31, 32, 41, 42, 43.
Antineoplastic (breast cancer)	12	12, 18, 19, 21, 25, 29, 30, 31, 32, 33, 41, 43.
Immunostimulant	10	1, 7, 18, 20, 23, 25, 30, 31, 32, 43.
Spasmolytic	7	18, 23, 30, 31, 40, 44, 45.
Antineoplastic (cervical cancer)	2	41,43.
Antineoplastic (ovarian cancer)	2	41, 43.

Compound: 1. 1,2,6-tris-O-galloyl-*β*-D-glucose; **2**. 2-hexenal; **3**. 3-hexen; **4**. 3-oxo-*α*-ionol; **5**. 4-oxo-*β*-ionol; **6**. 4-Hydroxy-*β*-ionone; **7.** Ascorbic acid; **8.** Benzaldehyde; **9.** Caffeic acid; **10.** Chlorogenic acid; **11.** Citral; **12**. Cycloartenol; **13**. Decanal; **14**. Ellagic acid; **15**. Ferrulic acid; **16**. Gentisic acid; **17**. Hexanal; **18**. Hyperoside; **19**. Kaempferol; **20**. Kaempferol 3-O-*β*-D-galactopyranoside; **21**. Kaempferol-3-O-*α*-L-arabinopyranoside; **22**. Methyl gallate; **23**. Nerol; **24**. Octanol; **25**. Pentagallolyl-D-glucose; **26**. Phenylacetaldehyde; **27**. Protocatechuic acid; **28**. Pulegone; **29**. Quercetin; **30**. Quercetin 3-O-*β*-D galactopyranoside; **31**. Quercetin 3-O-*β*-D glucopyranoside; **32**. Rutin; **33**. Squalene; **34**. Terpinolene; **35**. Tetradecanal; **36**. Vanillic acid; **37**. n Butanol; **38**. *p*-coumaric acid; **39**. *p*-hydroxybenzoic acid; **40**. *α* Tocopherol; **41**. *α*-Amyrin; **42**. *α*-Terpineol; **43**. *β*-Amyrin; **44**. *γ*-Tocopherol; **45**. *δ*-Tocopherol

Table 3. Rat acute oral toxicity of raspberry leaves compounds according to the LD_{50} values predicted by GUSAR

GHS Classification and Labelling of Chemicals (UNECE 2011)	Category 3	Category 4	Category 5	Non-toxic	Out of AD
LD_{50} (mg/kg bodyweight)	50-300	300-2000	2000-5000	≥5000	
The number with which the compounds are labeled in the table legend	46, 47	2, 12, 14, 16, 22, 27, 29, 36, 41, 43	1, 3, 4, 5, 6, 8, 9, 10, 11, 13, 15, 17, 18, 19, 20, 21, 23, 24, 26, 28, 30, 31, 32, 34, 35, 37, 38, 39, 40, 42, 44, 45	7, 25, 33	48, 49

Compound: 1. 1,2,6-tris-O-galloyl-*β*-D-glucose; **2**. 2-hexenal; **3**. 3-hexen; **4**. 3-oxo-*α*-ionol; **5**. 4-oxo-*β*-ionol; **6**. 4-Hydroxy-*β*-ionone; **7.** Ascorbic acid; **8.** Benzaldehyde; **9.** Caffeic acid; **10.** Chlorogenic acid; **11.** Citral; **12**. Cycloartenol; **13**. Decanal; **14**. Ellagic acid; **15**. Ferrulic acid; **16**. Gentisic acid; **17**. Hexanal; **18**. Hyperoside; **19**. Kaempferol; **20**. Kaempferol 3-O-*β*-D-galactopyranoside; **21**. Kaempferol-3-O-*α*-L-arabinopyranoside; **22**. Methyl gallate; **23**. Nerol; **24**. Octanol; **25**. Pentagallolyl-D-glucose; **26**. Phenylacetaldehyde; **27**. Protocatechuic acid; **28**. Pulegone; **29**. Quercetin; **30**. Quercetin 3-O-*β*-D galactopyranoside; **31**. Quercetin 3-O-*β*-D glucopyranoside; **32**. Rutin; **33**. Squalene; **34**. Terpinolene; **35**. Tetradecanal; **36**. Vanillic acid; **37**. n Butanol; **38**. *p*-coumaric acid; **39**. *p*-hydroxybenzoic acid; **40**. *α* Tocopherol; **41**. *α*-Amyrin; **42**. *α*-Terpineol; **43**. *β*-Amyrin; **44**. *γ*-Tocopherol; **45**. *δ*-Tocopherol; **46**. Sanguiin H-6; **47**. Lambertianin C; **48**. Lambertianin D; **49**. Pulegone.

Having in mind the oral consumption of raspberry leaves tea, we focused on prediction of rat oral acute toxicity potential of raspberry leaves compounds, reported to be present in extract according to Globally Harmonized System of Classification and Labelling of Chemicals-GHS [35]. Traditional oral consumption of raspberry leaves tea guided this research to analyze an acute oral toxicity of the

compounds. GUSAR software predicted that the largest number of compounds (32/49) belongs to the Category 5, ten compounds to the Category 4, two compounds to the Category 3 and 3 compounds were classified as non-toxic (Table 3). The recommended daily intake and proposed pharmaceutical form of raspberry leaf [34], alongside documented concentrations span of compounds from Category 3 and 4 in plant material [9], potential toxic levels of mentioned compounds could not be reached. A growing number of studies are turning to herbal remedies, such as *R. idaeus* leaves extract, for their medical requirements without realizing how effective they are or what other impact their constituents might have on the body outside the intended therapeutic effect [36].

3.5. Effect of Raspberry Leaf extract on HeLa Cell Viability and Proliferation

The detected HeLa cell viability after the treatment with different concentrations of extracts ranged from 65 to 108% (Figure 1A). Both hydroethanolic and hydromethanolic extracts of wild and cultivated raspberry leaves only in concentration of 1mg/mL showed statistically significant decrease in cell viability ($p<0.001$) compared to control. No statistically significant difference was detected between wild and cultivated raspberry leaves extracts of the same type in all tested concentrations (1 mg/mL, 0.1 mg/mL, 0.01 mg/mL, and 0.001 mg/mL).

The proliferation of HeLa cells after the treatment with tested extracts ranged from 84 to 108%. No statistically significant effect on HeLa cell proliferation was found between the tested concentrations (1mg/mL, 0.1 mg/mL, 0.01 mg/mL, and 0.001 mg/mL) of wild and cultivated raspberry extracts, compared to the control (Figure 1B).

There is not much data on the effect of raspberry leaf extract on human cervical carcinoma, which represents one of the women`s leading health problem worldwide. Based on the classification system proposed by Lönnroth and J. E. Dahl, the negative impacts of extracts on cell viability and proliferation are categorized as severely, moderately, slightly, or non-cytotoxic/non-antiproliferative. This classification depends on the activity relative to the control group: less than 30% for severe, between 30% and 60% for moderate, between 60% and 90% for slight, and greater than 90% for non-cytotoxic effects [37]. At the same time, stimulatory effects are also recognized, with positive impacts classified as non-stimulatory, mild, moderate, or pronounced. These categories correspond to activity relative to the control group being between 100% and 110%, 110% and 140%, 140% and 170%, and exceeding 170%, respectively [26].

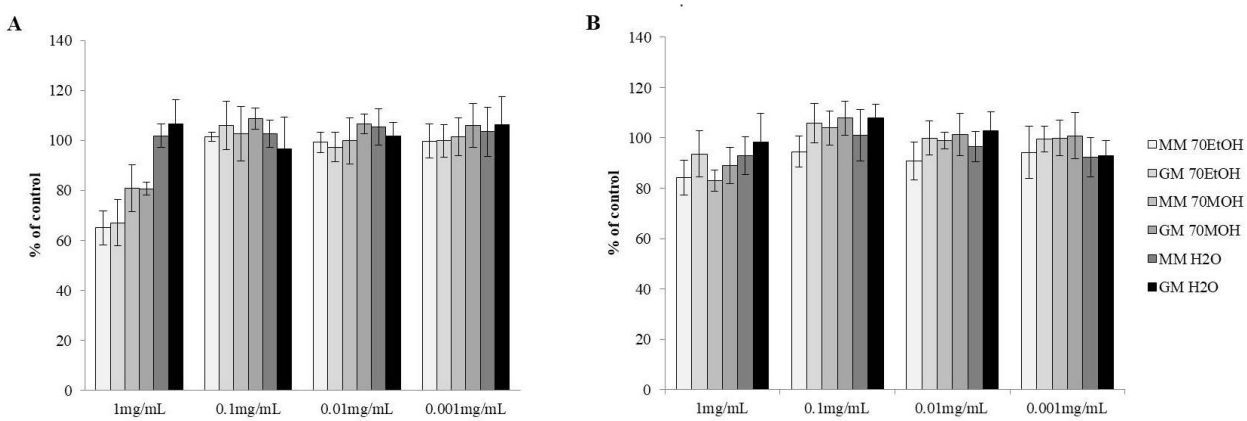

Figure 1. The effects of different concentrations of tested raspberry leaves extracts on A) viability; B) proliferation of HeLa cells. Results are presented as mean percentage ± SD and further compared using One-Way ANOVA followed by Tukey's *post hoc* test. * $p<0.05$

According to the obtained results and aforementioned classifications, only the highest concentrations of hydromethanolic and hydroethanolic extracts (1mg/mL) of both wild growing and cultivated raspberry showed slight, statistically significant, effect. Other tested extracts in tested concentrations showed no effect on HeLa cell viability (Figure 1).

Control (Figure 2A) and cells exposed to different extracts showed an adherent phenotype, with a large surface attached and wide and long cytoplasmic extensions. Only the cells treated with the highest concentrations of 1 mg/mL MM 70MOH and MM 70EtOH during the 24h of incubation showed a slight decrease in the adherent surface, and a decrease in width and extent of cytoplasmic extensions, with a negligible number of cells showing a non-adherent phenotype (Figure 2B). There was neither difference in the cell density, nor a difference in the number of dividing cells in the control group and other groups treated with extracts observed after 72h incubation. These findings indicate that all tested extracts, in all tested concentrations, show no noticeable effect on the proliferation of HeLa cells.

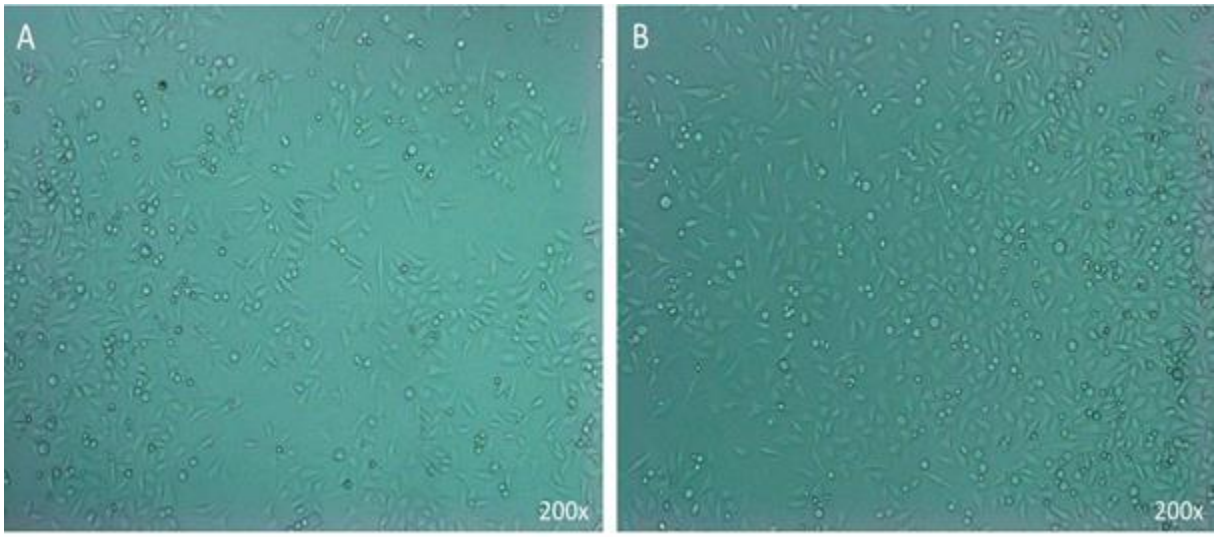

Figure 2. The effect of raspberry leaves extracts on viability of the HeLa cells. A) control B) cells treated with MM 70EtOH (1 mg/mL)

Regarding their therapeutic properties, there are evidence that cytotoxic properties of red raspberry leaves are cell type specific [38-42] which can be explained by different experimental designs, different concentrations of tested extracts and different characteristics of cell lines on which the tests were performed. The studies of Veljković et al., 2019 [1] and Durgo et al. 2019 [30] showed that effect on cell viability also varies between the plants of the same varieties collected on the different geographical locations which suggest that altitude, sun exposure, soil and irrigation influence phytochemical composition of raspberry leaves. The similar results obtained in the present study of wild and cultivated raspberries extracts are in concordance with these findings, although very mild effect on HeLa cell viability and proliferation was observed.

3.6. Influence on Uterus Contractility

Application of MM H_2O and GM H_2O in volumes spanning from 0.01-1 mg/mL failed to affect the contractions of the isolated rat uterus (Figure 3A). In the case of methanolic extract of wild and cultivated *R. ideaus* leaf extract (Figure 3B) statistically significant reduction in contraction force was noted only for the wild plant extracts in volumes exceeding 0.050 mg/mL. Furthermore, ethanolic extract of wild and cultivated *R. ideaus* leaves (Figure 3C) lead to a significant reduction in the contraction force of the isolated rat uterus when applied at volumes.

In the earliest studies application of *R. idaeus* leaf extract on isolated relaxed uteri of rabbit and guinea pig caused contraction. However, when the extract was applied to cat and dog isolated uteri suspended in a manner to produce contractile tone for some time, the relaxation effect was observed [43]. Thus, very little is known about whether raspberry tea actually induces labor in humans and if it does what is mechanism of action. Empirical evidence indicates that *R. idaeus* leaves may contract the uterus [43-46] or relax the uterus [4,43,47-48]..

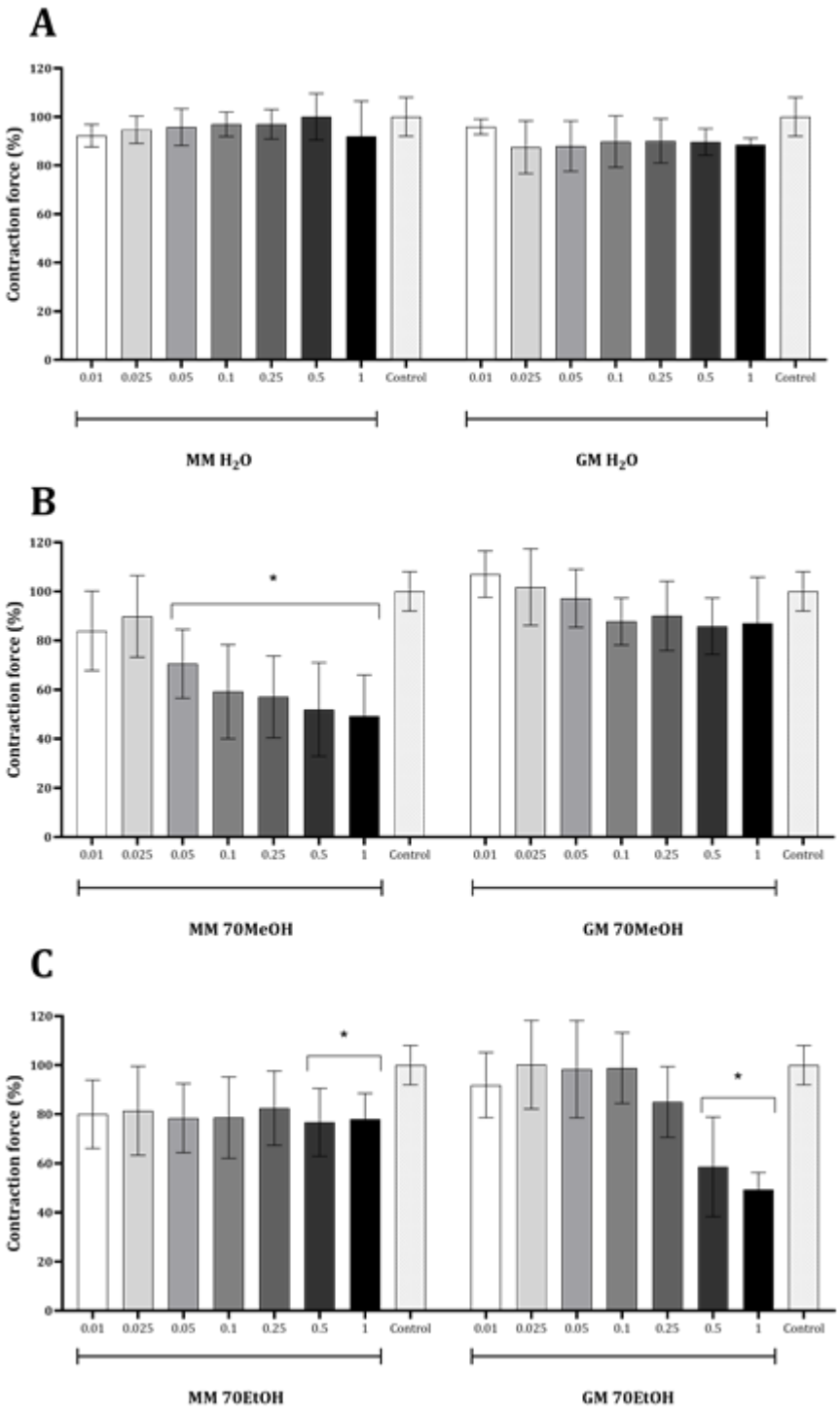

Figure 3. The effect of *R. ideaus* water (A), 70% methanol (B) and 70% ethanol (C) extracts applied in mg/mL on spontaneous uterine contraction. Data are given as mean ± SD (n=6). ANOVA followed by Tukey's *post hoc* test, *p<0.001 *vs.* control.

These apparent contradictory results may reflect variations in experimental parameters such as the species used, stage of pregnancy, whether the studies employed were *in vivo*, *in vitro*, or *in situ*, how *R. idaeus* leaves extract was prepared (water, alcohol, or oil based), as well as the applied concentrations of the extracts. Further mechanistic studies are designed to examine what might the

exact underlining mechanism of action be for the active extracts (Figure 3) which would encompass the application of specific stimulants in the presence of extract

The leaves of *R. idaeus* are rich in compounds that might affect smooth muscle activity [4,49], thus the extraction solvent might play an important role in the process of obtaining effective extract rich in bioactive constituents. The raspberry leaf extracts investigated in this study is a mixture of polyphenolic and non-polyphenolic compounds, which can interact with each other and cause different biological effects, depending on the time of exposure and concentration. According to studies conducted by both Beckett et al. (1954) [44] and Zheng et al. (2010) [45], using organic solvents to extract active constituents of raspberry leaves does not yield an extract that could produce a contractile response. This could suggest that more polar substances, particularly water-based preparations, might mediate the contractile responses observed *in vitro*. The aqueous extracts of *R. idaeus* leaves have been shown to induce concentration-dependent increase in contractile forces of isolated longitudinal strips of mouse uterus [50]. Although here prepared water extracts possess the highest amounts of phenolic compounds (Table 1) and were suggested in previous studies to be the ones that carry the activity they failed to produce any notable changes in the contraction patterns of the isolated rat uterus (Figure 3).

In silico prediction pointed out that large number of tested raspberry extract constituents might possess certain activity towards LOX and prostaglandin (PG) metabolism, e.g. kaempferol and quercetin with probability Pa>0.5. The LOX pathway is partially intertwined with PG synthesis which in turn is essential for cervical softening and contraction stimulation. Extract of *R. idaeus* in high concentration has been found to activate LOX [51] which in turn generates more PG. Certain polyphenols on the other hand limit the synthesis of PG [52]. Additionally, some polyphenolic compounds such as quercetin and catechins have been found to both enhance and inhibit production of cytokines [53]. The impact of raspberry extracts on NO may be one of the mechanisms via which they influence uterine contraction. During pregnancy, PG suppresses the generation of cervical NO while increasing the production of uterine NO [54]. According to *in silico* prediction all 45 tested compounds possess at least one activity related to NO signaling (Table 2). Thus, the detected decrease in contraction intensity could be potentially associated with a decrease in NO signaling hampered by the extract constituents.

All tested extracts from wild and cultivated raspberry leaves exhibited high polyphenol content and antiradical activity, with the hydroethanolic samples from wild *R. idaeus* leaves showing the strongest free radical scavenging ability. *In silico* analyzes predicted that a large number of raspberry leaves compounds possess anti-inflammatory, anti-apoptotic, anti-nociceptive, and NO signaling-related effects that may be associated with the course of pregnancy and childbirth. The traditional oral consumption of raspberry leaf tea prompted this study to analyze the acute oral toxicity of raspberry leaf compounds. According to the LD_{50} values predicted by GUSAR, no potentially toxic levels of the tested compounds could be reached with regular tea drinking. The results of the MTT test and the discrete morphological changes of the cells treated with the highest concentrations of the hydroethanolic and hydromethanolic extracts show that the tested extracts of cultivated raspberry v. Polka and wild raspberry in the area of Vlasina area have no significant effects on the viability and proliferation of HeLa cells. The impact of the extracts on spontaneous contraction of the isolated rat uterus was also modest. Although safety of use is unlikely to be a concern, even in the vulnerable group of pregnant women, further studies should be conducted to justify or negate the efficacy of the traditional application effectiveness of raspberry leaf tea for healthy pregnancy and easy delivery.

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Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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