Can yellow gentian (*Gentiana lutea*) be useful in protection against foodborne mutagens and food contaminants?

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Abstract. *Gentiana lutea* is a wellknown and respected medicinal plant that is used in many pharmacopoeias, mainly against different gastrointestinal disorders. The plant is under protection regimes in its natural habitats and for that reason is grown on plantations. In addition, it could be cultivated *in vitro*. The plants grown on plantation and in *in vitro* conditions were used to prepare methanolic and 50% ethanolic extracts of root and leaf/shoot, which were tested for antigenotoxic and antibacterial properties, against foodborne mutagens (heterocyclic aromatic amines PhIP and IQ) and food contaminants, respectively. The results obtained pointed out the excellent genoprotective effect (up to 78% inhibition of PhIP/IQ genotoxicity) based mostly on the antioxidative potential. The antibacterial effect was mainly weak; only the extracts of *in vitro* grown plant induced moderate activity against *Listeria monocytogenes* and *Staphylococcus aureus* (MICs ranged 0.15-5 mg/ml). In addition, the extracts’ potential to prevent biofilm formation by *L. monocytogenes* was very high (up to 90% inhibition). Taken together, the results obtained encourage further research that would be directed to the formulation of potent antigenotoxic and antibiofilm agents based on *G. lutea*.

1. Introduction

The need for safe and high-quality food is an imperative for the world population, and the reasons for that are numerous. Among others, ending hunger and poverty and promoting good health and well-being seem to be the most important [1]. To provide sustainable food processing, producers are inevitably faced with numerous problems that have to be resolved, in order to provide consumers with high quality food that complies with safety and security requirements, and simultaneously supports health [2]. The growth of microbial contaminants and the presence of mutagens in food are considered as among the most pronounced problems that need solutions.

2. Characterization of the problems: concepts of microbial contamination and food mutagens

Concerning microbial contamination, despite strict food protection measures implemented in developed countries, this problem still exists and the necessity to investigate new food preservatives, which are efficient and do not induce side effects on consumers’ health, is evident. Furthermore, the problem of foodborne illness is disproportionately higher among populations of developing, low- and middle income countries than in wealthy countries. The need for cheap and efficient means of food preservation in less wealthy nations is pronounced. As an illustration of the need to continuously combat this problem, the WHO report pointed out that annually, 600 million people became ill by ingestion of contaminated food, and among them as many as 420,000 casualties were estimated [3].

According to these facts, there is a serious need for the upgrading of food preservation techniques in order to provide green food processing that would consequently promote health [4]. Furthermore, being aware of side effects that could arise from synthetic additives/preservatives, the search for the potent antimicrobials of plant origin that could be used in foods is strongly advised [5].

Another important question that should be resolved considers the biological activity of food ingredients. Many of them are embedded with health-promoting properties – it is well known that bioactive constituents of fruits, vegetables and spices possess antioxidative, antimicrobial, antiviral,
anti-inflammatory, antirheumatic, lipid-lowering, antidiabetic, anticancer, hepato- and nephroprotective, and other beneficial effects [6]. However, some of foods’ bioactive compounds could induce harmful effects; there are also literature data indicating herbs’ toxicity and mutagenicity [7,8]. This paper will pay special attention to food mutagens.

Substances that can induce DNA damage and consequently lead to formation of mutations are designated as mutagens or genotoxic agents. Genotoxicity is defined as the ability of an agent to induce damage to genetic material, i.e. DNA molecule, or cellular components associated with the functionality and behavior of chromosomes [9]. By inducing genotoxicity, mutagens contribute to different genetic disorders and degenerative diseases, including hepatic, cardiovascular and neurodegenerative conditions, diabetes, chronic inflammation, arthritis and cancer. Genotoxic substances can also be found in food; some of them accidentally occur in food (such as aflatoxin, as a result of mould contamination), but the others are intentionally added, like food additives (such as boric acid and sunset yellow) [10-12]. Both of these food mutagen groups could be avoided by strict prevention of mould contamination, or by careful revision of permitted and prohibited food additives, which is periodically realized. However, there is one more group of food mutagens – the ones that are formed in foodstuffs during food processing, i.e. foodborne mutagens. They include three subgroups: polycyclic aromatic hydrocarbons, nitrosamines and heterocyclic aromatic amines (HAA) [12]. Although strict implementation of some codes of practice and standards, recommended by Codex Alimentarius Commission, could contribute to the reduction of foodborne mutagen levels, their presence in processed food cannot be completely avoided [13]. For that reason, the alternative strategy of using natural products with antigenotoxic properties in nutrition is recommended. There are numerous compounds of natural origin, mainly from edible and medicinal plants, that are well known for their antigenotoxic/genoprotective potential and could be designated as phyto-antimutagens [14,15].

3. Yellow gentian – use, biological activities, sources and chemical composition

Taking into account the above-mentioned facts, the aim was of this study was to explore and identify bioactive agents of plant origin that would be efficient both as antimicrobials and antimutagens. Our efforts directed us to great yellow gentian (Gentiana lutea), a medicinal plant which is recognized by many pharmacopoeias. It is used in pharmaceutical, cosmetic and food industries, in production of drugs and cosmetics, and as an additive in beverages and foods [16,17]. Reviews have reported numerous biological activities of this reputable folk remedy, such as antioxidative, antimicrobial, anti-inflammatory, antidiabetic, hepatoprotective, anticancer and radioprotective effects [18,19]. It is used in traditional medical preparations for gastrointestinal and menstrual disorders, treating wounds and arthritis, and as an antihypertensive agent [20,21].

However, due to the high demand for this medicinal plant, G. lutea is under protection regimes, both in the European Union and in Serbia [22,23]. Therefore, in order to preserve the plant in its natural habitats, G. lutea for commercial uses is cultivated on plantations [24]. Furthermore, with the aim to introduce G. lutea into in vitro plant tissue culture, preliminary attempts have been previously made [25,26], but cultivation of plants, both root and shoot, has been successfully undertaken by our research team [27].

Previous analysis of chemical composition pointed out that the main pharmacologically active compounds in G. lutea organs are secoiridoids, followed by iridoids, xanthones and C-glucosflavones. In addition, plant organs, mainly the ones in aerial plant parts, are rich in polyphenols, including flavonoids [18].

In order to study the antimutagenic and antimicrobial potentials of G. lutea substances, we prepared methanolic and 50% ethanolic extracts of root and leaf of the plantation grown plant, as well as methanolic extracts of root and shoot of the in vitro grown plant [27,28]. The various extracts (Table 1) and their chemical profiles, obtained by UPLC-PDA MS/MS analysis, and total polyphenols and flavonoids in the extracts (Table 2) are shown. The results presented in Table 2 confirmed the high contents of polyphenols and, among them of flavonoids, especially in the extracts of aerial plant parts (leaf and shoot). Furthermore, the contents of tested constituents did not differ significantly between the methanolic and 50%-ethanolic extracts of the same organs of plantation grown plant, at least for the
majority of the constituents. Iv-Met-S differed from all the other extracts – the high levels of bioactive constituents pointed to the constituents that were intensively produced in aerial parts (secoiridoids gentiopicroside, sweroside and swertiamarin, and the iridoid, loganic acid) remaining there only in the case of in vitro cultivation, while in the case of plantation grown plants, they were transported to the roots [27].

Table 1. Descriptive indication of labelled test substances – methanolic and 50%-ethanolic extracts of *G. lutea* grown in plantation and in vitro

| Plantation grown plants (P*) | In vitro grown plants (Iv) |
|-----------------------------|---------------------------|
| 50% Ethanolic-water extracts (Et) | Methanolic extract (Met) | Methanolic extract (Met) |
| Root (R) | Leaf (L) | Root (R) | Leaf (L) | Root (R) | Shoot (S) |
| Pg-Et-R | Pg-Et-L | Pg-Met-R | Pg-Met-L | Iv-Met-R | Iv-Met-S |

| Constituent (%) | Pg-Et-R | Pg-Et-L | Pg-Met-R | Pg-Met-L | Iv-Met-R | Iv-Met-S |
|-----------------|--------|--------|--------|--------|--------|--------|
| gentiopicroside | 5.05±0.291 | 1.14±0.147 | 4.42±0.339 | 1.528±0.170 | 1.949±0.101 | 11.463±0.098 |
| S/oroside | 2.080±0.270 | 0.207±0.034 | 1.999±0.052 | 0.232±0.033 | 0.184±0.005 | 3.495±0.125 |
| Swertiamarin | 0.631±0.032 | 0.301±0.036 | 0.551±0.017 | 0.302±0.007 | 0.046±0.005 | 1.338±0.056 |
| Loganic acid | 0.710±0.148 | 1.79±0.096 | 0.706±0.006 | 1.789±0.152 | 0.031±0.005 | 2.271±0.184 |
| Mangiferin | */* | 0.129±0.023 | / | 0.049±0.003 | / | 0.110±0.022 |
| Isogentisine | 0.259±0.086 | 0.231±0.053 | 0.136±0.019 | 0.049±0.002 | 0.022±0.011 | 0.039±0.007 |
| Homoorientin | 0.025±0.005 | 3.999±0.122 | 0.019±0.002 | 0.692±0.064 | / | 0.140±0.020 |
| Isovitexin | 0.013±0.005a | 3.038±0.487 | 0.006±0.003 | 0.739±0.033 | / | 0.114±0.001c |

**Total polyphenols** 25.8±3.2 53.4±3.5 22.7±3.3 44.7±3.5 23.9±1.7 50.8±0.5

**Total flavonoids** 1.4±0.1 20.7±1.8 1.12±0.1 18.2±2.7 1.8±0.4 21.6±3.1

The results are taken from our previous works [27,28]; Statistical significance was determined by comparing all the results and using one-way ANOVA with Tukey’s post hoc test. Values with different superscript letters in each row differ significantly (p<0.05); **a** The content is lower than the limit of determination.

4. Genoprotective activity of the *G. lutea* extracts against genotoxicity of selected HAAs

HAAs are considered potent mutagens that are formed during thermal processing and smoking of protein-rich foods, such as meat and fish. After metabolic activation by mammalian liver enzymes, they are converted into mutagenic and carcinogenic agents that play an important role in the etiology of various human cancers. How potent some of them are can be deduced from the observation that active carcinogenic concentrations can be at ng (10^-9 g) level [12]. The antigenotoxic potential of the extracts was assessed according to activity against the two HAA compounds selected: 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and 2-amino-3-methyl-3H-imidazo[4,5-f]quinolone (IQ). While PhIP is classified by the International Agency for Research on Cancer (IARC) as a possible carcinogen, IQ is declared as the most likely carcinogen harmful to human health [29].
Screening on this topic was performed by alkaline comet assay, which is routinely used to determine and quantify the DNA damage. Although only the in vivo alkaline comet assay is recommended by the OECD for genotoxicity determination, in vitro applications on different mammalian cell lines are widespread both in genotoxicity and antigenotoxicity testing [30]. In our investigation, the assay was performed on human hepatoma HepG2 cell line [27]. Preliminary data, concerning genotoxicity testing of PhIP and IQ, as well as of gentian extracts, were provided in order to determine the adequate concentrations of the mutagens, and to define the non-genotoxic concentration ranges of all tested extracts [27,28,31]. Then, co-treatment experiments involving both PhIP/IQ and each of the G. lutea extracts were performed; the results obtained indicate the framework of the plant’s antigenotoxic activity (Figure 1).

![PhIP-induced genotoxicity](image1)

**Figure 1.** The antigenotoxic effect of G. lutea extracts.

The inhibitions (%) of PhIP-induced (above) and IQ-induced (below) genotoxicity were determined in the alkaline comet assay, performed on human hepatoma HepG2 cell line. For all the extracts, antigenotoxic concentration ranges are specified.

As it can be seen from the figures, dose-dependent curves of antigenotoxicity were not determined for all extracts studied. On the contrary, for some of them a J-curve, indicating the highest beneficial effect at the lowest applied concentration, was observed. This detail, together with the fact that in higher concentration ranges all the extracts were genotoxic [27,28], suggests the hormesis phenomenon. The term hormesis refers to the situations characterized by a low-dose response that is opposite in effect to
that seen at high doses [32]. Actually, numerous agents in genotoxicology are defined as “Janus substances, which, applied at low doses act as antimutagens, but applied at higher ones act as mutagens” [33]. The fact that the tested gentian extracts act as Janus agents is cautionary; very careful analysis is required in order to clearly define the active genotoxic concentrations that could be applied for protection against IQ and PhIP mutagenicity.

Furthermore, since both PhIP and IQ mutagenicity is realized, at least partially, by oxidative DNA damage [34,35], we also quantified the extracts’ antioxidative potential. Results provided by DPPH assay, which determines the radical scavenging effect, show the extracts of aerial parts, i.e. Pg-Et-L, Pg-Met-L and Iv-Met-S, were embedded with higher capacity to quench radicals [27,28]. In addition, both ethanolic extracts (Pg-Et-L and Pg-Et-R) were confirmed to up-regulate Nrf2 transcriptional factor, being the master factor responsible for the expression of antioxidative enzymes in hepatoma HepG2 cells. Finally, the high antioxidative potential of ethanolic extracts, which was realized in the cells, was confirmed by measuring effects of ethanolic extracts on regeneration of the reduced form of glutathione in the HepG2 cells co-treated with PhIP/IQ and each of the Pg-Et-L/Pg-Et-R extracts [28]. These results pointed to the antioxidative properties of the extracts as being responsible for the observed antigenotoxicity. In other words, due to antioxidative action, the tested gentian extracts protect DNA from oxidative damage induced by IQ and PhIP.

5. Antibacterial and antibiofilm activity of the G. lutea extracts

Starting from literature data indicating the strong antimicrobial activity of G. lutea extracts [36], and bearing in mind the plant’s potential to mitigate gastrointestinal disorders, in further work, we screened for the antibacterial effect of the extracts on a palette of selected food contaminants. However, microdilution assay indicated only weak antibacterial activity which was not evident against all tested bacteria strains. The only exception was effect of the in vitro grown plants against Listeria monocytogenes and Staphylococcus aureus, where moderate antibacterial potential was determined. Table 3 shows the minimal inhibitory and bactericidal concentrations (MICs and MBCs, respectively) determined for the susceptible strains. Taking into account results presented in this Table and the fact that the tested strains of Escherichia coli and Shigella flexneri were not sensitive at all, the greater effect against Gram-positive than Gram-negative bacteria was evident.

Table 3. Antibacterial effect of G. lutea extracts*

| MIC/MBC values (mg/ml) | Pg-Et-R | Pg-Et-L | Pg-Met-R | Pg-Met-L | Iv-Met-R | Iv-Met-S |
|-----------------------|---------|---------|----------|----------|----------|----------|
| B. subtilis ATCC6633  | 5/10    | 5/10    | 5/10     | 2.5/5    | 5/10     | 1.25/2.5 |
| E. faecalis ATCC29212 | 5/10    | 5/10    | 5/10     | 2.5/5    | nd       | 5/10     |
| L. monocytogenes ATCC19111 | 10/nd | 10/nd   | nd       | 5/10     | 0.62/1.25 | 0.31/0.62 |
| S. aureus MSSA ATCC 25923 | 5/10    | 5/10    | nd       | 5/10     | 0.62/2.5 | 0.15/1.25 |
| S. aureus MRSA ATCC 43300 | nd**    | nd      | nd       | 5/10     | 5/10     | 5/10     |
| P. aeruginosa ATCC 15442 | 10/nd   | 10/nd   | 10/nd    | 10/nd    | nd       | nd       |

*Antibacterial activities of the extracts derived from plantation grown plants are taken from our previous work [37]; **nd – not determined in the applied concentration range (0.078-10 mg/ml)

Based on the results of the microdilution assay, we focused on L. monocytogenes and S. aureus and in further work, tested the extracts’ potential to prevent biofilm from being formed by these two bacteria strains. This direction was in accordance with the actuality of the problem of biofilms in food industry [38]. Preliminary unpublished data, provided by crystal violet assay, show that biofilm potential against S. aureus is not high (the maximum inhibition is 28%), while in the case of L. monocytogenes, the potential of the extracts to prevent biofilm formation is multifold higher; the inhibitions determined are up to 90%. Interestingly, the Iv-Met-R and Iv-Met-S extracts, which induced the highest antibacterial
potential in microdilution assay, were moderately active against biofilms and inhibited biofilm formation by maximally 35% (data not shown).

6. Conclusion
This investigation revealed that Gentiana lutea methanolic and 50% ethanolic extracts could be considered as the excellent genoprotective agents that protect against the genotoxicity of food borne mutagens, IQ and PhIP. The extracts’ antigenotoxic potential is at least partially based on their antioxidative properties. Results concerning antimicrobial effects indicated that some antibacterial potential exists, while the antibiofilm activity against L. monocytogenes was high. All data obtained encourage further investigation.

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