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Antioxidative properties and antigenotoxic potential of *Gentiana lutea* extracts against the heterocyclic aromatic amine 2-amino-1-methyl-6-phenylimidazo[4,5-*b*] pyridine, PhIP

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Abstract. Lipid oxidation that occurs in different types of food can cause alterations in nutritional qualities, flavour, texture and shelf life of foods. Furthermore, high temperature cooking of protein-rich food can lead to formation of heterocyclic aromatic amines capable of compromising the integrity of DNA molecules. To reduce these harmful effects, research has been focused on investigating plants as a source of potential natural food additives and preservatives. Thus, the aim of this study was to estimate antioxidant and antigenotoxic activities of 50% ethanolic-aqueous root and leaf extracts of the medicinal plant, *Gentiana lutea*. Antioxidative effect was investigated using the DPPH assay, while antigenotoxicity against the mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) was determined using *Salmonella* Typhimurium TA1535 in the SOS/*umuC* assay. Leaf extract showed high antioxidative effect with the ability to neutralize up to 87% of free radicals at 400 µg mL⁻¹. Antigenotoxicity testing revealed that both extracts exhibited remarkable genoprotective activity against PhIP-induced DNA damage, with the highest inhibition levels being 70% and 85% for root and leaf extracts, respectively. Results obtained are encouraging and suggest further research of *G. lutea* extracts as potential food preservatives and additives in improving food quality and human health.

1. Introduction

It is a known fact that diet and dietary habits can affect human health on different levels. One of the major problems when it comes to food safety is the free-radical mediated oxidation of foods that leads to quality deterioration of foods and consequently to impairing human health. Even though numerous synthetic antioxidants are used for food preservation, there are reports indicating the harmful effects of these additives [1]. Another problem comes from mutagens derived from food. One of the important classes of food mutagens are heterocyclic aromatic amines. These amines are formed during high temperature processing of protein-rich foods and require metabolic activation by liver enzymes in order to manifest their harmful effects. Their genotoxicity is manifested through forming covalent adducts with DNA molecules or generating reactive oxygen species, consequently leading to damage of biomolecules [2]. To counteract the negative effects of food oxidation and food mutagens, as well as to



satisfy consumer desire for natural and organic food preservatives, research is more focused on investigating different natural products as potential agents contributing to food safety.

It is well known that plants are an inexhaustible source of biologically active substances with various potential pharmacological activities. For that reason, medicinal and aromatic plants are being explored to a large degree as potential sources of effective, non-toxic and antioxidant compounds capable of improving food quality [3].

Gentiana lutea, yellow gentian, is a well known medicinal plant, widely used in folk medicine, as well as for the production of alcoholic and non-alcoholic beverages and pharmacological products [4]. Furthermore, rhizomes and roots of yellow gentian are used as an official drug by many pharmaceutical companies for the treatment of mild gastrointestinal ailments [5].

Taking into account the above, the aim of this study was to investigate the antioxidant activity of 50% ethanolic-aqueous root (GLR) and leaf (GLL) extracts of *G. lutea*. Furthermore, the antigenotoxic activity of extracts was estimated against 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) mutagen produced in thermally processed meat.

2. Materials and Methods

Plant material was obtained from the *Dr Josif Pančić* Institute for Medicinal Plant Research, Belgrade, Serbia, and extraction of plant material was done as previously described [6]. Antioxidant activity of extracts was measured through their ability to scavenge free radicals by applying the DPPH assay as described earlier [6]. Antigenotoxic effect of the extracts was investigated on the model organism *Salmonella* Typhimurium TA1535/pSK1002 in the SOS/*umuC* assay as previously described [7]. Prior to antigenotoxicity testing, a microdilution assay was done [7] and MIC values of the tested substances were obtained in order to avoid total inhibition of bacterial growth in the SOS/*umuC* assay. Furthermore, genotoxicity of extracts and PhIP mutagen was determined on *S. Typhimurium* in order to obtain non-genotoxic and genotoxic concentrations of the extracts and mutagen, respectively. Genotoxic potential was investigated without and with metabolic activation, provided by S9 fraction. After an appropriate incubation period, bacterial growth ratio (G) was evaluated by measuring optical densities (OD) at 600nm and using following formula: $G = \text{sampleOD}_{600} / \text{controlOD}_{600}$. Evaluation of G was additionally used to confirm lack of bacterial growth inhibition. A growth ratio less than 0.75 was selected as a marker of bacterial cytotoxicity. By measuring the absorbances (A) at 405nm, the DNA damage induction ratio (IR) was calculated using the formula: $IR = (\text{sampleA}_{405} / \text{controlA}_{405}) / G$, and $IR=2$ was selected as the threshold of genotoxicity.

Antigenotoxic activity of the extracts against PhIP was tested using non-genotoxic doses of the extracts and in the presence of S9 fraction, since PhIP mutagen requires metabolic conversion. Percentage of inhibition of PhIP-induced damage was calculated using the following formula: $I (\%) = 1 - (IR_{CT} / IR_M) \times 100$, where IR_{CT} and IR_M represent mean values of IR of co-treatment and sole mutagen treatment, respectively.

Statistical analysis was done using GraphPad Prism 6.01 software and one-way ANOVA with Dunnet's post hoc test was applied to test statistical significance ($p < 0.05$).

3. Results and Discussion

The ability of the extracts to scavenge free radicals, determined in the DPPH assay, revealed stronger antioxidant activity of leaf compared to root extract (Figure 1). At the highest applied concentration ($400 \mu\text{g mL}^{-1}$), GLL extract scavenged up to 87% of DPPH radicals [6]. The high capacity of the gentian extracts to neutralize free radicals is in line with previously published data [8, 9], confirming the good antioxidant activity of *G. lutea*. In addition, the root extract of *G. lutea* successfully reduced lipid oxidation and colour changes in beef patties [10], suggesting its potential as a food preservative.

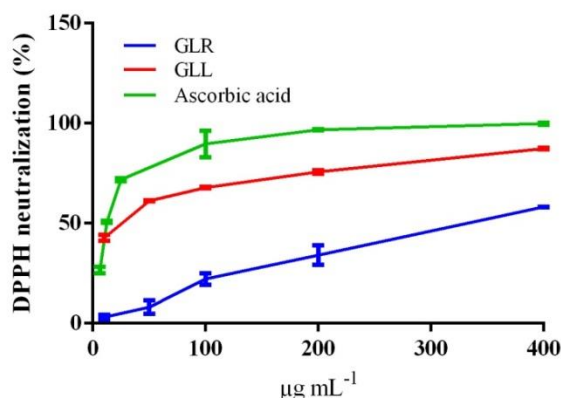


Figure 1. DPPH radical scavenging activity of *G. lutea* extracts

Results are expressed as mean values of neutralization (%)±standard deviations.

Ascorbic acid was used as positive control. GLR – root extract; GLL – leaf extract.

Furthermore, pre-screening of *S. Typhimurium* survival in the microdilution assay indicated that concentrations up to 2.5 mg mL⁻¹ and 50 µg mL⁻¹ for the extracts and PhIP, respectively, were non-cytotoxic, and could be used for further genotoxicity testing. The absence of cytotoxicity of tested substances was confirmed in SOS/*umuC* assay by determining the growth ratio (Figure 2).

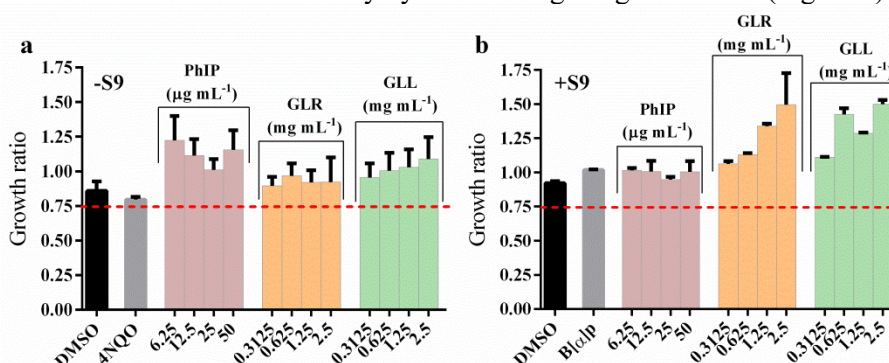


Figure 2. Effect of *G. lutea* extracts and PhIP on bacterial growth in SOS/*umuC* assay without (a) and with (b) metabolic activation (S9)

Results are expressed as mean values of growth ratio±standard deviations. DMSO (10%) was used as solvent control; 4NQO (0.5 µg mL⁻¹) and B[a]p (50 µg mL⁻¹) were used as positive controls. Red line positioned on 0.75 represents a threshold, below which the effect is considered cytotoxic. GLR – root extract; GLL – leaf extract.

Results of genotoxicity testing in the SOS/*umuC* assay showed that none of the extracts exhibited genotoxic potential in the absence of metabolic activation (S9) (Figure 3a). On the other hand, after testing with the addition of S9 fraction, both extracts showed genotoxic potential at certain concentrations (Figure 3b), indicating that they act as promutagens, i.e. they require metabolic activation in order to manifest their mutagenicity. As will be discussed below, the fact that the antigenotoxic agents can act in certain conditions and high concentrations as mutagens is not rare. Considering PhIP, as expected, it was only genotoxic in the presence of S9 fraction.

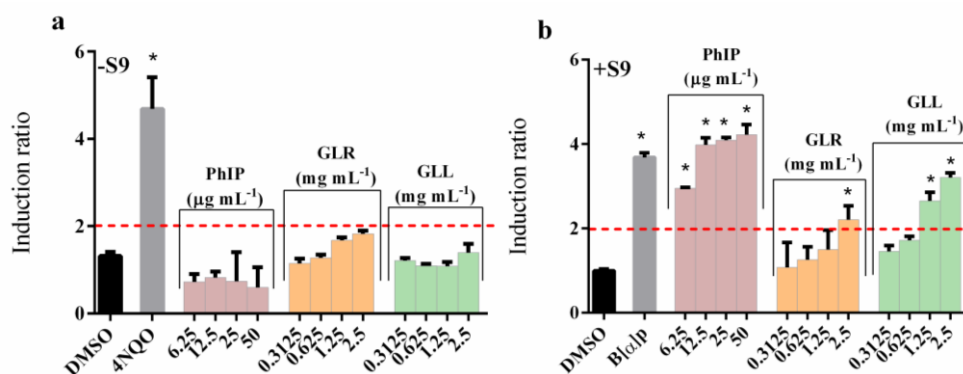


Figure 3. Effect of *G. lutea* extracts and PhIP on induction ratio in SOS/*umuC* assay without (a) and with (b) metabolic activation (S9).

Results are expressed as mean values of induction ratio \pm standard deviations. DMSO (10%) was used as solvent control; 4NQO ($0.5 \mu\text{g mL}^{-1}$) and B[a]p ($50 \mu\text{g mL}^{-1}$) were used as positive controls. Red line positioned on 2 represents a threshold, above which the effect is considered genotoxic. Statistical significance in regard to DMSO was tested using one-way ANOVA with Dunnet's post hoc test (* $p < 0.05$). GLR – root extract; GLL – leaf extract.

In further work, only non-genotoxic doses of the extracts were used for antigenotoxicity testing against PhIP, applied at a concentration of $12.5 \mu\text{g mL}^{-1}$. Results of antigenotoxicity revealed that both extracts exhibited strong genoprotective activity (Figure 4). The highest inhibition of PhIP-induced DNA damage was 70% and 85% for GLR and GLL, respectively. Similar results, in terms of genoprotective effect, were recorded for the extracts of *Gentiana asclepiadea* and *Gentiana cruciata* [11, 12]. It was shown that these extracts are capable of reducing DNA damage caused by hydrogen peroxide and the mutagen ethyl methanesulfonate.

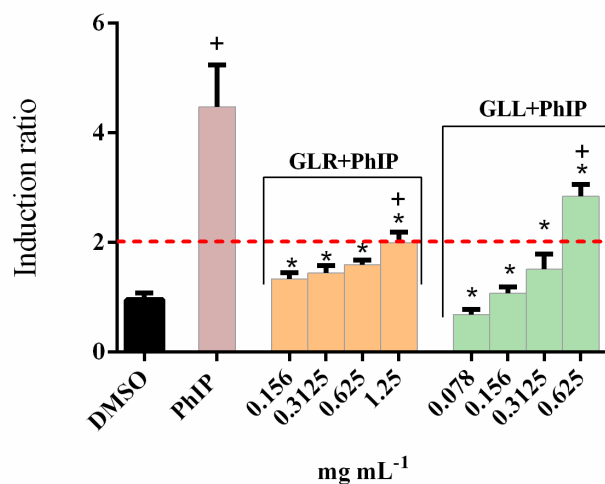


Figure 4. Antigenotoxic potential of *G. lutea* extracts against PhIP ($12.5 \mu\text{g mL}^{-1}$) in the SOS/*umuC* assay.

Results are expressed as mean values of induction ratio \pm standard deviations. DMSO (10%) was used as solvent control. Red line positioned on 2 represents a threshold, above which the effect is considered genotoxic. Statistical significance was tested using one-way ANOVA with Dunnet's post hoc test; $p < 0.05$, + statistical significance in regard to DMSO; * statistical significance in regard to PhIP. GLR – root extract; GLL – leaf extract.

In our study, interestingly, both extracts were more efficient in reducing DNA damage at lower concentrations. Furthermore, we detected dual genotoxic/antigenotoxic nature of extracts. Generally, the extracts showed genotoxic effect when applied in higher doses, but at lower concentrations provided strong protection against PhIP mutagen. This kind of result can be explained by the hormesis phenomenon, which refers to situation where toxic substances provoke beneficial biological responses

when applied in low doses [13]. In addition, the response curve in the form of the letter “J”, obtained in our study, is not uncommon for antimutagenesis studies [14].

The strong genoprotective activity demonstrated in our study can be partially explained by free radical scavenging activity of the extracts. Taking into account that PhIP can induce oxidative damage to DNA [15], the observed antioxidant activity of the extracts surely contributed to the detected antigenotoxicity. However, one should bear in mind that different secondary metabolites and interactions between them, as well as other mechanisms, can be responsible for antigenotoxic potential, which requires additional investigations.

4. Conclusion

Data obtained in this study indicate that 50% ethanolic-aqueous root and leaf extracts of *G. lutea* possess good antioxidative activity and strong capacity to reduce DNA damage induced by the thermally produced heterocyclic aromatic amine, PhIP. These results are promising and recommend further research of yellow gentian as a potential natural food preservative. However, the observed dual genotoxic/antigenotoxic effect of the extracts cannot be excluded and requires additional investigation in order to ensure the safe usage of *G. lutea* in the food industry.

Acknowledgement

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