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# Determination of naphthoquinones in invasive alien plants *Impatiens glandulifera* Royle and *I. balfourii* Hook.f. from Croatia

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**Abstract:** The study aimed to develop an HPLC method for a simultaneous determination of 2-hydroxy-1,4-naphthoquinone (2-HNQ) and 2-methoxy-1,4-naphthoquinone (2-MNQ) in plant material. The method was implemented to determine naphthoquinones (NQs) in leaves and flowers of the invasive alien species *Impatiens glandulifera* and *I. balfourii* collected in Croatia. Two NQs extraction methods from plant material were compared: decoction and ultrasonic extraction. Validation parameters indicate that the developed method is reliable for the simultaneous determination of 2-HNQ and 2-MNQ in plant material. The decoction extraction obtained a higher NQs yield than ultrasonic extraction. In the leaves and flowers of *I. glandulifera* and *I. balfourii* only 2-MNQ was detected. Both, *I. glandulifera* and *I. balfourii*, had higher 2-MNQ concentrations in flowers than in leaves. A significantly higher 2-MNQ concentration was found in *I. glandulifera* compared to *I. balfourii*. Therefore, *I. glandulifera* can be considered a rich source of 2-MNQ whose antitumor potential is established.

**Keywords**: invasive alien species, 2-hydroxy-1,4-naphthoquinone, 2-methoxy-1,4-naphthoquinone, novel therapeutics, HPLC.

## INTRODUCTION

NAPHTHOQUINONES (NQs) *e.g.*, 2-hydroxy-1,4-naphthoquinone (2-HNQ, lawsone), 2-methoxy-1,4-naphthoquinone (2-MNQ, lawsone methyl ether), and 5-hydroxy-1,4-naphthoquinone (5-HNQ, juglone), are a diverse group of chemicals, present in plants and microorganisms.[1,2] NQs are well known for their biological activities; 2-HNQ and 2-MNQ are found to have antibacterial, antifungal, antiviral, and antitumor effects.[3–5] As such, NQs have the potential to be developed as novel therapeutics.[1,2,6,7]

In Asia, *Impatiens balsamina* L. (Balsaminaceae) is used in traditional medicine in the treatment of rheumatism, fractures, swelling, superficial infections, and fingernail inflammation.[3,4] Among biologically active compounds isolated from *I. balsamina* are NQs believed to contribute to biological activity.[3,4,8] Ding *et al.* (2008) confirmed antitumor activity of *I. balsamina* leaves extract on a human hepatocellular carcinoma cell line (HepG2) and connected antitumor activity with the presence of 2-MNQ in the leaves extract.

In Europe, several species of the genus *Impatiens* are present, of which *Impatiens noli-tangere* L. is the only native species, while others are neophytes from Asia and North America.[9,10] In Croatian flora five taxa are found, including *I. glandulifera* Royle and *I. balfourii* Hook.f.[9] *I. glandulifera* is naturalised alongside waterways and in forests, and is considered a highly invasive species, while *I. balfourii* can colonize open habitats and is also considered invasive in Croatia.[9–11]

Studies conducted in France (Alsace) and Czechia (Ceske Budejovice) confirmed the presence of NQs in plant material of some *Impatiens* species growing in Europe.[12,13]

In these studies, conventional methods for extraction of NQs were used, and NQs were detected by HPLC. However, there is no data on the presence of NQs in *Impatiens* species grown wild in Croatia and the level of NQs in *I. balfourii* was not reported in the literature. Since *Impatiens* species contain high levels of NQs that have the potential to be developed as novel therapeutics, the aim of this study was to determine NQs levels in the two most widespread invasive alien species of the genus *Impatiens* in Croatia.

The objective of this study was to determine NQs in plant material (leaves and flowers) of *I. glandulifera* and *I. balfourii* grown wild in Croatia. A reliable HPLC method for simultaneous quantification of 2-HNQ and 2-MNQ in plant material was optimised and two plant extract preparation procedures were tested. The NQs levels in *I. glandulifera* and *I. balfourii* plant material collected in Croatia are reported for the first time.

# EXPERIMENTAL

## Chemicals and Preparation of Standards

2-HNQ (2-hydroxy-1,4-naphthoquinone, 98 % purity) and 2-MNQ (2-methoxy-1,4-naphthoquinone, 98 % purity) standards were procured from Sigma Aldrich (St. Louis, USA). Ethanol (96 %, *p.a.* grade) used for extraction was from Kemika (Zagreb, Croatia). Methanol and acetic acid used for HPLC mobile phase were of HPLC grade and were obtained from Kemika (Zagreb, Croatia). MilliQ water (18.2 MΩ $cm^{-1}$) was used for mobile phase preparation.

2-HNQ and 2-MNQ standards stock solutions in concentration 20 mg $mL^{-1}$ were prepared in HPLC grade methanol. Working standards (in the concentration range 2-100 μg $mL^{-1}$) were prepared by diluting standard stock solutions with HPLC grade methanol.

## Plant Material Collection

The *I. glandulifera* and *I. balfourii* leaves and flowers were collected in the continental part of Croatia (Čučerje, near the city of Zagreb) during the flowering season (August and September) of 2016. The samples obtained were authenticated at the Faculty of Science University of Zagreb.

Afterward, the leaves and flowers were air-dried protected from the sun and stored in a brown paper bag at room temperature (25 °C) in the dark until extract preparation.

## Preparation of Plant Extracts

For each plant sample, two extraction methods were used: decoction and ultrasound-assisted extraction (ultrasonic extraction). For the decoction procedure, 500 mg of the grounded air-dried plant material was decocted in 10 mL of 96 % ethanol (*v*/*v*) under reflux conditions for 45 minutes. The ultrasonic extraction consisted of two steps. In the first step, 500 mg of the grounded air-dried plant material was extracted by ultrasound in 5 mL of 96 % ethanol (*v*/*v*) for 15 minutes at 40 °C, after which the extract was filtered through cotton wool. In the following step, the cotton wool was washed with 5 mL of 96 % ethanol (*v*/*v*) and the ultrasonic extraction process was repeated under the same conditions. The extracts from the first and the second ultrasonic extraction were then combined. Prior to HPLC analysis, 1 mL of the ethanolic extract was filtered through 0.2 μm PTFE syringe filter.

## HPLC Analysis

The HPLC analysis was performed on HPLC Agilent 1100 (Santa Clara, CA, USA), which consisted of a gradient pump, autosampler, and diode-array detector (DAD). Data were acquired and processed by ChemStation for LC 3D software. Separation of 2-HNQ and 2-MNQ was achieved using the analytical column LiChrospher RP-C18 (150 mm × 4.6 mm, particle size 5 μm; Merck, Darmstadt, Germany). Mobile phase consisted of methanol (A) and 2 % acetic acid (B); gradient elution: 0–10 min 25 % B; 10–20 min 32 % B; 20–35 min 45 % B; 35–42 min 25 % B, with a flow rate set to 1 mL $min^{-1}$. The injection volume was 20 μL. Chromatograms were recorded at 280 nm. The retention time of 2-HNQ was 16.3 min and 2-MNQ was 22.1 min. NQs were quantified based on a calibration curve prepared from respective standards (2-HNQ or 2-MNQ).

# RESULTS AND DISCUSSION

## Validation of the HPLC Method

The HPLC method for quantification of 2-HNQ and 2-MNQ in plant extracts was validated and the results are presented in Table 1.

To test method linearity, 2-HNQ and 2-MNQ standards in a concentration range from 2 to 100 μg $mL^{-1}$ were used. For each calibration point, standard was injected into the HPLC instrument at least three times and calibration curves were obtained by plotting the peak area against the concentration of each standard. Obtained calibration curves of both standards (2-HNQ or 2-MNQ) were linear. The correlation coefficient ($R^2$) for 2-HNQ and 2-MNQ were 0.9841 and 0.9826, while their calibration curves were $y = 1 \times 10^{-5}x - 0.0028$ and $y = 1 \times 10^{-5}x - 0.0030$, respectively.

The method precision (expressed as intra- and inter-day precision) was tested by injecting 2-HNQ and 2-MNQ standards at the lowest concentration (2 μg $mL^{-1}$; Figure 1) into the HPLC instrument in six replicates on three consecutive days. The obtained relative standard deviation (RSD) for intra-day precision was below 5 % and for inter-day precision was below 10 % for both, 2-HNQ and 2-MNQ.

**Table 1.** Validation data of HPLC method for assessment of 2-hydroxy-1,4-naphthoquinone (2-HNQ) and 2-methoxy-1,4-naphthoquinone (2-MNQ).

| Validation parameter | 2-HNQ | 2-MNQ |
|---|---|---|
| Calibration curve intercept | −0.0028 | −0.0030 |
| Correlation coefficient ($R^2$) | 0.9841 | 0.9826 |
| LOD / μg mL$^{-1}$ | 0.1 | 0.5 |
| LOQ / μg mL$^{-1}$ | 0.1 | 0.5 |

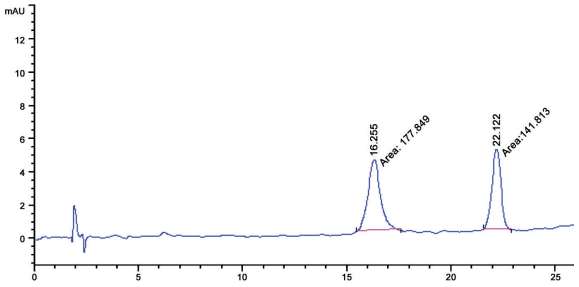


**Figure 1.** Chromatogram of 2-hydroxy-1,4-naphthoquinone (2-HNQ; retention time 16.3 min) and 2-methoxy-1,4-naphthoquinone (2-MNQ; retention time 22.1 min) standards at 2 μg mL$^{-1}$ concentration.

The method sensitivity is given as limit of detection (LOD) and limit of quantification (LOQ). LOD and LOQ were calculated as the 3 times and 10 times of the signal-to-noise ratio of the standard of the lowest concentration, respectively. For both, 2-HNQ and 2-MNQ, the calculated LOD was 0.1 μg mL$^{-1}$ while LOQ was 0.5 μg mL$^{-1}$.

Validation parameters indicate that the method is linear, precise, and sensitive and allows simultaneous determination of both NQs, and therefore can be used for quantification of 2-HNQ and 2-MNQ in plant extracts.

## Quantification of NQs in *I. glandulifera* and *I. balfourii* Plant Material

Species from the *Impatiens* genera produce a high amount of NQs, predominantly 2-HNQ and 2-MNQ.[8,13] Previous studies conducted on *Impatiens* species plant material collected in Europe confirm the presence of 2-HNQ and 2-MNQ in *I. glandulifera*.[12,13] However, until now the level of NQs was not investigated in *I. balfourii*. Moreover, there is no data on the NQs levels of *I. glandulifera* and *I. balfourii* growing wild in Croatia. Therefore, in this study plant material (leaves and flowers) of both plants were collected (August-September, during the flowering season) and 2-HNQ and 2-MNQ levels were determined.

For the extraction of NQs from plant material (grounded air-dried leaves or flowers) in this study ethanol was used, as was done similarly in studies of other researchers.[3,11,12] Two types of extraction procedures were compared, decoction as the conventional method and ultrasonic extraction, as the non-conventional method. The non-conventional procedures such as ultrasound-assisted extractions, are considered environmentally friendly due to the lower volume of organic solvents used, reduced operational time, better yield and better quality of extract obtained.[14,15] Results indicate that a higher NQs yield is obtained using decoction in comparison to ultrasound-assisted extraction. Decoction of flowers of both plant species, *I. glandulifera* and *I. balfourii*, yield nearly double 2-MNQ amount in comparison to ultrasonic extraction. In the *I. glandulifera* flowers, the level of 2-MNQ was 60.6 ± 1.9 μg mL$^{-1}$ after decoction, and ultrasonic extraction yield 36.6 ± 1.3 μg mL$^{-1}$ of 2-MNQ (Table 2). The 2-MNQ levels in the *I. balfourii* flowers were 2.2 ± 0.3 μg mL$^{-1}$ and 0.7 ± 0.05 μg mL$^{-1}$, for decoction and ultrasonic extraction, respectively (Table 2). The extraction efficiency depends on matrix properties, extraction solvent, temperature, pressure, and extraction duration.[14,15] The higher yield of NQs by decoction extraction can be explained by increased temperature for 45 minutes under reflux conditions.

Results are presented in Table 2. Each sample is injected to the HPLC in triplicates, and the results are expressed as mean value ± standard deviation. Regardless of the extraction procedure applied, in leaves and flowers of *I. glandulifera* collected in Croatia, only 2-MNQ was found, while 2-HNQ was not detected. In *I. glandulifera* leaves and flowers collected in Alsace and around Ceske Budejovice higher levels of 2-MNQ in comparison to 2-HNQ was detected.[12,13] In leaves, stems, or flowers of *I. glandulifera* collected in Alsace throughout the season (from May to October), Lobstein *et al.* (2001) observed higher levels of

**Table 2.** The concentration of 2-methoxy-1,4-naphthoquinone (2-MNQ) in *I. glandulifera* and *I. balfourii* plant material extracts prepared by decoction and ultrasonic extraction.

| Species | sample | Extraction procedure | 2-MNQ / μg mL$^{-1}$ |
|---|---|---|---|
| *I. glandulifera* | leaves | Decoction | 20.6 ± 1.4 |
| | leaves | Ultrasonic | 17.5 ± 0.7 |
| *I. glandulifera* | flowers | Decoction | 60.6 ± 1.9 |
| | flowers | Ultrasonic | 36.6 ± 1.3 |
| *I. balfourii* | leaves | Decoction | n.d. |
| | leaves | Ultrasonic | n.d. |
| *I. balfourii* | flowers | Decoction | 2.2 ± 0.3 |
| | flowers | Ultrasonic | 0.7 ± 0.05 |

2-MNQ in comparison to 2-HNQ. In the study of Tříska *et al.* (2013) in *I. glandulifera* leaves collected around Ceske Budejovice, regardless of the year of collection, type of drying, storage procedure, and solvent used for extraction (methanol or water), higher levels of 2-MNQ than 2-HNQ were detected. Thus, it can be concluded that 2-MNQ is the major NQ in *I. glandulifera* leaves and flowers.

In leaves of *I. glandulifera* collected around Ceske Budejovice concentration of 2-MNQ was from 69 to 1301 mg $kg^{-1}$.[13] Detected concentration of 2-MNQ in leaves of *I. glandulifera* collected in Alsace was in the range 0.017–0.68 % of dry weight (0.17 g $kg^{-1}$–6.8 g $kg^{-1}$) while in flowers the range was 0.11–0.83 % of dry weight (1.1–8.3 g $kg^{-1}$).[12] In this study, the concentration of 2-MNQ in the leaves of *I. glandulifera* was 20.6 μg $mL^{-1}$ (the equivalent of 400 mg $kg^{-1}$ of dry weight) (Table 2). The detected level of 2-MNQ in leaves of *I. glandulifera* collected in Croatia was higher than the level of 2-MNQ reported by Lobstein *et al.* (2001), however, it was approximately the same concentration as 2-MNQ reported by Tříska *et al.* (2013) in leaves of *I. glandulifera* collected around Ceske Budejovice.

A significantly higher concentration of 2-MNQ was detected in flowers than in leaves of *I. glandulifera* collected in Croatia: 60.6 μg $mL^{-1}$ (approximately 1200 mg $kg^{-1}$ of dry weight) (Table 2). Previous studies noted that the level of NQs in *I. glandulifera* depends on plant organ.[12,13,16] Throughout the annual vegetative cycle, a variation in the 2-MNQ level was observed; young leaves were particularly rich in NQs and the decrease of 2-MNQ concentration was observed with plant age (by the end of the growing season their concentration decreases).[12,16] At the beginning of the growing season Lobstein *et al.* (2001) detected higher NQs levels in the leaves, while in the flowering period NQs were localised in the flowers. Since plant material (leaves and flowers) was collected during the flowering season, herein results confirm that during the flowering season 2-MNQ is localised in flowers of *I. glandulifera* (higher level of 2-MNQ is detected in flowers than in leaves). Although 2-MNQ concentration in *I. balfourii* was low, it can be noted that during the flowering season 2-MNQ is localised in flowers of this species as well (Table 2). Block *et al.* (2019) explained the higher NQs concentrations in *I. glandulifera* flowers by a possible spread reduction of pathogenic fungi by pollinators; NQs in *I. glandulifera* flowers could influence the nectar microbiome due to its antifungal activity and thereby reducing the spread of pathogenic fungi by pollinators.

To our knowledge this is the first study that investigated level of NQs in *I. balfourii* plant material. Similar to *I. glandulifera* leaves and flowers, 2-HNQ was not detected in *I. balfourii* leaves and flowers. In *I. balfourii* flowers the 2-MNQ level was 2.2 μg $mL^{-1}$ (approximately 44 mg $kg^{-1}$ of dry weight), while in leaves 2-MNQ was not detected (Table 2). The detected level of 2-MNQ in *I. balfourii* flowers was 30 times lower than in *I. glandulifera* flowers. Lobstein *et al.* (2001) compared the level of 2-HNQ and 2-MNQ in several European species of the genus *Impatiens*: *I. capensis* Meerb, *I. glandulifera*, *I. noli-tangere*, and *I. parviflora* DC collected in Alsace during the flowering season. In flowering aerial parts of all species, 2-HNQ was in higher concentration than 2-MNQ, except for flowers of *I. glandulifera* for which the opposite was true (2-MNQ was in a higher concentration than 2-HNQ). The level of 2-MNQ in flowers of *I. glandulifera* was significantly higher in comparison to other *Impatiens* species *e.g.* the level of 2-MNQ in flowers of *I. noli-tangere* was 20 times lower (0.030 % of dry weight *vs.* 0.631 % of dry weight of *I. glandulifera*). As already mentioned in flowers of *I. balfourii* as in flowers of *I. glandulifera* 2-MNQ was only detected, and in comparison to other European species of the genus *Impatiens* investigated by Lobstein *et al.* (2001) the level of 2-MNQ in *I. balfourii* flowers was higher than in *I. parviflora* but lower than in *I. capensis* and *I. noli-tangere.* Results of this study confirm the variability of NQs concentration among various *Impatiens* species and confirm high 2-MNQ level in *I. glandulifera*. Therefore, *I. glandulifera* can be considered a promising source of 2-MNQ whose antitumor activity is observed earlier.[3]

## CONCLUSION

This study describes a reliable method for simultaneous determination of 2-HNQ and 2-MNQ in plant material. Validation parameters confirm that the applied method is linear, sensitive, and precise. It is demonstrated that a higher NQs yield from plant material into ethanol is gained by decoction compared to ultrasonic extraction. This is the first study that studied 2-HNQ and 2-MNQ levels in leaves and flowers of *I. glandulifera* and *I. balfourii* collected in Croatia. It was found that both species contain higher levels of 2-MNQ compared to 2-HNQ and that in both species, during the flowering season, 2-MNQ is present in higher levels in flowers compared to leaves. Additionally, it was determined that *I. glandulifera* has higher levels of NQs compared to *I. balfourii* confirming *I. glandulifera* as a rich source of 2-MNQ. The results indicate the potential use of invasive alien plants that are otherwise difficult to manage.

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