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An Early Quantitative Approach to Phytochemical Composition of the Aqueous Extract of *Triticum vulgare*

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Research Article

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ABSTRACT

Plant extracts revealed advantageous properties in wound healing and skin repair. In particular, the aqueous extract of *Triticum vulgare* (TVE), which is widely recognized to speed up healing processes, has been so far used in cutaneous creams, gauzes and vaginal creams, solutions and pessaries under the brand name Fitostimoline®. Recently, it has been demonstrated that the TVE largely comprises poly- and oligosaccharides and it is involved in tissue repair. Thus, since many of the documented TVE beneficial effects cannot be merely ascribed to poly/oligosaccharidic components, an exploration of the low-weight phytochemical constituents accountable for such anti-inflammatory and anti-oxidant properties has been performed. Eight bioactive metabolites, namely Allantoin, S-cis-Abscissic acid, Ferulic Acid-D-Glucoside, Luteolin, Jasmonic Acid, 3-(4-hydroxy-3-methoxyphenil)-Propionic Acid, 2-Methoxy-Benzoquinone and 2,6-Dimethoxy-Benzoquinone, were selected and quantified through an accurate UPLC-MRM-MS analysis. The TVE matrix was found to be rich in many of them and thus, they can be recognized as responsible for its anti-inflammatory and anti-oxidant bioactivity.

INTRODUCTION

Plant extracts have shown beneficial properties in wound healing, helping the skin repair through the enhancement of cell-cell and cell-matrix connections and adjuvating immunomodulation processes [1]. In this scenario, the aqueous extract of *Triticum vulgare* (TVE), obtained from the whole germinated plant and containing mainly poly/oligosaccharidic components, has many biological properties, acting as bioactive complex on wound repairing factors [2,3]. This extract is currently an active component of several medicinal products under the brand name Fitostimoline® (Farmaceutici Damor S.p.A., Naples, Italy). In fact, TVE-based products speed up healing processes

both in cutaneous and not cutaneous models, thanks to a complex and not yet fully understood mechanism of action [4]. Up to now, it has been reported that TVE increases collagen I and elastin expressions and positively modulates integrin and acquaporin-3, occurring in a better remodeling of dermal tissue during healing [5]. TVE is frequently used for the treatment of decubitus ulcers, venous leg ulcers, burns and more generally for re-epithelialization or tissue regeneration, prompting tissue repairing processes, stimulating chemotaxis and fibroblastic maturation [6].

Recent studies on *in vitro* and *in vivo* skin lesion models demonstrated that TVE possesses anti-inflammatory properties since it has been shown to reduce nitric oxide, IL-6, PGE2 and TNF and to modulate protein-kinase B and matrix metalloproteinases 9 protein expression in BV-2 cells [7,8].

Thus, to elucidate TVE role involved in the complex mechanism of wound healing processes, have selected some low molecular weight phytochemical constituents, conceivably occurring in TVE, which could be responsible for such regenerative, anti-inflammatory and anti-oxidant properties. Thus, eight bioactive metabolites, namely Allantoin, S-cis-Abscissic acid, Ferulic Acid-D-Glucoside, Luteolin, Jasmonic Acid, 3-(4-hydroxy-3-methoxyphenil)-Propionic Acid, 2-Methoxy-Benzoquinone and 2,6-Dimethoxy-Benzoquinone, were identified and selected to be quantified through an accurate UPLC-MRM-MS analysis.

MATERIALS AND METHODS

TVE extracts were prepared as follows: 3 ml of each sample were snap frozen in dry ice/acetone, lyophilized and dissolved in 300 µl of H_2O/ACN 90/10.

Calibration curves were prepared as mixtures (in H_2O/ACN 90/10), ranging from 125 nM to 100 µM for each molecule.

The MRM-MS method was build-up infusing standard solutions of the compounds (0.2 µg/µl in H_2O/ACN 50/50) into a Sciex 6500 QTRAP mass spectrometer (ABSciex, Foster City, CA, USA) at a flow rate of 10 µl/min. Full scan and Collision Induced Dissociation (CID) spectra were acquired to optimize spectral parameters for each compound. Thus, a comprehensive MRM method was obtained and the transitions are reported in Supplementary Table 1, alongside with the Declustering Potential (DP), Entrance Potential (EP), Collision Energy (CE), and Collision Cell Exit Potential (CXP) parameters.

The quantitative analysis was carried out injecting 10 µl of the standard mixture and/or of TVE extracts on a Shimadzu Nexera LC system equipped with a Synergi Fusion-RP column (2.5 µm, 100 Å, 50 × 2 mm; Phenomenex, Torrance, CA, USA) and interfaced with a Sciex 6500 QTRAP mass spectrometer. The mobile phases were H_2O 0.1% formic acid (A) and ACN 0.1% formic acid (B) at a flow rate of 0.400 ml/min, using the following gradient: 1 min at 3% B, 1 min to 15 min from 3% to 90% B, 15 min to 18 min held at 90% B and back to 3% B. Sciex 6500 QTRAP operated in negative MRM scan mode and Analyst software (version 1.6.2, ABSciex, Foster City, CA, USA) was exploited for data acquisition and processing.

For the quantification of 2-methoxy-benzoquinone and 2,6-dimethoxy-benzoquinone, Girard's reagent T (Sigma Aldrich-Merck, Saint Louis, USA) was used.

Thus, 20 µl of each extract were added with 400 µl of MeOH, 30 µl of acetic acid and 40 µl of a 750 µg/µl Girard's reagent T solution and were incubated overnight at 25°C, under continuous shaking and in the dark.

A calibration curve of 2-methoxy-benzoquinone and 2,6-dimethoxy-benzoquinone was obtained in the same experimental conditions, ranging from 0.5 µM to 300 µM. Samples were then dried under vacuum and re-dissolved in H_2O/ACN 90/10 and 10 µl were analyzed as described : the two compounds……

The two compounds were separated using the following gradient; 0.5 min at 0% B, 0.5 min to 3 min from 0% to 5% B, 3 min to 5 min from 5% to 95% B, 5 min to 7 min held at 95% B and then back to 0% B. Sciex 6500 QTRAP was operated in positive MRM scan mode, and the transitions of the two analyzed metabolites are reported in Supplementary Table 1, alongside their DP, EP, CE, and CXP.

Linearity was determined by the correlation values of calibration curves (Supplementary Table 2). The Limit of Quantification (LOQ) and the Limit of Detection (LOD) were evaluated injecting a series of progressively diluted standard solutions until a signal-to-noise ratio of 10 and 3 were achieved, respectively.

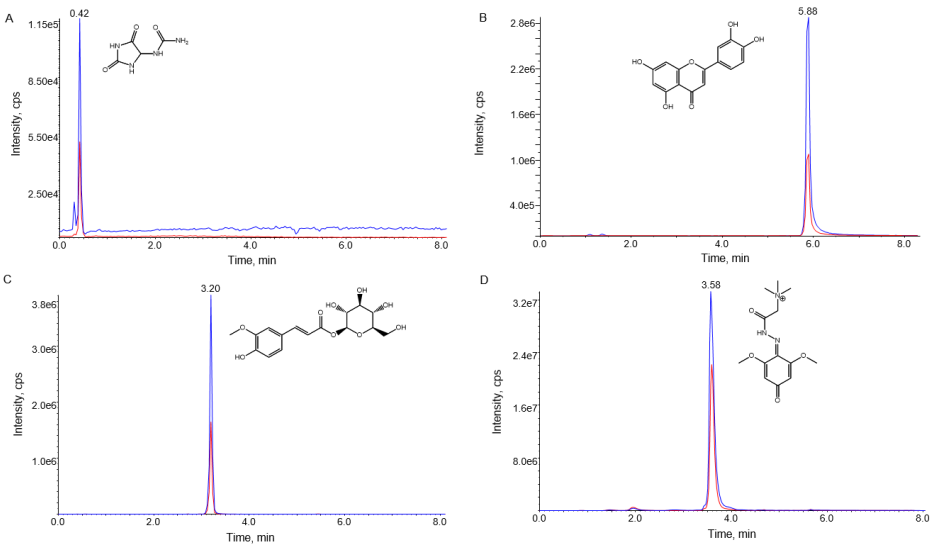
RESULTS AND DISCUSSION

To deepen TVE phytochemical profile, eight low molecular weight active molecules have been quantified by a targeted metabolomic approach, utilizing UPLC coupled to Multiple Reactions Monitoring (MRM-MS).

To correctly assign spectral parameters to each compound, their MS optimization was performed by infusing the relative standard solutions in the ESI-QTRAP system and the obtained data were exploited to build-up two selective and sensitive MRM-MS methods (Supplementary Table 1). More in details, six over eight metabolites such as Allantoin, S-cis-Abscissic acid, Ferulic Acid-D-Glucoside, Luteolin, Jasmonic Acid and 3-(4-hydroxy-3-methoxyphenil)-Propionic Acid were simultaneously quantified via an experimental procedure interfaced with a negative polarity UPLC-MRM-MS method. Besides, 2-Methoxy-Benzoquinone and 2,6-Dimethoxy-Benzoquinone were submitted to a reaction with Girard's reagent T, an acetyl hydrazide trimethylammonium chloride commonly exploited to convert ketones into the corresponding imines bearing a quaternary ammonium, showing a much better MS ionization capability. This easy, fast and complete reaction allowed us to simply quantify the quinones with higher sensitivity and confidence through a positive polarity UPLC-MRM-MS method (Supplementary Table 1).

Thus, TVE extracts were snap-frozen in dry ice/acetone, lyophilized and then re-dissolved in H_2O/ACN to either quantify metabolites or to be submitted to the reaction with Girard's reagent T (high molar excess, 25°C, overnight) for the analysis of the two quinones. Figure 1 reports some exemplificative chromatograms of the 3 out of the 6 metabolites obtained injecting the unreacted standard mixture (panels A-C) and of 2,6-dimethoxy-benzoquinone corresponding imine obtained after Girard's T reaction (panel D).

Figure 1. Exemplificative traces of 4 of TVE analyzed metabolites, namely allantoin (A), luteolin (B), ferulic acid-D-Glucoside (C) and 2,6-dimethoxy-benzoquinone modified by Girard's T reagent (D). For each metabolite, two transitions are reported.



TVE extracts quantification was achieved selecting the best transition for each metabolite, as the one showing the highest intensity and signal-to-noise ratio. Another transition, when possible, has been used for an unambiguous identification. Following, three different TVE samples were analyzed in triplicate and the obtained results, expressed as mean ± standard deviation of µg of each analyte per litre of extract, are reported in Table 1 alongside the corresponding Limit of Quantification (LOQ) and Limit of Detection (LOD).

As it can be observed, Ferulic Acid-D-Glucoside, 2-Methoxy-Benzoquinone and 2,6-Dimethoxy-Benzoquinone resulted as the most abundant low-molecular weight components of TVE extracts, whereas allantoin, 3-(4-hydroxy-3-methoxyphenil) propionic acid, S-cis-abscissic acid, Luteolin and Jasmonic Acid were present in lower amounts.

Table 1. UPLC-MRM-MS quantitative analysis results. For each compound, the concentration in TVE extracts is expressed as the mean ± standard deviation of the three production lots analyses, as µg/l. Furthermore, retention time, Limit of Quantification (LOQ) and Limit of Detection (LOD) are also reported.

Compound	Retention time (min)	TVE concentration (µg/l)	LOQ (µg/l)	LOD (µg/l)
Allantoin	0.42	134 ± 3	0.004	0.0013
S-cis-abscissic acid	4.98	0.14 ± 0.05	0.0066	0.0022
Ferulic acid D-Glucoside	3.2	2153 ± 175	0.1782	0.0594
Luteolin	5.87	0.15 ± 0.03	0.1431	0.0477
Jasmonic acid	5.42	0.33 ± 0.02	0.3154	0.1051
3-(4-Hydroxy-3-methoxyphenil) propionic acid	3.28	4.5 ± 0.7	0.5882	0.1961
2,6-Dimethoxy-benzoquinone imine derivative	3.58	602 ± 74	0.0282	0.0094
2-Methoxy-benzoquinone imine derivative	4.16	10821 ± 1410	0.5493	0.1831

CONCLUSIONS

In this communication, we have reported the quantification of a few low molecular weight TVE phytochemicals to lay basics for a better understanding of TVE potential beneficial properties and to shed light on its mechanism of action. Our UPLC-MRM-MS analysis showed how the analyzed compounds, 2-Metoxy-Benzoquinone, Ferulic Acid-D-Glucoside, 2,6-Dimethoxy-Benzoquinone and Allantoin, contribute for the major part to the extract composition.

As thoroughly reported in literature, Ferulic acid, its derivative such as glucosides and its reduction products are abundant phenolic phytochemicals showing a great anti-oxidant activity [9-11], primarily related of scavenging of free radicals, binding transition metals (e.g., iron and copper) and preventing lipid peroxidation. They can exert such anti-oxidant effects by forming a phenoxy radical, whose stable resonance structures causes propagation of chain reactions initiated by free radicals [12]. Furthermore, the ferulic part of the molecule is able to chelate metal ions, such as Cu(II) or Fe(II) [13,14] and to up-regulate the heme oxygenase-biliverdin reductase system, thus prompting the production of the endogenous free radical scavenger bilirubin [15-18].

Next, quinones consist of a class of bioactive compounds with promising potential, mainly as anti-oxidants since they are the universal mediators of electron transport; besides, it is well known that they are components for anti-cancers drugs. In particular, 2,6-Dimethoxy-Benzoquinone exerts a strong *in-vitro* cytotoxicity against human tumor cell lines. Recent data [19] also suggest that both 2-Metoxy-Benzoquinone and 2,6-Dimethoxy-Benzoquinone possess also anti-proliferative and anti-metastatic on human cancer cell lines; moreover, both are able to kill tumor cells by the induction of apoptosis *via* the caspase-poly [ADP-ribose] polymerase-pathway [20].

TVE extracts also contain Allantoin, which can be considered as a free-radical scavenger with anti-oxidant properties [21]. Besides, Allantoin can reduce erythema on irritated skin [22,23].

In between the other recognized metabolites, Jasmonic Acid has been reported to exhibit anti-cancer, anti-inflammatory and anti-aging assets acting on extracellular matrix remodeling and promoting growth factor activities and skin wound healing [24,25].

Regarding Luteolin, this flavonoid is able to decrease adverse photobiological effects in the skin by acting as a first line of defense and it has also anti-oxidative and anti-inflammatory activities on keratinocytes and fibroblasts [26], also decreasing the prototypic inflammatory cytokine IL-6 [27].

Finally, the isoprenoid stress-associated phytohormone Abscissic Acid has recently been known to own stress-related functional characteristics which contribute to improve inflammatory defense in living systems also thanks to its anti-oxidative properties [28]. Besides its anti-inflammatory and immunostimulant action, other remarkable Abscissic Acid properties are the anti-angiogenic one [29] and the stimulation of the mesenchymal stem cells, which may open a new avenue for its potential use in the field of regenerative medicine [30,31].

Taken together, our quantification results show that the TVE, which is well-known to be mainly composed by oligo and polysaccharides, is also rich of small phytochemicals, each endowed with many beneficial properties such as anti-oxidant, anti-inflammatory and anti-cancer ones. This is the first research report in which low molecular weight components of TVE (constituting the active ingredients of the worldwile exported brand Fitostimoline®) are identified and quantified, disclosing important features on its chemical composition and shedding light on its whole mechanism of action.

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