British Journal of Pharmaceutical Research
11(4): 1-8, 2016, Article no.BJPR.25785
ISSN: 2231-2919, NLM ID: 101631759

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Cytotoxic and Hepatoprotective Effects of *Bupleurum flavum* Flavonoids on Hepatocellular Carcinoma HEP-G2 Cells

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Authors' contributions

This work was carried out in collaboration between all authors. Authors RG, ND, SR and VL designed the study, wrote the protocol and wrote the first draft of the manuscript. Authors SK and VM managed the literature searches, analyses of the study performed the spectroscopy analysis and author DZD managed the experimental process and author RG identified the species of plant. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BJPR/2016/25785
Editor(s):
(1) Dongdong Wang, Department of Pharmacogonosy, West China College of Pharmacy, Sichuan University, China.
Reviewers:
(1) Bechan Sharma, University of Allahabad, Allahabad, India.
(2) Mingfeng Qiu, Shanghai Jiao Tong University, P.R. China.
Complete Peer review History: http://sciencedomain.org/review-history/14298

Original Research Article

Received 20th March 2016
Accepted 16th April 2016
Published 22nd April 2016

ABSTRACT

Aims: In this study, we aimed at investigating the possible cytotoxic and hepatoprotective effects of crude extract, flavonoid mixture and pure flavonoids isolated from the aerial parts of *Bupleurum flavum* Forsk. (Apiaceae) native to Bulgaria.
Methods: For the first time flavon C-glycoside vicenin-2 was isolated from *B. flavum.* Its structure was identified by LC-ESI/MS/MS analysis in positive ion mode. Cytotoxic effects of vicenin-2, as well as the known flavonoid narcissin and *B. flavum* crude extract at concentrations ranging from

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25 to 400 µg/ml were tested on cultured hepatocellular carcinoma HEP-G2 cells for 72 h by MTT assay. *In vitro* hepatoprotective activities of *B. flavum* flavonoid mixture (BFF), and rutin and narcissin isolated from BFF, were evaluated on epirubicin-induced damage.

Results: IC_{50} of 141.7 µM, 195.7 µM and 369.3 µg/ml was calculated for vicenin-2, narcissin and crude extract, respectively. Curcumin was used as positive control with IC_{50} 17.90 µM. At a concentration 0.4 mg/ml of rutin, the strongest cytoprotective effect was verified, discerned by statistically significant (P = 0.05) increase in cell viability by 43.5%. However, incubation with narcissin had no significant hepatoprotective activity.

Conclusions: Vicenin-2 showed the most prominent cytotoxic effect at the highest tested concentration. Rutin in BFF had function for the observed protection against oxidative damage caused by the epirubicin. Therefore, *B. flavum* flavonoids are worth investigating for the potential development of agents against hepatocellular carcinoma.

Keywords: Bupleurum flavum; vicenin-2; rutin; narcissin; hepatocellular carcinoma; cytotoxicity.

1. INTRODUCTION

Bupleurum L. (Apiaceae) species are important ingredients in many multi-herb remedies in traditional Chinese medicine with liver-protecting activity [1,2]. For instance, hepatoprotective and antioxidant properties of herbal prescriptions formulated with roots of *B. fruticosum* L. (Apiaceae) [3], *B. kaoi* Liu (Chao et Chuang) [4], and *B. scorzonerifolium* Willd. [5] have been shown on carbon tetrachloride-, d-galactosamine-, and lipopolysaccharides-induced injuries in rat hepatocytes. Indeed, saikosaponins from *Bupleurum* spp. are thought to be potential hepatoprotective agents against acute and chronic liver injury [6]. Previous reports have shown that some saponins from *Bupleurum* species suppress hepatocarcinoma [7] and improve chemotherapy [8].

To facilitate effective resource utilization, Nakahara et al. [9] have investigated aerial parts of *B. falcatum* L. and *B. rotundifolium* L., which are commonly discarded, for saponin content and hepatoprotective activity. Together with the saponins, the high concentrations of polyphenols significantly contribute to the observed hepatoprotective effects of *Bupleurum* spp. It has been shown that aerial parts from *B. falcatum*, *B. kaoi*, *B. rigidum* L. and *B. fruticescens* L. have scavenging activity against DPPH, ABTS and superoxide anion radicals [10-12]. Furthermore, a leaf infusion of *B. kaoi* decreased the hepatotoxicity of paracetamol and CCl_4 in rat hepatocytes, as indicated by increased viability of damaged cells [10]. Indeed, the majority of herbal drugs comprising polyphenols have been reported to exert antioxidant activity by scavenging of free radicals and reducing ability, and in this way to protect against various induced oxidative stress [13]. There are literature data revealing the high flavonoids/total polyphenols ratio as one of the features responsible for antioxidant activity [14]. It has been shown that flavonoids combined with some conventional chemotherapeutics agents exerted synergistic inhibitory effect on tumor growth or alleviated toxic side effects [15]. Moreover, phytochemicals such as flavonoids and saponins were reported to have great potential in antiHIV-1 chemotherapeutics [16].

Bupleurum flavum Forsk. is an annual plant of the Eastern Mediterranean area (Bulgaria, Greece, and Turkey) [17]. A previous study of the aerial parts of *B. flavum* led to the isolation of five lupane-type triterpenoids, one lignan and eight flavonoids [18]. Recently, we revealed a significant free radical scavenging activity of the aerial parts from *B. flavum* using the DPPH and ABTS methods, as well as ferric reduction ability (FRAP) [19]. Administration of *B. flavum* flavonoid mixture (BFF), consisting of rutin and narcissin, and pure flavonoids, isolated from the same mixture, markedly counteracted the harmful effects of both carbon tetrachloride (CCl_4) and tert-butylhydroperoxide (*t*-BuOOH) in rat hepatocytes as indicated by the elevation of glutathione (GSH) and reduction of lactate dehydrogenase (LDH) and malondialdehyde (MDA) [20]. In addition, a stronger antioxidant effect of BFF in microsomes was evidenced in comparison with pure flavonoids rutin and narcissin, as well as the natural hepatoprotector silymarin.

As a part of our ongoing investigation on *B.flavum*, the present study reports the isolation and structural elucidation of the C-flavonoid vicenin-2. The study was aimed at investigating the cytotoxic effects of the crude extract and pure flavonoids narcissin and vicenin-2 on

hepatocellular carcinoma HEP-G2 cells. In addition, we evaluated the potential cytoprotective effect of the studied compounds on epirubicin-induced damage in the same cell line.

2. MATERIALS AND METHODS

2.1 Plant Material, Isolation of Vicenin-2 and Preparation of Solutions for Cell Viability Assay

Aerial parts of *B. flavum* were collected in August 2006 at the Black Sea coast (St. Vlas region) (42°42′00″ N – 27°48′00″ E). The plant was identified by one of us (R.G.). Voucher specimen of plant material (SOM 171498) was deposited at Institute of Biodiversity and Ecosystems Research, Bulgarian Academy of Sciences, Sofia, Bulgaria.

Air-dried powdered parts of *B. flavum* (25 g) were extracted with aqueous methanol (250 ml, 80%, v/v) by sonication (2 × 15 min each time) to yield the crude extract. The crude extract was fractionated by low pressure liquid chromatography over a C18 column (310 x 25 mm, 40-63 µm, Merck, Germany) using initial passage of water (100 mL) and then a step-gradient elution to give 8 flavonoid fractions. The binary solvent system consisted of water (solvent A) and methanol (solvent B), and separation was achieved using the following step-gradient: 20% B (100 ml), 70% B (600 ml) and 100% B (100 ml). Fractions 1-4 were concentrated in vacuo at 40°C and further purified by a repeated low pressure liquid chromatography to give vicenin-2 (8 mg, 96% HPLC). In addition, rutin, narcissin and *B. flavum* flavonoid mixture (BFF) were obtained as described earlier [20]. The crude extract, BFF and individual flavonoids were dissolved in DMSO and concentration was adjusted at 20 mg/ml as the stock solutions which were then filtered through a 0.22 µm filter (Millipore, Bedford, MA) and stored at 4°C. For the cell viability assay, the stock solutions were appropriately diluted.

2.2 LC-MS/MS Analysis

The LC-MS/MS analysis was performed by LTQ Orbitrap® Discovery mass spectrometer (Thermo Scientific Co, USA) equipped with Surveyor® Plus HPLC system consisted of a binary gradient pump, autosampler and PDA detector (Thermo Scientific Co, USA) and electrospray ionization module IonMax® (Thermo Scientific Co, USA). LC separation was carried out on XBridge C18 column (I.D 2.1 × 150 mm, 3.5 µm particle size) (Waters, Germany) by a gradient elution at room temperature. Eluent A was 0.1% formic acid in water, and eluent B was 0.1% formic acid in acetonitrile. The gradient program was as follows: 0% B for 3 min, 0-50% B for 7 min, 50% B for 1 min, 50-0% B for 1 min, and 0% B for 3 min. The flow rate was 250 µl/min, and the injection volume was 10 µl. For the MS and MS/MS experiments the instrument was set to collect data in positive ion mode at resolution settings of 30,000. Full scan spectra over the m/z range 150-2000 were acquired using the following MS parameters: spray voltage, 3.8 kV; sheath gas flow rate, 35; auxiliary gas flow rate, 8; sweep gas flow rate, 2; capillary voltage, 36 V; capillary temperature, 250°C; and tube lens 125 V. Normalized collision energy used for vicenin-2 was set to pulsed Q collision induced dissociation (PQD) 35%. Data were acquired and processed using XCalibur® software package (Thermo Scientific Co, USA), version 2.0.7. Mass Frontier 5.1 Software program (Thermo Scientific Co, USA) was used to assist spectrum interpretation.

2.3 Cells and Cell Culture

HEP-G2 cells were obtained from the German Collection of Microorganisms and Cell Cultures (Leibnitz-Institut-DSMZ GmbH, Braunschweig, Germany). They were grown under standard conditions: RPMI-1640 medium supplemented with 10% fetal bovine serum in humidified atmosphere with 5% CO_2 at 37°C. Cells were propagated using 25 cm^2 tissue culture flasks.

2.4 Determination of Cell Viability on HEP-G2 by MTT

The cell viability, after continuous exposure to the tested flavonoids – narcissin, vicenin-2, rutin, BFF, and *B. flavum* crude extract, was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay [21]. Exponentially growing cells were seeded in 96-well microplates (100 µl/well) at a density 1 x 10^5 cells and allowed to attach to the surface of the well for 24h at 37°C. Thereafter, the medium was discarded and cells were treated with various concentrations of the studied flavonoids or with combination of the toxicity-inducing agent, 0.625 µM epirubicin and the referred compounds (final concentration 0.2 and 0.4 mg/ml) for up to 72 h. In order to estimate the

cytotoxic effect of solvent for the flavonoids (DMSO), in all experiments, the control was treated with 1% (v/v) DMSO. The solvent was non-cytotoxic. For each concentration, a set of at least eight wells was used. After the treatment, 10 µL MTT solution (10 mg/ml in PBS) aliquots were added to each well. The microplates were further incubated for 4 h at 37℃ and the MTT formazan crystals formed were dissolved through addition of 110 µl/well 5% formic acid (in 2-propanol). The absorption was read on a microprocessor-controlled Labexim LMR-1 microplate reader at 580 nm.

2.5 Statistical Analysis

Half-maximal cytotoxic concentrations (IC50) were calculated with the GraphPad Prism 5 program (Graph Pad Software). A non-linear regression curve fitting model Log (inhibitor) versus normalized response—Variable slope was used:

$$Y = \frac{100}{1 + 10^{(\log IC50 - X)\,HillSlope}}$$

where X is the log of dose or concentration; Y is the normalized response, 100% down to 0%, decreasing as X increases and LogIC50 is the same log units as X. HillSlope: Slope factor or Hill slope, unitless. HillSlope describes the steepness of the family of curves. A HillSlope of -1.0 is standard, and should be considered constraining the Hill Slope to a constant value of -1.0. A Hill slope more negative than -1 (say -2) is steeper.

Results from the viability tests are expressed as mean values from at least eight independent experiments. The cell survival data were normalized as percentage of the untreated control set as 100% viability. The statistical analysis included the Student's t-test whereby values of P = 0.05 were considered as statistically significant.

3. RESULTS AND DISCUSSION

3.1 Identification of Vicenin-2

The identification of vicenin-2 was based on the combination of LC-MS and LC-MS/MS data. The ESI-MS spectrum, of the investigated compound, exhibited a protonated molecular ion $[M+H]^+$ at m/z 595.16656 in positive ion mode (calculated for $C_{27}H_{31}O_{15}$) indicating a molecular formula $C_{27}H_{30}O_{15}$ and corresponding to molecular mass of 594 Da. Upon PQD fragmentation, the precursor ion with m/z 595.16656 produced ions at m/z 577.13269 $[M+H-18]^+$, m/z 511.11078 $[M+H-(30+54)]^+$, m/z 505.15899 $[M+H-90]^+$, m/z 475.02509 $[M+H-120]^+$ and base peak at m/z 457.02100 $[M+H-(120+18)]^+$ indicating cross-ring cleavages of sugar residues, whose fragmentation pattern is characteristic for flavones di-C-glycosides. The loss of 120 U is attributed to the presence of hexose as C-glycosylation sugar. The formation of the product ions at m/z 355.08667 $[M+H-(120+120)]^+$ and 379.06720 $[M+H-(150+30+18)]^+$ demonstrated that the compound possesses two hexose moieties (glucoses). Based on the detection of low intensive ion at m/z 271.01982, the aglycone was assigned accordingly as apigenin (MW 270). Comparison to the literature data [22] allowed the characterization of this compound as 6,8-di-C-glucosylapigenin, also known as vecenin-2 (Fig. 1).

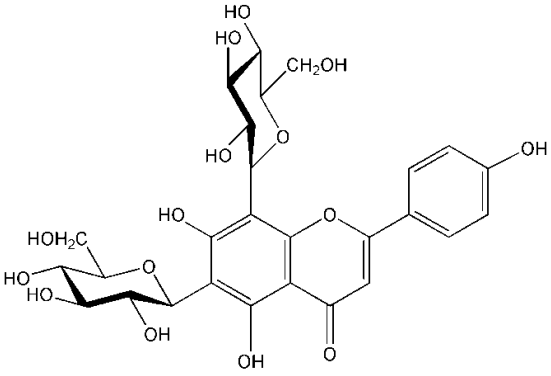

Fig. 1. Structure vicenin-2

3.2 Cytotoxicity Assay of Flavonoids

The cytotoxic activity of vicenin-2 and narcissin was evaluated in HEP-G2 cell line using MTT dye reduction assay. Vicenin-2 seems to be the most cytotoxic to cells with IC_{50} 141.7 (109.8 to 182.9 µM) µM while IC_{50} of narcissin is 195.7 µM (177.9 to 215.2 µM) (Fig. 2).

Similar experiment was conducted to test the effect of *B. flavum* crude extract on HEP-G2 cells. Viability was reduced significantly at the concentration between 100 and 400 µg/ml (P = 0.05) and an IC_{50} of 369.3 µg/ml (321.6 to 424.2 µg/ml) was calculated for the extract. Even though both vicenin-2 and narcissin showed a cytotoxic activity, it has to be noted, that their potential is lower than those of the well-known

cytotoxic agent curcumin, used as a positive control (IC_{50} 17.48 µM [14.63 to 20.87 µM]). The survival rate of HEP-G2 cells after 72 h of incubation with 0.4 and 0.2 mg/ml narcissin, rutin and *B. flavum* flavonoid mixture (BFF) is shown in Table 1. Narcissin and BFF administered alone at the higher concentration demonstrated statistically significant cytotoxicity (P = 0.05). Conserning rutin and BFF, the viability was always above 50% at the studied concentrations (Table 1).

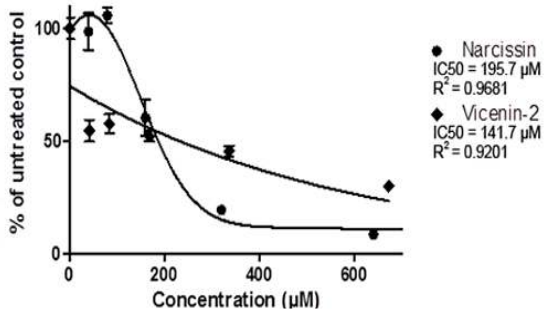

Fig. 2. Dose-response curves from non-linear regresion (log(inhibitor)/normalized response) of narcissin and vicenin-2

Table 1. Cell viability (HEP-G2 cells) in epirubicin (Epi)-induced damage assayed by the MTT test. Cells were treated with narcissin (N), rutin (R), *B. flavum* flavonoid mixture (BFF), and in combination with Epi

Group	Cell viability[1] (%)
Control	100.0±5.5
0.625 µM Epi	56.0±4.1*
0.4 mg/ml N	60.3±4.7*
0.2 mg/ml N	110.7±3.3
0.4 mg/ml N + Epi	42.4±3.7*
0.2 mg/ml N + Epi	62.4±3.9
0.4 mg/ml R	90.1±3.3
0.2 mg/ml R	96.4±4.1
0.4 mg/ml R + Epi	99.5±3.4#
0.2 mg/ml R + Epi	76.7±3.8#
0.4 mg/ml BFF	83.2±5.4*
0.2 mg/ml BFF	90.2±4.5
0.4 mg/ml BFF + Epi	73.4±4.4#
0.2 mg/ml BFF + Epi	75.3±4.3#

[1] *% of untreated control*

[*] *values significantly different from the control, P = 0.05*

[#] *values significantly different from Epirubicin, P = 0.05*

3.3 Hepatoprotective Effect Assay

Afterwards, we assayed rutin, narcissin, and the flavonoid mixture (BFF) consisting of both of them, for their possible hepatoprotective effect on epirubicin-induced damage in HEP-G2 cells. Cells incubation with epirubicin resulted in statistically significant reduction of cell viability by 56.0±4.1% (Table 1).

Our data demonstrated that after 72 h incubation of treated cells, either with rutin or BFF in combination with epirubicin, cell viability increased significantly (P = 0.05) as compared to that of the toxic agent (Table 1). BFF had the same effect at both tested concentrations on Epirubicin-induced damage but significant. However, incubation with narcissin at 0.4 and 0.2 mg/ml in combination with epirubicin had no significant hepatoprotective activity.

In this paper, we show for the first time that *B. flavum* extract and flavonoids narcissin and vicenin-2 isolated from the same extract affect hepatocellular carcinoma HEP-G2 cells viability (Fig. 2). In addition, rutin, and to a lesser extent *B. flavum* flavonoid mixture (BFF) consisting of rutin and narcissin, show significant hepatoprotective effect judged by increased cell viability after epirubicin-induced injury (Table 1). For the first time flavon C-glycoside vicenin-2 was isolated from *B. flavum*. In keeping with the report of Zhang et al. [23] the presence of vicenin-2 seems to be characteristic of *Bupleurum* genus.

Rutin was the main flavonoid in BFF, being present at 197.58 mg/g dry BFF, while the content of narcissin was substantially lower (75.74 mg/g) [20]. Narcissin was the principal flavonoid (24.21 mg/g dry weight) in the total *B. flavum* extract [20]. Rutin, isoquercitrin and isorhamnetin-3-glucoside were also evidenced in concentrations 21.83±0.66, 0.46±0.007 and 0.40±0.02 mg/g, respectively.

To test the biological effects of *B. flavum* extract, BFF and individual flavonoids, we applied HEP-G2 cell line as a cell model for antineoplastic activity and free radical liver tissue damage. Recently, a number of flavonoids have been shown to possess hepatoprotective activity against *t*-BuOOH-, tacrine- and hydrogen peroxide (H_2O_2)-induced cytotoxicity in HEP-G2 cells [24]. Evidence came from the dramatic decrease of oxidative stress of HEP-G2 cells through different mechanisms, such as detoxification of H_2O_2, inhibition of ROS generation, and removal of generated ROS [25]. Rutin is a promising agent due to its protective activity against H_2O_2- and doxorubicin-induced

DNA damage [26,27]. On the other hand, rutin did not have any effects on *t*-BuOOH-induced DNA damage in HEP-G2 cells [28]. In our previous study, both narcissin and rutin suppressed hepatic injury in CCl_4 and *t*-BuOOH toxicity models with isolated rat hepatocytes [20]. In addition, the BFF was more potent as cytoprotective and antioxidant agent as compared with the positive control silymarin at an equal concentration. In line with these findings our study showed that incubation of HEP-G2 with rutin ameliorated epirubicin-induced damage discerned by the increase in cell viability (Table 1). Incubation with BFF in combination with epirubicin also attenuated the decrease of cell viability. Narcissin in BFF produced an antagonistic effect to rutin in the protection against oxidative damage caused by the epirubicin.

Generally, studies of cytotoxicity and genotoxicity showed that the majority of flavonoids, such as catechins, genistein, daidzein and rutin, demonstrated negative results in these tests [29]. *In vitro* studies have revealed that rutin at 810 μM reduced significantly the survival of HTC (hepatoma of Rattus norvegicus) cells, after 72 h of treatment [30]. Kim and Jang (2009) observed pro-oxidant activity of flavonoids such as quercetin judged by induced apoptosis in HEP-G2 cells after prolonged treatment with high concentrations (100 μM) [25]. Our study indicated that both BFF and rutin did not show any cytotoxicity towards HEP-G2 cells at the tested concentrations (Table 1). In contrast, the cytotoxic effect of vicenin-2 and narcissin in concentration-depended manner was found, with a stronger activity of vicenin-2 (Fig. 2). The *B. flavum* crude extract displayed a significant cytotoxic effect at the highest concentration.

4. CONCLUSION

To the best of our knowledge, flavon C-glycoside vicenin-2 was isolated for the first time from the aerial parts of *Bupleurum flavum* Forsk. native to southeastern region of Bulgaria. Vicenin-2 and narcissin isolated from the *B. flavum* aerial parts were shown to have substantial concentration dependent antitumor activity against HEP-G2 cells *in vitro*. Rutin and BFF were found to exert hepatoprotective effects after anthracycline-induced cell damage. Therefore, *B. flavum* flavonoids are worth investigating for the potential development of agents against hepatocellular carcinoma.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
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