https://doi.org/10.31925/farmacia.2019.6.17

ORIGINAL ARTICLE

CHEMICAL COMPOSITION AND ANTI-INFLAMMATORY ACTIVITY OF *KITAIBELIA BALANSAE* BOISS.

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Manuscript received: March 2018

Abstract

Aerial part extracts of *Kitaibelia balansae* Boiss. were examined for their phenolic contents, as well as anti-inflammatory activities. The chemical compositions of the extracts were analysed by spectrophotometric and chromatographic (HPLC/MS/MS and HPLC-PDA) approaches. To measure anti-inflammatory activity, the effects of the extracts on nitric oxide (NO) and Tumour Necrosis Factor-α (TNF-α) levels were measured in lipopolysaccharide (LPS) treated murine macrophage cell line (RAW 264.7). The ethyl acetate fraction was found to be abundant in phenolic compounds (259.78 ± 15.07 $mg_{GAE}/g_{extract}$). Rutin was identified as the major compound in all the extracts. The ethyl acetate fraction potentially inhibited NO and TNF-α production (37.26 μM and 1781.524 pg/mL, respectively, 6 h) in LPS induced cells.

Rezumat

Extractele obținute din părți aeriene de *Kitaibelia balansae* Boiss. au fost teste privind conținutul fenolic și efectul antiinflamator. Compoziția chimică a extractelor a fost determinată folosind metode spectrofotometrice și cromatografice (HPLC/MS/MS și HPLC-PDA). Au fost determinate valorile NO și TNF-α folosind o linie de celule murine de macrofage (RAW 264.7) tratate cu lipopolizaharide (LPS). Fracția de acetat de etil s-a dovedit a fi abundentă în compuși fenolici (259,78 ± 15,07 $mg_{GAE}/g_{extract}$). Rutinul a fost identificat drept compus principal în toate extractele. Fracția de acetat de etil a inhibat producția de NO și TNF-α (37,26 μM și, respectiv, 1781.524 pg/mL, 6 h) în celulele tratate cu LPS.

Keywords: *Kitaibelia balansae*, *Malvaceae*, HPLC/MS/MS, anti-inflammatory

Introduction

Medicinal and aromatic plants contain bioactive compounds with specific (bio) chemical or organoleptic properties that enable the use of in the pharmaceutical field [1]. Phytochemicals that are naturally occurring in the plants have various therapeutic properties, and also, they are effective in the treatment of chronic and even infectious diseases. Now there is a need for the new, safe, potent, non-toxic anti-inflammatory plant-based drugs because of the long-term use of non-steroidal anti-inflammatory drugs (NSAIDS) adverse side effects [16].

Kitaibelia balansae Boiss. is a member of *Malvaceae* and is a tall herb with spreading-pilose stems. It is endemic to Turkey and it differs from the European *K. vitifolia* Willd. in its lanceolate (not ovate) stipules and triangular (not trifid) leaves [6]. To our knowledge there are no studies related to chemical composition and biological properties of this plant. The other member of the genus *Kitaibelia vitifolia* Willd. is endemic to Serbia is well known for its anti-oxidant and antimicrobial activity. Many studies have illuminated the chemical composition of *K. vitifolia* and the results have shown that rosmarinic acid, apigenin, chrysoeriol, kaempferol, quercetin 3-*O*-*β*-glucopyranoside and 3-*O*-rutinoside were present in this specie. Rosmarinic acid has been reported as the major phenolic compound and it was expressed that this compound is responsible for the antioxidant effects of *K. vitifolia* [13, 14].

To date, the biological activity and chemical composition of *K. balansae* has not been investigated, and in view of the lack of information on this specie, it is important to investigate the secondary metabolites found in endemic *K. balansae* and to question the possible anti-inflammatory activity of the plant. For all these reasons we aimed to: 1) investigate the chemical composition of *K. balansae* aerial part 70% methanol extract and its *n*-butanol and ethyl acetate fractions with spectrophotometric and chromatographic techniques (HPLC and LC/MS/MS); 2) examine the potential anti-inflammatory effects of *K. balansae* extracts on LPS induced macrophage cell lines.

Materials and Methods

Plant Material, Chemicals and Cell Line

Aerial parts of *K. balansae* were collected in June 2015, in Central Anatolia Region of the province of Hadım-Konya, and plant specimen was identified by Prof. Dr. Osman Tugay from Selçuk University and stored at Selçuk University, Art and Science Faculty Herbarium, Turkey (KNYA 26906). All standards were purchased from Sigma Chemical Company (St. Louis, MO, USA). Indomethacin was purchased from Deva Holding AŞ (İstanbul, Turkey). Cell line (RAW 264.7) has been obtained from the collection of American Type Culture Collection (ATCC Manassas, VA, USA).

Extract Preparation

Air dried *K. balansae* herb (135 g) was grounded and extracted with 750 mL of 70% methanol in a water bath (40°C) with mechanical shaking, followed by filtration. The same procedure was repeated two times using the same plant residue and the obtained filtrates were combined. The solvent of combined extract was removed *in vacuo* (40°C). 20 g of 70% methanol extract (kbMeOH) was obtained, which was then fractionated with ethyl acetate and *n*-butanol. Both ethyl acetate (kbEtOAc) and *n*-butanol (kbBuOH) fractions were concentrated *in vacuo*. All extracts were stored at -20°C after lyophilisation.

Total Phenolic and Flavonoid Contents

The Folin-Ciocâlteu method using gallic acid was used to determine total phenolic content [21]. Aluminium chloride colorimetric assay using catechin was used to determine total flavonoids content [25].

Chemical composition analysis with LC-MS/MS and HPLC systems

LC-MS/MS analysis were carried out with Shimadzu 20A HPLC system coupled to an Applied Biosystems 3200 Q-Trap LC-MS/MS instrument equipped with an ESI source operating in negative ion mode. Octa-decyl silica gel analytical column (GL Science Intersil ODS 250 × 4.6 mm, i.d., 5 µm particle size) was used for the chromatographic separation with 0.3 mL/min flow rate. The method we used was presented in a previous article [17].

HPLC experiments were performed with Agilent HP1100. Reverse-phase Mediterrenean-C18 analytical column (250 x 4.6 mm i.d., 5 µm particle size) was used to perform separations operating at 22°C with 1 mL/min flow rate. Extracts were prepared at 1 mg/mL concentration prior to injection. Components were identified by comparison of their retention times to those of authentic standards under identical analysis conditions and UV spectra using our in-house PDA library. The method we used was presented in a previous article [17].

Cell culture and cell viability-MTT assay

The murine macrophage RAW 264.7 cell line was cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with foetal bovine serum (FBS), streptomycin, and penicillin 10%, 100 µg/L, 100 IU/mL, respectively, at 37°C with 5% CO_2.

The procedure consisted in the technique described by Ilieva *et al*. The cells were treated with extracts ranging in concentration between 25 - 400 µg/mL. Absorbance was measured at 570 nm with a multi-well ELISA reader [10].

Measurement of NO and TNF-α level

The RAW 264.7 cells were seeded 5×10^5 cells/well in 12 well plates and cultured for 24 h. 100 µg/mL concentrated kbMeOH, kbEtOAc and kbBuOH extracts of *K. balansae* were prepared in DMEM without FBS, to give a total volume of 500 µL *per* well of plate. Then media of each well were removed, media containing extracts were replaced. After 3 h and 6 h treatment with extracts, cells were induced with 1 µg/mL of LPS for 24 h. Indomethacin was used as a reference drug at 25 µM concentration in the experiments. Culture supernatants were collected after treatment of LPS.

Culture supernatants and Griess reagent [0.1% N-(1-naphthyl)-ethylene diamine, 1% sulphanilamide in 5% phosphoric acid] were mixed with the equal volume (50 µL). The absorbance was taken at 550 nm with ELISA after 10 min incubation. Sodium nitrite was used as a standard between 2.5 - 100 µM concentrations [18].

TNF-α cytokine level was quantified using enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's instructions (eBioscience Inc., San Diego, CA).

Statistical analysis

Data are given as mean values ± 95% confidence interval. SPSS software, version 12.0 was used for all statistical analyses. ANOVA procedures were used to assess variance analysis. Significance of differences between means were determined by comparison test of Tukey's pairwise, $p < 0.05$ level.

Results and Discussion

Total Phenolic and Flavonoid Contents

The plant raw extracts often contain significant amounts of carbohydrates and/or lipid material and the crude extracts may therefore have a low concentration of phenolics [5]. The crude extract was partitioned into fractions with ethyl acetate and *n*-butanol to concentrate and obtain polyphenol-rich fractions. Based on the values of total phenol and flavonoid content, the fractions have higher total phenol and flavonoid content than crude 70% methanol extract (Table I).

Table I

Yields of extracts**,** total phenols and flavonoids data for *K. balansae* extracts

Extracts* and standards	Yield	Total Phenols[a] [$mg_{GAE}/g_{extract}$]	Total Flavonoids[b] [$mg_{CA}/g_{extract}$]
kbMeOH	17% (24.16g)	110.350 ± 4.92	39.443 ± 0.55
kbEtOAc	14.6% (2.92 g)	259.780 ± 15.07	90.693 ± 1.56
kbBuOH	31.7% (6.34g)	146.447 ± 0.18	51.287 ± 4.57

kbMeOH = 70% methanol extract; kbEtOAc = ethyl acetate fraction; kbBuOH = *n*-butanol fraction.

[a] = Total phenols expressed as mg gallic acid/gram (dry weight) of extract. [b] = Total flavonoids expressed as mg catechin/gram (dry weight) of extract. Values are given as means ± standard error (n = 3).

Chemical composition analysis with LC-MS/MS and HPLC systems

The qualitative-quantitative analyses of the extracts carried out using LC/MS/MS and HPLC systems and the results presented in Table II and Table III, respectively. In the present work, organic acids, phenolic acids and flavonoids were detected in a negative ionization mode. At the beginning of the analysis, $[M-H]^-$ ion at *m/z* 179 as well as a fragment ion at *m/z* 135, revealing the compound to be caffeic acid [3]. A very polar organic acid such as malic acid was detected in accordance with the literature [7]. A hydroxycinnamic acid derivative (compound 3) was identified as coumaric acid for its specific MS^2 product ions (m/z 163 and 119) [8]. Molecular ion $[M-H]^-$ (*m/z* 385) was defined as feruloylglucaric acid by the MS/MS fragment ions *m/z* 209 and *m/z* 193 [23]. Rutin was suggested for the precursor ion at *m/z* 609. The MS and MS/MS spectra showed product ions $[M-H]^-$ at *m/z* 301 and *m/z* 271 [22]. Compound with a retention time of 20 min., having *m/z* 593 $[M-H]^-$ molecular ion was cleaved into *m/z* 285 and 255 product ions in MS/MS analysis and as a result, it was determined that this compound was kaempferol coumaroyl hexoside [20]. Similarly, compound with a retention time of 21.2 min, which has a molecular ion *m/z* of 447 $[M-H]^-$ and *m/z* 285 (kaempferol $[M-H]^-$ Glc) and *m/z* 273, 257,151 fragment ions, defined as kaempferol -3-*O*-glucoside [22].

After the loss of a glucose unit (-162 Amu) from $[M-H]^-$ ion at *m/z* 463, fragment ion [quercetin-$H]^-$ at *m/z* 301 obtained and this was identified as quercetin glucoside [22]. At 20.3, a peak with *m/z* 623 fragmented to *m/z* 315 and 300 which was in agreement with the fragmentation pattern of methyl-quercetin-rhamnoside-hexoside [2]. Other detected quercetin glycoside was determined to be quercetin acetyl hexoside $[M-H]^-$ at *m/z* 505) relative to the product ions corresponding to the losses of hexosyl moieties (-162 Amu) and the acetyl residue (-42 Amu) [9]. Kaempferol-3-*O*-glucoside was subsequently sequenced with the losses of aglycone fragment ions *m/z* 285 (284), *m/z* 255, *m/z* 227 from $[M-H]^-$ with glucose [4]. The ion at [M−H] - *m/z* 489 was identified as kaempferol-3-O-acetylglucoside in the parent fragment *m/z* 285 (after loss 204 Amu: acetyl-hexose). *m/z* 519, MS^2 product ions at 315 and 300, corresponding to the initial loss of 204 Amu: acetylhexoside [11] and then 15 Amu: a methyl group, was assigned as methyl quercetin acetylhexoside [15]. At 28.6, a peak with *m/z* 301 fragmented to *m/z* 228, 179 and 151 which was consistent with the fragmentation pattern of quercetin [15].

Regarding the HPLC analysis of *K. vitifolia*, rosmarinic acid was found to be the main component, and lower content was observed for caffeic acid and *p*-hydroxy benzoic acid [13]. In both species, *K. balansae* and *K. vitifolia*, caffeic acid, ferulic acid, *p*-coumaric acid and quercetin were found similar, but major phenolic compounds are different from each other**.**

Table II

LC/MS/MS results of *K. balansae* extracts

t_R (min)	$[M-H]^-$ (*m/z*)	Ms/Ms (*m/z*)	Identification	Extracts
8.5	179	135	Caffeic acid	kbBuOH; kbEtOAc; kbMeOH
9.9	133	115	Malic acid	kbMeOH
15.69	163	119	*p*-Coumaric acid	kbMeOH
17.2	385	209, 191	Feruloylglucaric acid	kbBuOH
18.5	609	301, 271	Rutin	kbBuOH; kbEtOAc; kbMeOH
19.7	593	285, 255, 227	Kaempferol coumaroyl hexoside	kbBuOH; kbEtOAc; kbMeOH
20.0	463	300, 271	Quercetin glucoside	kbBuOH; kbEtOAc; kbMeOH
20.3	623	315, 300	Methyl-quercetin-rhamnoside-hexoside	kbBuOH; kbEtOAc; kbMeOH
20.68	505	463, 300, 271	Quercetin acetylhexoxide	kbBuOH; kbEtOAc; kbMeOH
21.2	447	284, 255, 227	Kaempferol-3-*O*-glucoside	kbMeOH; kbEtOAc
22.34	489	327, 285, 227	Kaempferol-3-*O*-acetylglucoside	kbBuOH; kbEtOAc; kbMeOH
23.1	519	315, 300 285, 271	Methyl quercetin acetylhexoside	kbBuOH; kbEtOAc, kbMeOH
28.6	301	228,179,151	Quercetin	kbEtoAC

kbMeOH = 70% methanol extract; kbEtOAc = ethyl acetate fraction; kbBuOH = *n*-butanol fraction

Table III

HPLC results of *K. balansae* extracts

Compounds	Extracts* kbMeOH	kbEtOAc	kbBuOH
Caffeic acid	t.a	0.294 ± 0.017	t.a
***p*-Coumaric acid**	t.a	0.602 ± 0.014	t.a
Rutin	25.909 ± 0.408	177.032 ± 3.500	83.121 ± 0.225
Quercetin	n.d	t.a	n.d

***** kbMeOH = 70% methanol extract; kbEtOAc = ethyl acetate fraction; kbBuOH = *n*-butanol fraction.
[&] = mg/g, mean ± SD, n.d. = not detected; t.a. = trace amount.

Cell Viability

In order to measure NO and TNF-α levels before cell viability assay, the MTT test was performed. The results obtained by the MTT cell viability assay are given in Figure 1. In this study, a 50 µg/mL concentration which gives better cell viability percentages has been used for further *in vitro* anti-inflammatory investigations.

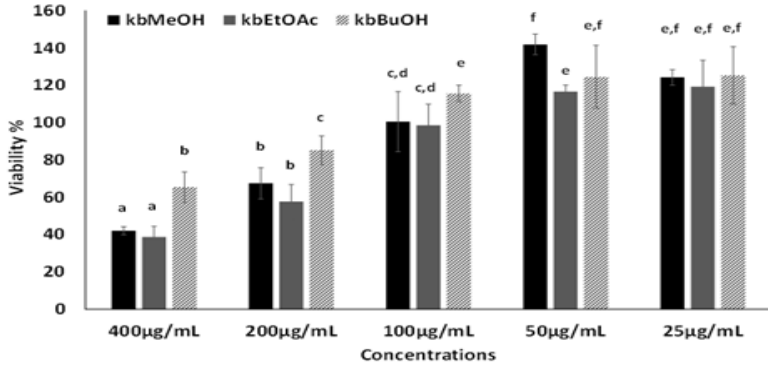

Figure 1.

Cytotoxic effects of *K. balansae* extracts on RAW 264.7 cells.

kbMeOH = 70% methanol extract; kbEtOAc = ethyl acetate fraction; kbBuOH = *n*-butanol fraction. Each value is the mean ± standard error of triplicate analysis. Values within a column followed by the same letter and number (a-f) are not significantly ($p > 0.05$) different.

Measurement of NO and TNF-α level

LPS induction of iNOS and production of NO are important inflammatory responses of macrophage cells. NO excessive induction leads to various deleterious responses such as tissue damage, septic shock, apoptosis and necrosis [12].

The results of nitric oxide measurement revealed that *K. balansae* extracts pre-treatment provides a time-dependent effect on LPS-stimulated NO production in RAW 264.7 cells. LPS application significantly increased nitrite release in control group (70.36 ± 3.12 µM) ($p < 0.05$) compared to control conditions without LPS (9.58 ± 0.36 µM). The results are given in Table IV. However, LPS-induced nitrite release reduced significantly by the pre-treatment with 50 µg/mL *K. balansae* extracts, ($p < 0.05$), especially with ethyl acetate extract (49.16 ± 2.66 µM 3 h, 37.76 ± 1.20 µM 6 h), at 3 h and 6 h, compared to LPS alone. The results of this study highlighted that the duration of treatment of extracts affected nitrite release from macrophages. In 6 h treatment, 70% methanol extract and ethyl acetate fraction showed significant anti-inflammatory effects compared to 3 h pre-treatment ($p < 0.05$).

Table IV

Inhibitory effects of *K. balansae* extracts on NO levels and TNF-α production in RAW 264.7 cells

Extracts	Nitrite concentration (µM) 3 h	6 h	TNF-α (pg/mL) 6 h
kbMeOH	52.1 ± 0.35^{a}	45.23 ± 2.77^{b}	2053 ± 90.36^{a,b}
kbEtOAc	49.16 ± 2.66^{a,b}	37.76 ± 1.20^{c}	1781.52 ± 37.75^{a}
kbBuOH	63.56 ± 0.80^{d}	54.7 ± 1.31^{a,d}	2263.85 ± 84.95^{b}
Indomethacin	15.14 ± 1.45^{e}	17.7 ± 1.58^{e}	205 ± 7.01^{c}
Control	8.43 ± 0.25^{f}	9.58 ± 0.36^{f}	863.41 ± 28.53^{d}
LPS group	65.41 ± 2.45^{g}	70.36 ± 3.12^{g}	2654.82 ± 138.18^{e}

kbMeOH = 70% methanol extract; kbEtOAc = ethyl acetate fraction; kbBuOH = *n*-butanol fraction.
Each value is the mean ± standard error of triplicate analysis. Values within a column followed by the same letter and number (a-g) are not significantly ($p > 0.05$) different.

TNF-α is an important inflammatory mediator that activates leucocytes, enhances neutrophils and endothelial adhesion of monocytes, and promotes the synthesis of eicosanoids, which increases the migration of inflammatory cells in the intracellular matrix [10]. Pre-treatment of *K. balansae* extracts, decreased the LPS-induced releasing of the inflammatory cytokine TNF-α levels in RAW 264.7 cells (Table IV). LPS application increased TNF-α release in control group (2654.82 ± 138.18 pg/mL) compared to control groups without LPS (863.41 ± 28.53 pg/mL) ($p < 0.05$). This increasing was significantly reduced by pre-treatment with ethyl acetate extract (1781.52 ± 37.75) at a concentration of 50 µg/mL at 6 h, compared to LPS alone. No extract was found to be as active as indomethacin, an indole derivative nonsteroidal anti-inflammatory drug (positive control).

LPS application to RAW 264.7 cells increases the release of inflammatory mediator NO and pro-inflammatory cytokine TNF-α, compared to LPS (-) control group. However, pre-treated cells with ethyl acetate extract released significantly less NO and TNF-α. The anti-inflammatory effect of ethyl acetate extract is related with the major phenolic compound, rutin, while several studies have found that rutin has an anti-inflammatory effect [19, 24].

Conclusions

The anti-inflammatory effect of the extracts investigated is closely related to their chemical composition. Compounds directly responsible for such biologically active compounds are polyphenols. However, *in vivo* studies needed to elucidate the mechanisms involved in the anti-inflammatory properties of *K. balansae*, and our future research will focus on *in vivo* studies.

Acknowledgement

Erciyes University Scientific Research Projects (THD-2016-6600) supported this research.

Conflicts of interest

The authors declare no conflict of interests.

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