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Communication

The flavonoid rich black currant (*Ribes nigrum*) gemmotherapy extract prevents microglial body swelling in hippocampus and reduces serum TNF-α level: pilot study

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Abstract: The fruits and leaves of the black currant (BC, *Ribes nigrum*) contain phytochemicals with therapeutic benefits. The current paper reports on a standardized BC gemmotherapy extract (BC-GTE) prepared from fresh buds, and details the extract specific flavonoid content, antioxidant, and anti-inflammatory properties, respectively. The main HPLC identified aglyka flavonoids were luteolin, quercetin, apigenin and kaempferol, while all together, at about 133 phytonutrients could be detected in the reported BC-GTE, so that the BC specific presence of many compounds was reported for the first time.

We also demonstrated that in adult male Wistar rats pretreated with BC-GTE, and assessed after the LPS injection, the body size modifications of the activated microglial cells in the hippocampal CA1 region were not apparent, and no elevated serum specific TNF-α levels were seen under such LPS induced inflammatory conditions. The specific flavonoid content, and the LPS induced inflammatory model based experimental data are all suggesting that the assessed BC-GTE seems to feature anti-neuroinflammatory property, holding the promise of a novel GTE based complementary therapeutic approach.

Keywords: *Ribes nigrum*, gemmotherapy, neuroinflammation

1. Introduction

The black current (BC, *Ribes nigrum*) emerges as a superfood due to its phytonutrient profile and the related health effects (1). Some BC extracts prepared from fruits or leaves could elicit anti-inflammatory and antioxidant properties, respectively. A study on BC fruit juice had shown to lower TNF-α gene transcription in LPS induced cultured macrophages (2). Other studies carried out on diet-induced obese mice, demonstrated that a BC fruit extract reduced obesity-induced inflammation in adipose tissue and splenocytes by lowering TNF-α transcription (3). Furthermore, BC fruit extract repressed obesity associated M1 polarization of both murine and human macrophages by reducing the expression of pro-inflammatory genes like TNF-α (4).

In the current paper, we are describing for the first time the assessment of a black current based GTE obtained from fresh buds of the planta. Besides its substantial antioxidant activity, the assessed BC-GTE featured an aglyka flavonoid profile rich in luteolin, quercetine, apigenin and kaempferol. Moreover, we demonstrated that the BC-GTE administration could suppress the transformation of microglial cells in hippocampus, while the level of serum-specific TNF-α appeared reduced in the LPS induced rat model for neuroinflammation.

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2. Materials and methods

2.1. Preparation of BC-GTE

Fresh buds of BC were harvested annually around the February-March period in Cluj (Romania), from an organic crop culture (ECOINSPECT certificate Ro-008), during three consecutive years. Each time the extractions were performed at cold by periodic mixing of the fresh vegetal material with the solvent (96 % vol. ethanol - 100 % glycerol = 1:1) for 20 days. The extraction ratio was 1:20, while the extracted solutions were separated from the vegetal rests by decantation and pressing at 400 atm. The obtained BC-GTEs could be stored at room temperature for at least 2 years without affecting their total flavonoid content and antioxidant activity.

2.2. UHPLC-ESI-MS analysis of BC-GTE

A Dionex Ultimate 3000RS UHPLC system equipped with Thermo Accucore C18 column, 100/2.1 with a particle size of 2.6 μm was coupled to a Thermo Q Exactive Orbitrap mass spectrometer equipped with an electrospray ionization source (ESI), and the measurement accuracy was within 5ppm.

2.3. UHPLC-ESI-MS analysis of BC-GTE

The total flavonoid content (TFC) was evaluated by the spectrophotometric method as described by Romanian Pharmacopoeia (1993) at 430 nm, with $AlCl_3$ as coloring agent. The flavonoids were determined quantitatively using a Shimadzu Nexera-i HPLC equipped with Fortis C18 columns, 150*2.1 mm*3 μm, and an UV-Vis DAD detector was used at 360 nm. The elution was performed with a solvent gradient (see Supplementary Table 1). The standards for the identification of apigenin, kaempferol, luteolin and quercetin were obtained from Phytolab (Germany), while for their quantitation, calibration curves were compelled (see Supplementary Table 2). The antioxidant capacity of the BC-GTE was evaluated using methods like FRAP (5), CUPRAC (6), superoxide radical and the xanthinoxidase inhibition (7). All assessments were performed in technical triplicates.

2.4. Animals and experimental design

Eighteen male, eight months old Wistar rats were housed in groups (2 animals/cage), so that a standard laboratory diet and water was provided ad libitum at 12-h light/dark cycle. The animals were randomly divided into three groups from which two of them, namely the saline group (n=6) and the control group (C, n=6) received a diluted stock solution that contained 40% ethanol, 40% vegetable glycerin and 20% water. The third group (BC-GTE, n=6) specific drink was a diluted stock solution of BC-GTE having 40% ethanol, 40% vegetable glycerin and 20% BC bud material extract. The content of drinking vessels was replaced on daily basis with 250 ml freshly prepared drinks, while the above mentioned stock solutions were diluted 1:7500. All experimental procedures on animals were approved by the Animal Examination Ethics Council of the Animal Protection Advisory Board at the Semmelweis University (Budapest, Hungary) that fully complied with the principles of EU Directive 2010/63/EU for animal experiment.

2.5. The LPS induced inflammatory reaction

After a four weeks long BC-GTE administration period, there were collected blood samples from the right foot saphenous vein. During the following day, the animals were administered intraperitoneal a dose of 1 mg/kg body weight LPS (Lipopolysaccharide from Escherichia Coli, Sigma-Aldrich Co., USA) and saline injection, respectively. Three hours after injection, blood samples were collected from the left foot saphenous vein and centrifuged at 1500-x g for 15 minutes at 4°C, and following the supernatant removal, the samples were kept on ice until assayed.

 doi:10.20944/preprints202210.0435.v1

2.6. Quantification of the serum specific TNF-α levels

A commercial TNF-α ELISA kit was used, according to the manufacturer's protocol (Invitrogen Co., USA).

2.7. Immunohistochemistry analysis

72 h post-inoculation with LPS, the animals were sacrificed by transcardial perfusion with saline containing heparin under CO_2 anesthesia. The obtained brain samples were processed for immunohistochemical evaluation, and stained with the IBA-1 antibody as described by (8).

2.8. Analysis of microglial activation

Two immunohistochemically labeled pictures per rat were obtained from the hippocampal region specific CA1 area (200x, Leica Application Suite V4.12). In each picture, there was drawn a square covering the equivalent of 50.000 μm^2 in the case of the original microscopic section, and from 5 to 10 microglial cells were analyzed in each square. The morphological features of the immunostained microglia were assessed by image analysis software (Fiji, National Institute of Mental Health, USA).

2.9. Statistical analysis of the rat-based experiments

The Statistica 13.5.0.17 soft was applied to assess the laboratory animal experiments specific data. The immunohistochemistry and ELISA results were evaluated using Student's t-test. All numerical data were represented as mean ± SEM. Differences were considered significant for $P < 0.05$.

3. Results and Discussion

3.1. The BC-GTE contains important flavonoids and features relevant *in vitro* antioxidant properties

The HPLC-ESI-MS study on the BC-GTE revealed 133 phytonutrients that belonged predominantly to the flavonoids, while other chemical compounds like carboxylic acids, amino acids, vitamins, alkaloids, esters, terpenes and others were also present (Table 1). Interestingly, the BC specific presence of some phytonutrients like acacetin, ampelopsin, apigenin, astragalin, eriodictyol, genkwanin, isorhamnetin, narcissin, naringenin, rhamnetin, sakuranetin, tetrahydroxychalcone, galloylglucose, phloretin, phlorizin, tetrahydroxy-methoxy chalcone and abscisic acid were confirmed for the first time through our GTE qualitative analysis. Surprisingly, no anthocyanins could be revealed in the BC-GTE, though others already reported their presence in BC fruits.

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Table 1. The phytonutrient profile of BC-GTE.

Chemical classification	Bioactive compounds
Alkaloids	Kynurenic acid
	Trigonelline
Amino acids	4-Guanidinobutyric acid
	4-Hydroxyisoleucine
	Asparagine
	Glutamic acid
	Isoleucine or Leucine
	Phenylalanine
	Proline
	Threonine
	Tryptophan
Others	Hydroxybenzaldehyde
	Indole-4-carbaldehyde
Esters	Ethyl gallate
	Sinapoyl-methoxyphenol
Flavonoids	Acacetin
	Ampelopsin (Ampeloptin, Dihydromyricetin)
	Apigenin
	Astragalin (Kaempferol-3-O-glucoside)
	Catechin
	Chrysoeriol
	Dihydrokaempferol (Aromadendrin, Katuranin)
	Dihydroxy-dimethoxyflavone isomer 1
	Dihydroxy-dimethoxyflavone isomer 2
	Dihydroxy-dimethoxyisoflavan
	Dihydroxy-methoxyflavanone-O-hexoside isomer 1
	Dihydroxy-methoxyflavanone-O-hexoside isomer 2
	Dihydroxy-trimethoxyflavone isomer 1
	Dihydroxy-trimethoxyflavone isomer 2
	Epicatechin
	Epigallocatechin
	Eriodictyol
	Genkwanin (Apigenin-7-O-methyl ether)
	Hydroxy-dimethoxyflavone
	Hydroxy-tetramethoxyflavone (Retusin)
	Hydroxy-trimethoxyflavone (Salvigenin)
	Hyperoside (Quercetin-3-O-galactoside, Hyperin)
	Isoquercitrin (Hirsutrin, Quercetin-3-O-glucoside)
	Isorhamnetin
	Isorhamnetin-3-O-glucoside
	Isorhamnetin-3-O-rutinoside (Narcissin)
	Isorhamnetin-O-hexoside isomer 1
	Isorhamnetin-O-hexoside isomer 2
	Isorhamnetin-O-hexoside-O-pentoside

 doi:10.20944/preprints202210.0435.v1

	Kaempferol
	Kaempferol-3-O-rutinoside (Nicotiflorin)
	Kaempferol-O-hexoside
	Kaempferol-O-hexoside-di-O-deoxyhexoside
	Kaempferol-O-hexoside-O-pentoside-O-deoxyhexoside
	Kaempferol-O-pentoside
	Luteolin
	Myricetin
	Myricetin-3'-O-glucoside (Cannabiscitrin)
	Myricetin-3-O-rutinoside
	Myricetin-O-(malonyl)glucoside
	Myricetin-O-arabinoside
	Myricetin-O-xyloside
	Naringenin
	Naringenin-6,8-di-C-glucoside
	Pentahydroxyflavanone
	Pentahydroxyflavanone isomer 1
	Pentahydroxyflavanone isomer 2
	Pentahydroxy-methoxyflavon-O-hexoside isomer 1
	Pentahydroxy-methoxyflavon-O-hexoside isomer 2
	Pentahydroxy-methoxyflavon-O-rutinoside
	Prodelphinidin B
	Prodelphinidin C isomer 1
	Prodelphinidin C isomer 2
	Prodelphinidin C isomer 3
	Prunin (Naringenin 7-O-glucoside)
	Quercetin
	Quercetin-3-O-rutinoside-7-O-glucoside
	Quercetin-di-O-hexoside
	Quercetin-O-(acetyl)hexoside
	Quercetin-O-(coumaroyl)hexoside
	Quercetin-O-hexoside-di-O-deoxyhexoside
	Quercetin-O-hexoside-O-pentoside-O-deoxyhexoside
	Quercetin-O-pentoside isomer 1
	Quercetin-O-pentoside isomer 2
	Rutin (Quercetin-3-O-rutinoside)
	Sakuranetin (4',5-Dihydroxy-7-methoxyflavanone)
	Taxifolin (Dihydroquercetin)
	Tetrahydroxychalcone (Butein)
	Tetrahydroxy-dimethoxyflavone isomer 1
	Tetrahydroxy-dimethoxyflavone isomer 2
	Tetrahydroxy-dimethoxyflavone-O-hexoside isomer 1
	Tetrahydroxy-dimethoxyflavone-O-hexoside isomer 2
	Tetrahydroxy-dimethoxyflavone-O-hexoside-O-deoxyhexoside
	Tetrahydroxyflavanone-O-hexoside

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	Tetrahydroxyflavone-O-(pentosyl)hexoside
	Trihydroxy-dimethoxyflavone
	Trihydroxy-methoxyflavone
	Trihydroxy-trimethoxyflavone-O-hexoside
	Trihydroxy-trimethoxyflavone-O-hexoside isomer 1
	Trihydroxy-trimethoxyflavone-O-hexoside isomer 2
	Caffeic acid
	cis-Aconitic acid
	Dihydroxy-methoxybenzoic acid isomer 1
	Dihydroxy-methoxybenzoic acid isomer 2
	Ferulic acid
	Hydroxyhexadecanoic acid (hydroxypalmitic acid)
	Jasmonic acid
	trans-Aconitic acid
	Tuberonic acid
	α-Linolenic acid
	5-O-p-Coumaroylnigrumin
	Caffeoylglucose isomer 1
	Caffeoylglucose isomer 2
	Caffeoylglucose isomer 3
	Chlorogenic acid (3-O-Caffeoylquinic acid)
Carboxylic acids	Chryptochlorogenic acid (4-O-Caffeoylquinic acid)
	Coumaroyl-glucose isomer 1
	Coumaroyl-glucose isomer 2
	Coumaroyl-glucose isomer 3
	Coumaroylquinic acid isomer 1
	Coumaroylquinic acid isomer 2
	Coumaroylquinic acid isomer 3
	Feruloylquinic acid isomer 1
	Feruloylquinic acid isomer 2
	Feruloylquinic acid isomer 3
	Galloylglucose isomer 1
	Galloylglucose isomer 2
	Neochlorogenic acid (5-O-Caffeoylquinic acid)
	Phloretin
	Phlorizin (Phloridzin)
	Tetrahydroxy-methoxy chalcone
	Tuberonic acid glucoside
Terpenes	Abscisic acid (ABA)
	Geranylgeraniol
	Adenine (B4)
Vitamins	Nicotinic acid (Niacin,B3)
	Pyridoxine (B6)
	Riboflavin (B2)

Our study noticeably indicates that the flavonoids are the most numerous phytonutrients in the BC-GTE as we could identify at about 80 of them including both aglyka and glycoside types. The most important aglyka are the luteolin, quercetine and apigenin, all

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together quantitatively representing 91.7 % from all flavonoids, whereas the presence of kaempferol looks diminished (see Table 2 and Supplementary Figure 1). Noteworthy, the BC-GTE showed relevant *in vitro* antioxidant properties as demonstrated by all four used methods.

Table 2. BC-GTE flavonoid content and antioxidant activity

BC-GTE Flavonoids content					BC-GTE Antioxidant capacity			
Total flavonoids [mg/ml]	Apigenin [mg/ml]	Kaempferol [µg/ml]	Luteolin [mg/ml]	Quercetin [mg/ml]	FRAP[1]	CUPRAC[1]	Superoxid radical inhibition[2]	Xanthin-oxidase inhibition[3]
0.82 ± 0.013	0.152 ± 0.002	3.9 ± 0.04	0.34 ± 0.003	0.26 ± 0.003	672 ± 7.1	4565 ± 48.8	4512 ± 49.6	41 ± 0.4
	18.5 %		41.5 %	31.7 %				

[1] mM TE/100 ml extract
[2] µM TE/100 ml extract
[3] mg AE/ml extract

3.2. The BC-GTE pretreatment overcomes the swelling of microglial cell body, following LPS activation

It is well documented that the activated microglial cells did play a determinative role in brain inflammation, leading to the swelling of microglial cell body, thickening of proximal processes, and reduction of distal ramifications (9). Analyzing 72h post-LPS injection, the hippocampal CA1 area of the rats, we could observe that the body size of microglia appeared significantly reduced in the case of BC-GTE pretreatments as compared to BC-GTE untreated controls ($P < 0.001$), (Fig. 1B-C; Table 3). The LPS injected control animals have significantly greater body sizes as compared to the non-LPS induced saline group ($P < 0.01$), an effect that indicates the inflammatory potential of the LPS. Moreover, no significant differences are apparent among all samples regarding the microglia number, and the thickening of proximal processes of microglial cells in all analyzed samples (Fig. 1B). Therefore, our observations are suggesting that the pre-treatment with BC-GTE could efficiently prevent/suppress the microglial activation induced swelling of cell body in the hippocampus upon LPS induced neuro-inflammation.

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Table 3. The cell body sizes and diameter of microglias.

	Saline	Control	BC-TGE
Cell body size (μm²)	30.16073	65.13714	35.06247
Diameter	6.19849	9.10918	6.68323

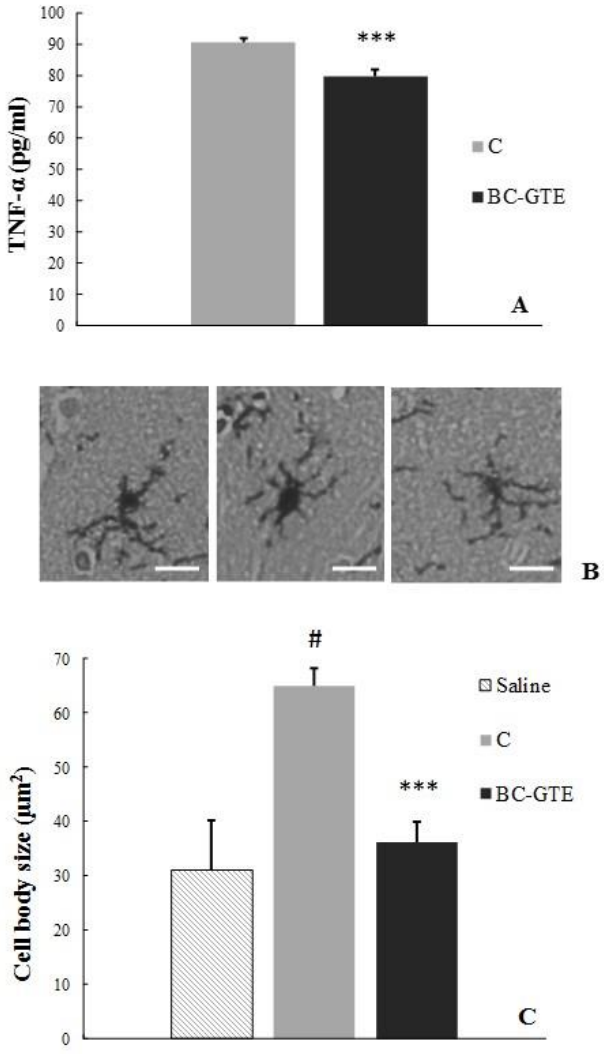

Figure 1. Experimental data presenting the physiological effects of BC-GTE on LPS-induced adult rats. The changes of peripheral TNF-α levels (A) and microglial cell body swelling (B, C). Abbreviations: C-control group; BC-GTE-extract treated animal lot; (***) $P < 0.001$ control vs. BC-GTE group; (#) $P < 0.01$ control vs saline group. Scale bar: 10 μm.

3.3. The BC-GTE pretreatment leads to diminished serum TNF-α levels

Noteworthy, some flavonoids identified in BC-GTE when assessed independently were featuring immunomodulatory and anti-inflammatory effects (9). Other studies were indicating that the stimulation of the peripheral innate immune system through LPS injection could activate microglial cells, and their morphological changes would be accompanied

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by secreting pro-inflammatory cytokines like TNF-α (10), leading to neuroinflammation (11). To further substantiate the anti-inflammatory effect of BC-GTE, we set to analyze the serum specific TNF-α level after LPS injection. In the BC-GTE pretreated animals, the serum-specific concentration of TNF-α appears significantly lowered as compared to the BC-GTE non-pretreated control individuals (Fig. 1A). Moreover, we could not detect TNF-α (i) in the "saline" group where animals were pretreated with the solvent mix of the BC-GTE and the LPS got replaced with the saline injection; (ii) in control animals prior to LPS injection; (iii) in animals pretreated with BC-GTE prior to LPS injection (data not shown). Our results are indicating that the pretreatment with BC-GTE prevented the raise of circulating TNF-α levels following LPS injection, further suggesting that the BC-GTE would possess an anti-inflammatory effect.

3.4. The BC-GTE features anti-neuroinflammatory effect

Several reported mice and rats-based experiments clearly demonstrated that the peripheral inflammatory LPS stimuli would cause brain specific microglial activation associated with an increased TNF-α protein level (13, 14). Such an experimental approach successfully recapitulates neuroinflammation in laboratory conditions, and therefore, it would serve as a valuable tool for defining novel therapeutic strategies to treat/prevent neurodegenerative diseases with inflammatory components (15). Our experiments are indicating that the BC-GTE administered prior to LPS injection prevents to some extent the transformation of hippocampal microglia, which is further corroborated by the lower serum specific TNF-α level. To our knowledge, we are the first to report the flavonoid rich composition of the BC-GTE and the associated anti-neuroinflammatory effect. Though more studies are needed to define the cellular mechanism(s) of such protective effects, our results are suggesting that the BC-GTE based complementary therapeutic approach holds the promise of a multiple therapy with increased efficiency in case of neurodegenerative diseases.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, s2 and s3. Figure S1: The HPLC chromatogram of BC-GTE; Table S1: Solvent gradient; Table S2: Calibration curves data.

Author Contributions: TT and CN designed and performed the animal experiments. EmM, N-KO and ZC carried out the chemical experiments. TT, EmM and EnM analyzed data. TT, N-KO, ZC and EnM wrote and edited the manuscript.

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Institutional Review Board Statement: The animal study protocol was approved by the Animal Examination Ethics Council of the Animal Protection Advisory Board at the Semmelweis University (Budapest, Hungary) that fully complied with the principles of EU Directive 2010/63/EU for animal experiment.

Conflicts of Interest: The authors declare no conflict of interest.

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