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Effect of berberine and Berberis aetnensis C. Presl. alkaloid extract on tissue transglutaminase in primary astroglial cell cultures

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Effect of berberine and *Berberis aetnensis* C. Presl. alkaloid extract on tissue transglutaminase in primary astroglial cell cultures.

Short title: Effect of berberine and *B. aetnensis* alkaloid extract on TG2

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Abstract

Berberis aetnensis C. Presl. is a bushy-spiny shrub common on Mount Etna (Sicily, Italy), containing various alkaloids with several pharmacological properties. Herein, we assessed the effect of berberine and of the alkaloid extract of *B. aetnensis* roots on the glutamate-evoked tissue transglutaminase (TG2) up-regulation in rat astrocyte primary cultures, used as an *in vitro* model of excitotoxicity. Our findings show that the alkaloid extract of *B. aetnensis* roots consists mainly of berberine. Furthermore, berberine and the alkaloid extract of *B. aetnensis* roots were able to restore the oxidative status modified by glutamate and the levels of TG2 to control values. We found that berberine or the alkaloid extract of *B. aetnensis* roots are able to ameliorate excessive production of glutamate, protein misfolding and aggregation, mitochondrial fragmentation, and neurodegeneration. Thus, we suggest that berberine and the alkaloid extract of *B. aetnensis* roots, may represent a natural therapeutic strategy in the neuropathological conditions associated to excitotoxicity.

Keywords: berberine; *Berberis aetnensis* C. Presl.; tissue transglutaminase; glutamate; excitotoxicity; astrocytes.

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INTRODUCTION

Several findings demonstrated that some plants of the genus *Berberis,* and in particular *Berberis vulgaris* and *Berberis koreana*, have beneficial effects on the neural system, suggesting a potential use for treatment of some neuronal disorders, such as epilepsy and convulsion (Fatehi et al., 2005; Tomosaka et al., 2008; Yoo et al., 2008). It has been reported that these effects are mainly due to their content in berberine, an alkaloid which possesses a wide range of biological activity (Kulkarni and Dhir, 2007; Kupeli et al., 2002; Singh and Kakkar, 2009) and is a scavenger of intracellular free radicals (Wartenberg et al., 2003). Berberine also potentiates the nerve growth factor (NGF)-induced differentiation in neural cells (Shigeta et al., 2002), and has potent neuroprotective effects against ischemic damage in cerebral ischemia/reperfusion models (Cui et al., 2009; Yoo et al., 2006; Yoo et al., 2008).

In previous researchers, we showed that *Berberis aetnensis* C. Presl., a bushy-spiny shrub which is endemic to the volcano Mount Etna (Sicily, Italy), possessed antimicrobial activity (Iauk et al., 2007; Musumeci et al., 2003). However, its alkaloid content and the use in traditional medicine are still unknown.

It has been shown that glutamate excitoxicity, a pathological process due to overactivation of receptors for the excitatory neurotransmitter glutamate, is involved in the neuronal loss associated with many acute (stroke, ischemia) and chronic diseases (Alzhéimer's and Parkinson's diseases), mainly by activation of specific ionotropic and metabotropic receptors (Doble, 1999). In particular, in differentiated astrocytes, a brief exposure to glutamate causes cell swelling, whereas a prolonged incubation induces cell damage, resulting from calcium overload, glutathione (GSH) depletion and reactive oxygen species (ROS) production (Matute et al., 2002). Consequently, several calcium-

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dependent enzymes implicated in cellular stress response, as well as tissue transglutaminase (TG2), may be activated, causing mitochondria impairment, decrease in ATP levels, and subsequent neuronal cell death (Sandhu et al., 2003). TG2 is a truly multifunctional protein expressed predominately within neurons involved in many physiological as well as pathological functions of the brain (Lesort et al., 2000). Several lines of evidence suggest that aberrant TG2 activity, although not causative, may contribute to the inexorable decline associated with different neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases (Gentile and Cooper, 2004).

Herein, we assessed the composition of the alkaloid extract of *Berberis aetnensis roots* and its effect and of berberine on glutamate-induced TG2 up-regulation in primary astroglial cell cultures, used as an *in vitro* model of excitotoxicity.

MATERIALS AND METHODS

Plant material

Roots of *B. aetnensis* C. Presl. were collected on the slopes of the volcano Mount Etna (1800-1900 m) thanks to the Commandant of the Regional Forest Corps Detachment of Catania-Nicolosi (Sicily, Italy), Warrant Officer Gianluca Ferlito.

A voucher specimen of the plant no. 15/03 was deposited in the herbarium of the Pharmacobiological Department of the University of Messina (Italy).

Preparation and chromatographic analysis of the alkaloid extract

Air-dried, powdered roots (50 g) of *B. aetnensis* were extracted as previously reported (Suau et al., 1998), and the alkaloids were isolated, identified and quantified by HPLC. Gradient-elution HPLC of the alkaloid residue (4.62 g), and reference compounds

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(berberine, berbamine, palmatine, jatrorrhizine and oxyacanthine), was carried out on a Hewlett Packard liquid chromatograph (HPLC 1100) equipped with quaternary solvent pump, an autosampler, a thermostat and a photo diode array detector. A Spherisorb S5ODS1 reversed-phase column (250 × 4.6 mm i.d. with 5.0 µm particle size) with a ODS guard-column (4 × 20 mm i.d.) was used. The procedure used was the same as previously described (Yoo et al., 2006).

Cell cultures

Primary cultures of astrocytes were prepared from the neocortex of newborn albino rat brains (1–2-day-old Wistar strain) as reported (Campisi et al., 2003). Cells were maintained at 37.0±0.1°C in a 5% CO_2/95% air humidified atmosphere for 2 weeks and the medium changed every 3 days. Cells were characterized at 14 *days in vitro* (DIV) by immunofluorescent staining for Glial Fibrillary Acidic Protein (GFAP, Chemicon, Prodotti Gianni, Milan, Italy), an astroglial marker of growth, maturation and differentiation (Campisi et al., 2003). All efforts were made to minimize both the suffering and number of animals used. All experiments conformed to the guidelines of the Ethical Committee of the University of Catania, Italy.

Treatment of the cells

Astroglial cell cultures at 14 DIV were treated with different concentrations of berberine (Sigma, Milan, Italy) (10 and 20 µM) or of the alkaloid extract of *B. aetnensis* roots (5, 10 and 20 µg/ml), in absence or in presence of glutamate (500 µM) for 24 h (Campisi et al., 2004). To assess the involvement of glutamate receptors, a lot of these cultures were incubated 20 min prior to glutamate exposure with 100 µM 1-[4-

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aminophenyl]-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine (GYKI 52466), a well known inhibitor of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate (KA) receptors (Campisi et al., 2003).

Cytotoxicity assessment

MTT test was used to evaluate cell viability (Campisi et al., 2004). To verify the occurrence of necrotic cellular death, lactate dehydrogenase (LDH) release was performed in the culture medium and in the cellular lysates (Campisi et al., 2004).

Glutathione measurement

Total glutathione intracellular content (GSH+GSSG), was chemically determined as previously described (Chen et al., 2000).

ROS levels

The intracellular peroxide levels were performed according to the method by Chen et al. (2000).

Western Blot Analysis

Analysis of TG2 was performed with monoclonal antimouse against the protein (NeoMarkers, Fremont, CA) followed by incubation for 1 hr with horseradish peroxidase–conjugated antimouse IgG (Campisi et al., 2003). TG2 expression was visualized by chemiluminescence with an ECL kit (Amersham Pharmacia Biotech, Milan, Italy) after autoradiography film exposure. Blots were scanned and quantified

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with an AlphaImager 1200 System (Alpha Innotech, San Leandro, CA). The results were expressed as the percentage of TG2 increase over that in the control.

Statistical analysis

All data were statistically analysed using one-way ANOVA, followed by Newman–Keuls post hoc test. All values are presented as means ± S.D. of 5 experiments. The Student t-test was used to compare the values of each assay; differences were considered statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

The alkaloid extract of *B. aetnensis* roots was characterized by HPLC analysis. We found that the major compound in the extract was berberine (376 mg/g), and that it also contains smaller amounts of berbamine (35 mg/g) and jatrorrhizine (33 mg/g), and even less amounts of palmatine (12 mg/g), oxyacanthine (9 mg/g) and other alkaloids.

Primary rat neocortical astroglial cell cultures at 14 DIV, that is when confluent, were characterized by immunofluorescent staining with the glial marker GFAP (Campisi et al., 2003). About 90-95% of cells were GFAP positive, indicating that cultures were highly enriched in astrocytes (data not shown).

Preliminary assays by MTT test and morphological observations have been made to establish the suitable concentrations of berberine and of the alkaloid extract of *B. aetnensis* roots for the cultures both in absence or in presence of 500 μM glutamate for 24 h (Campisi et al., 2004). Berberine (10 and 20 μM) or the *B. aetnensis* alkaloid extract (5-20 μg/ml) treatment did not significantly affect cellular viability (data not shown). No significant changes in LDH release was found (data not shown). This result

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demonstrated that berberine or the alkaloid extract of *B. aetnensis* roots did not induce necrotic cellular death in astroglial cell cultures. Consequently, in the experiments 10 and 20 μM berberine and 5, 10 and 20 μg/ml *B. aetnensis* alkaloid extract concentrations were chosen.

In order to verify if berberine or the alkaloid extract of *B. aetnensis* roots were able to restore the oxidative status modified by glutamate, the GSH levels and the intracellular ROS production were evaluated. Glutamate treatment induced a significant increase in GSH depletion (Fig. 1) and ROS levels (Fig. 2) when compared with the respective untreated ones used as a control. In cultures primed with GYKI 52466, a selective inhibitor of AMPA/KA receptors, glutamate effects on GSH and ROS levels were diminished (Figs. 1, 2). The present data indicate that stimulation of cultured astrocytes with high concentrations of glutamate results in oxidative stress, as indicated by the reduction of GSH levels and overproduction of intracellular ROS levels. The altered redox status is consistent with a transporter-mediated toxicity evoked by glutamate (Matute et al., 2002).

Berberine and the alkaloid extract of *B. aetnensis* roots were able to counteract the effect of glutamate, restoring, in a dose-dependent manner, GSH and ROS levels similar to control values (Figs. 1, 2). The effect of the *B. aetnensis* extract was slightly greater compared to berberine. Furthermore, 20 μM berberine and 20 μg/ml extract, which contain a very similar amount of berberine, showed values similar to those obtained after priming with GYKI 52466, followed by the addition of glutamate (Figs. 1, 2).

We assessed the effects of berberine and the alkaloid extract of *B. aetnensis* roots on TG2 expression in 14 DIV astroglial cell cultures in the absence or in the presence of 500 μM glutamate. TG2 expression was evaluated by Western blot analysis (Fig. 3).

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Immunoblots of control cultures showed low levels of TG2. Glutamate treatment induced a marked increase in TG2 protein levels (Fig. 3a). Densitometric analysis of TG2 expression confirmed that the protein was up-regulated by glutamate injury (Campisi et al., 2004) (Fig. 3b).

The pre-incubation with 10 and 20 μM berberine was able to counteract glutamate-dependent increase in TG2 expression (Fig. 3). In particular, we observed that the levels of protein, at 20 μM, were similar to untreated control values. When cultures were primed with *B. aetnensis* alkaloid extract at all concentrations (5, 10, 20 μg/ml) followed by the addition of glutamate, there was a dramatic reduction in the glutamate-induced increase in TG2 levels (Fig. 3). Its effects appeared slightly greater than that of berberine (Fig. 3).

In our experimental model, we demonstrated that the pre-incubation of primary rat astroglial cell cultures with berberine or the alkaloid extract of *B. aetnensis* roots, were able to counteract, in a dose dependent manner, the effects of glutamate restoring the levels of TG2 to control values. The causative effect of oxidative stress on the rises in TG2 expression in primary astroglial cell cultures was confirmed by the inhibition of ROS production and intracellular GSH depletion through addition of berberine or the alkaloid extract of *B. aetnensis* roots. In particular, we found, for the first time, that the extract was able to counteract glutamate-dependent increase in TG2 expression also at the lower concentration (5 μg/ml) (Fig. 3).

The protective effect against glutamate toxicity of berberine and the alkaloid extract of *B. aetnensis* roots appeared stronger than that of the synthetic antioxidant compounds used in our previous researches (Campisi et al., 2004).

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These results are in agreement with other recent findings demonstrating that berberine, exerts neuroprotective effect against ischemic damage in mouse organotypic hippocampal slice cultures stimulated by NMDA (Cui et al., 2009), attenuates transient ischemia-induced up-regulation of type 1 NMDA receptor expression, and reduces calcium influx in the hippocampus of gerbils (Yoo et al., 2008). Furthermore, it has been demonstrated that berberine exhibits neuroprotective action against *in vitro* ischemia induced neuronal damage in the hippocampus at least in part by the suppression of Bcl-2 phosphorylation (Cui et al., 2009). In addition, berberine decreased ROS levels and inhibited angiogenesis in embryoid bodies, indicating that the compound was acting as a scavenger of intracellular free radicals (Wartenberg et al., 2003). Berberine was also able to block the generation of ROS in hypoxia-treated mesenchymal stem cells exerting potent antiapoptotic activities by inhibiting the generation of ROS (Zhang et al., 2009). Recently, it has also been demonstrated that berberine exerted antidepressant-like effect modulating brain biogenic amines (norepinephrine, serotonin or dopamine) and further, the antidepressant-like effect of berberine in the forced-swim test involved an interaction with the L-arginine-NO-cGMP pathway (Kulkarni and Dhir, 2007).

Thus, the most significant findings of our study are that berberine and the alkaloid extract of *B. aetnensis* roots were able to counteract the effects of glutamate restoring the levels of TG2 to control values and also the intracellular oxidative status modified by neurotransmitter. Furthermore, we found that berberine represents the major alkaloid of the alkaloid extract of *B. aetnensis* roots. This finding is in agreement with other reports on the alkaloids from the roots of *Berberis* species (Ivanovska and Philipov, 1996; Yeşilada and Küpeli, 2002).

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It is possible to assume that berberine or the alkaloid extract of *B. aetnensis* roots are able to counteract glutamate uptake-induced impairment of cystine/glutamate antiporter leads to depletion of GSH content and biochemical alterations, resulting in the delayed toxic effect for primary astrocyte cultures (Chen et al., 2000; Kato et al., 1992).

Thus, we suggest that berberine and the alkaloid extract of *B. aetnensis* roots, which mostly contains berberine, ameliorating the excessive production of glutamate, protein misfolding and aggregation, mitochondrial fragmentation, may represent a natural therapeutic strategy in the neuropathological conditions associated to excitotoxicity. However, other studies are now in progress to better clarify the molecular mechanisms of berberine and the alkaloid extract of *B. aetnensis* roots, and to provide other information on their regulatory mechanisms of TG2 gene transcription evoked by excitotoxicity.

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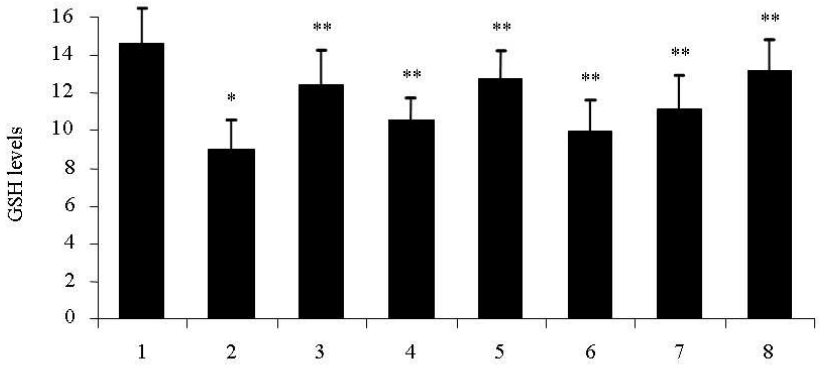

Fig. 1 - GSH levels in primary rat astroglial cell cultures at 14 DIV exposed to glutamate (500 μM for 24 h) in presence or absence of GYKI 52466 or of berberine (BER, 10 and 20 μM)/B. aetnensis alkaloid extract (BAE, 5-20 μg/ml). GSH levels are expressed as GSH nmoles/mg protein.
1: control; 2: glutamate; 3: glutamate + 10 μM GYKI 52466; 4: glutamate + 10 μM BER; 5: glutamate + 20 μM BER; 6: glutamate + 5 μg/ml BAE; 7: glutamate + 10 μg/ml BAE; 8: glutamate + 20 μg/ml BAE.
*$p<0.05$ vs. control. **$p<0.05$ vs. glutamate.

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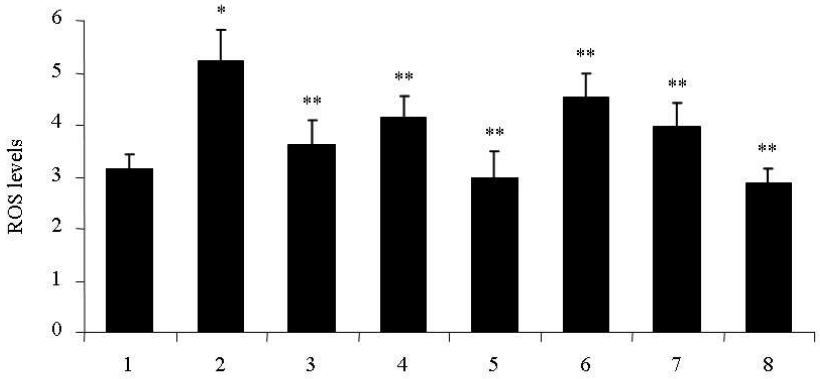

Fig. 2 - ROS levels in primary rat astroglial cell cultures at 14 DIV exposed to glutamate (500 μM for 24 h) in presence or absence of GYKI 52466 or of berberine (BER, 10 and 20 μM)/B. aetnensis alkaloid extract (BAE, 5-20 μg/ml). ROS levels are expressed as nmoles of dichlorofluoresceine produced/30 min/mg protein.
1: control; 2: glutamate; 3: glutamate + 10 μM GYKI 52466; 4: glutamate + 10 μM BER; 5: glutamate + 20 μM BER; 6: glutamate + 5 μg/ml BAE; 7: glutamate + 10 μg/ml BAE; 8: glutamate + 20 μg/ml BAE.
*p<0.05 vs. control. **p<0.05 vs. glutamate.
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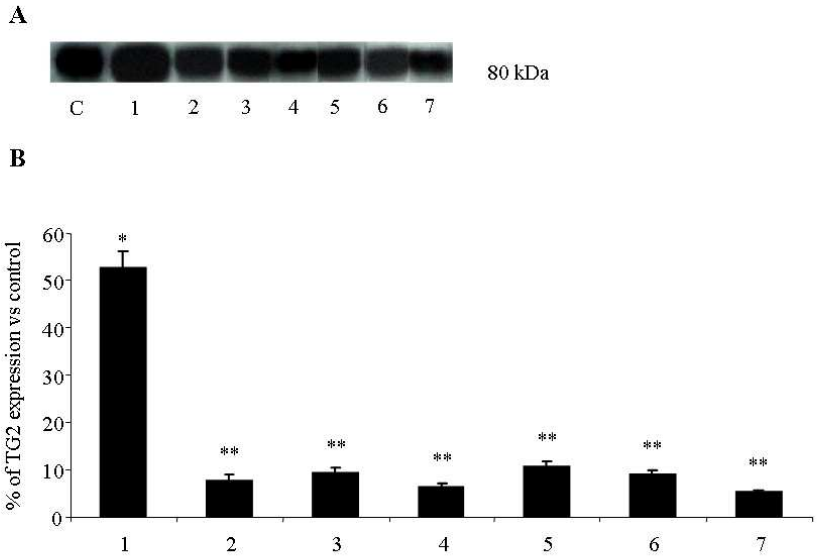

Fig. 3 - Representative immunoblot (A) and densitometric (B) analyses of TG2 expression in 14 DIV primary rat neocortical astroglial cell cultures exposed to glutamate (500 μM) for 24 h that show the effect of exposure to berberine (BER, 10 and 20 μM)/B. aetnensis alkaloid extract (BAE, 5-20 μg/ml). The results of densitometric analysis are expressed as the percentage of increase in TG2 over that in the control.
1: glutamate; 2: glutamate + 10 μM GYKI 52466; 3: glutamate + 10 μM BER; 4: glutamate + 20 μM BER; 5: glutamate + 5 μg/ml BAE; 6: glutamate + 10 μg/ml BAE; 7: glutamate + 20 μg/ml BAE.
*p< 0.05 vs. control. **p< 0.05 vs. glutamate.

83x59mm (300 x 300 DPI)