Aqueous extract of *Launaea acanthodes* induces glutamate uptake and GABA release in astrocyte cell culture via a ROS scavenging mediated process

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**Abstract**

*Launaea acanthodes* is extensively used in the semi-arid region of Iran for treatment of seizure. However, the underlying mechanism has not been studied well. In our previous study we showed that *Launaea acanthodes* extract could effectively stimulate GABA release from PC12 cell culture. The critical role of astrocytes in epileptic brain in regulation of neurotransmitter balance in central nervous system encouraged us to investigate the effect of *Launaea acanthodes* extract on GABA and glutamate release from astrocytes. Our results indicated that LA extract could stimulate both glutamate uptake and GABA release by astrocytes. The results confirmed this fact that GABA release by astrocytes in response to LA treatment is a glutamate uptake-dependent process. We showed that stimulation of GABA release by *Launaea acanthodes* is a gene expression based process which depends on glutamate uptake. We propose that glutamate uptake via glutamate transporter 3 could activate expression of glutamate decarboxylase which in turn transforms uptaken glutamate into GABA.

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**1. Introduction**

Epileptic activity of brain occurs in response to several conditions including brain injuries (Vespa et al., 1999). To date, multiple mechanisms concerning this situation have been explained (Takano and Coulter, 1937). While, imbalance in neurotransmitter release has been described as one of the most important mechanisms (Bradford, 1995). Among myriad number of neurotransmitters, imbalance in release of gamma-aminobutyric acid (GABA) and glutamate, an inhibitory and an excitatory neurotransmitter respectively, plays the crucial role in initiation and continuity of epileptic activity (Bradford, 1995). However, the exact mechanisms governing unregulated release of these neurotransmitters is unknown.

The participation of astrocytes in neurotransmitter release and uptake during seizure has been studied extensively and investigation the exact role of astrocytes in inducing or inhibiting seizure is of great interests to neuroscientists (Clasadonte and Haydon, 2010; Coulter and Steinha, 2015). To date, many efforts have been done in order to illustrate the importance of reactive astrocytes in treatment and protection of brain from epileptic attacks. Morcos et al. have shown the accumulation of astrocytes in seizure areas of brain (Morcos et al., 2003). Following studies indicated that the release of glutamate by astrocytes may exacerbate the epileptic situation (Parpura and Haydon, 2009). However, the exact role of reactive astrocytes in such situation is controversial. In this ground, several studies have focused on the ability of astrocytes to release and uptake both glutamate and GABA (Le Meur et al., 2012).

Even though, glutamate plays a key role in induction of seizures and its uncontrolled release is accompanied with many nervous system dysfunctions, GABA has the same importance, as well. Decrease in GABA level will results in highly excited and firing neuron and neural circuits which causes brain dysfunction (Fritschy et al., 1999). Regulation of GABA level in brain is another function of astrocytes and the importance of astrocytes in epilepsy has been studied widely concerning this issue (Fritschy et al., 1999).

*Launaea acanthodes* (LA) is a medicinal plant native to semi-arid regions of central Iran (Moalem, 2009). LA extract as herbal tea is...
used widely in these area for treatment of mental disorders. Several studies have indicated the antioxidant, anti-hyperglycemic and anti-convulsing activity of hydro-alcoholic extract of LA (Khan, 2012; Moalem, 2009; Mohammadi et al., 2016). In previous studies we have shown the stimulatory effect of Aqueous extract of LA on GABA release by PC12 cells. Hence, we decided to assess the effect of LA extract on GABA and glutamate release from astrocytes as an essential group of central nervous system cells.

Astrocyte culture was treated with various concentrations of LA extract and the amount of Glutamate and GABA was measured using high performance liquid chromatography. Our experiments showed that treatment of astrocytes with LA extract will increase GABA release, while has a contradictory effect on glutamate release via its uptake. This study was complementary to our previous studies concerning the effect of LA extract on PC12 cells and illustrates the mechanism of action of this medicinal plant.

2. Material and methods

2.1. Cell culture

Astrocytes were cultured in DMEM medium containing 10% fetal bovine serum under 5% CO2. Cell cultures were treated with various concentrations of LA extract and LA toxicity and cell viability were measured using MTT assay (Stockert et al., 2012). ROS level in treated and untreated cells was also measured using DCFH-DA assay (Wu and Yotnda, 2011).

Morphology of treated and control cells was determined using phase-contrast microscopy.

2.2. HPLC analysis

GABA release was measured by derivatization GABA in culture medium via 2-hydroxynaphthaldehyde. Briefly, 1 ml culture medium was aspirated from treated cell culture and was reached to 100 mM HCl and centrifuged at 20,000 g for 15 min the supernatant was neutralised by addition of 100 mM NaOH and 1 mM 2-hydroxynaphthaldehyde was added to solution. The reaction was conducted by incubation of the solution at 80 for 15 min the solution was cooled to ambient temperature, 5 ml methanol was added and solution was filtered through a 0.2 syringe filter.

Derivatized GABA was separated on a reverse phase SB-C18 column using a Shimadzu HPLC instrumentation. The sample was eluted by a buffer gradient system containing methanol (buffer B) and water (buffer A) (Hayat et al., 2014).

Glutamate measurement was carried out using the method of Moraes et al. via o-phthaldehyde derivatization (Moraes et al., 2012). The protein content of cell culture was precipitate by 100 mM of HCl and following centrifugation. The acidic solution was neutralized by 100 mM NaOH and addition of 100 mg/ml OPA and 100 mM borate buffer pH 9.5. The reaction mixture was incubated at room temperature and was analyzed using Shimadzu fluorescence HPLC system. The mobile phase was composed of phase a containing 50 mM sodium acetate, methanol 5% and 2-propanol (pH 5.67) and methanol 70% as phase B. A gradient mixture of two phases was used for elution. The column was eluted with 100% phase A at zero time, after 20 min 50%, and back to 100% at 25 min. The fluorescence signal was recorded by excitation at 340 nm and detector was set up at 460 nm. All HPLC results were normalized relative to cell count.

3. Results and discussion

The critical role of astrocytes for proper functioning of central nervous system is obvious (Clasadonte and Haydon, 2010). Astrocytes are able to participate in uptake and release of a wide range of neurotransmitters and mediate neural signal propagation in CNS (Sahlinger et al., 2014). Defective cooperation of astrocytes has been reported in several brain dysfunctions (Colangelo et al., 2014). One of the widely studied situations is the epileptic activity of brain (Coulter and Steinha, 2015). Participation of astrocytes in regulation of neural circuit firing has been investigated from many aspects and their role in neurotransmitter release during seizures has been detected as one of the most important mechanisms. It has been shown that astrocytes mediate GABA and Glutamate release and uptake in epileptic brains (Clasadonte and Haydon, 2010). However. The exact role of astrocytes in epilepsy is controversial.

LA is commonly used in central area of Iran for treatment of neurological disorders especially epilepsy. The mechanisms underlying LA role in controlling brain dysfunction have been investigated extensively. Several groups have reported that the anti-convulsant activity of LA originates from the high content of phenolic compounds in aqueous-alcoholic extract of LA (Moalem, 2009). In our previous study we described a new mechanism governing the antiepileptic activity of LA based on stimulation of GABA release from PC12 cells. In this study we investigated the effect of LA extract on release and uptake of two critical neurotransmitters in controlling epilepsy by astrocytes which has been shown to play a critical role in seizures.

Treatment of astrocytes with LA extract was followed by viability tests. Viability of treated cells was measured after 24, 48 and 72 h. MTT test results indicated that cell viability was increased by increase in LA concentration up to 3 mg/ml in a time-dependently manner (Fig. 1a). It means that the effect of low concentrations of LA is mediated via gene expression regulation and causes increase in cell proliferation which has been reported previously. In LA concentrations more than 3 mg/ml the cell viability was decreased in comparison to control culture. Comparison of viability of cells treated with higher concentrations of LA than 3 mg/ml showed no significant change in various time
periods. It can be concluded that the effect of high concentrations of LA is short term process and is mediated mostly through the effect of toxic secondary metabolites on enzymatic processes inside the cells.

The antioxidant activity of LA extract could mediate higher proliferation rate of treated astrocyte culture. Measurement of ROS level showed that by addition of LA extract up to 3 mg/ml the ROS level decreases, while in the presence of higher concentrations of extract the ROS level in cells increased rapidly (Fig. 1b). The higher level of ROS in high concentration of extract would be due to the presence of secondary metabolites in LA extract which could interfere with mitochondria activity and causes mitochondria dysfunction.

Morphology studies on treated and untreated astrocytes with various concentrations of AELA after 72 h showed that cell growth has increased in samples treated with 3 mg/ml AELA, while has decreased in cultures treated with 5 mg/ml AELA that is in accordance with MTT results (Fig. 2).

After treatment of astrocyte culture with 3 mg/ml of LA extract for various periods the cell culture medium was removed and derivatized as was stated in experimental section and GABA and glutamate content was analyzed using HPLC.

Measurement of glutamate content of astrocyte culture after treatment with various concentrations of LA showed that glutamate content of cell culture decreased after treatment (Fig. 3b). Glutamate uptake should be the mechanism underlying...
enhanced proliferation of astrocytes after treatment with LA extract (Le Meur et al., 2012). The hypothesized mechanism was assessed by comparison of astrocytes viability after treatment with LA in medium supplemented with and without glutamate (data not shown). Higher proliferation rate of astrocytes in the presence of glutamate showed that LA mechanism in stimulation of astrocyte proliferation is partially glutamate dependent. The observed role of glutamate in enhancing LA effect on astrocyte should be mediated via activation of metabotropic glutamate receptor 5 in astrocytes by active compounds in LA extract.

Inhibition of glutamate uptake in high concentrations of extract could be explained by this fact that glutamate uptake by astrocytes is inhibited by high concentration of ROS which has been produced in response to high concentrations of LA extract (Sorg et al., 1997).

Analysis of GABA release by HPLC showed that the GABA release was increased after treatment of astrocytes with LA (Fig. 3d). It can be concluded from the figure that the GABA release increases related to treatment time.

We hypothesized that GABA release is mediated via a gene expression pattern change in treated astrocytes which is mediated itself through glutamate uptake. We assume that this phenomenon mostly depends on expression of glutamate decarboxylase that convert the glutamate to GABA and has been shown to participate in GABA release signaling pathways. The kinetic study on glutamate release and GABA uptake showed that GABA release depends on glutamate uptake (Fig. 4). It is worth mentioning that the glutamate uptake and GABA release rate increases after 16 h of treatment which shows dependency of glutamate uptake and GABA release on a gene expression pattern.

4. Conclusion

Here we introduce another mechanism underlying LA activity against mental disorders. Our results showed that LA extract at concentrations lower than 3 mg/ml could enhances glutamate uptake and GABA release by astrocytes. We showed that GABA release is a glutamate uptake-dependent process in which glutamate is converted to GABA by glutamic acid decarboxylase enzyme. The quickness of this process in reducing the excitatory and increasing inhibitory neurotransmitters at the same time is responsible for effective activity of LA against epileptic activity of brain.

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References


