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Combination of *Zizyphus jujuba* and silymarin showed better neuroprotective effect as compared to single agent in MCAo-induced focal cerebral ischemia in rats

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ABSTRACT

Ethnopharmacological relevance: Traditionally, *Zizyphus jujuba* is used for anticonvulsant, hypnotic-sedative, anxiolytic, tranquilizer, antioxidant and anti-inflammatory properties. Likewise silymarin is popularly used for its potent antioxidant and hepatoprotective effects. Stroke being a multifactorial disease with unsatisfactory treatment outcomes, necessitates development of multimodal therapeutic interventions. Thus, we evaluated the therapeutic benefits of herbal combination of *Z. jujuba* and silymarin in a focal cerebral ischemia model.

Aim of the study: To evaluate the neuroprotective potential of hydroalcoholic extract of *Z. jujuba* (HEZJ) fruit and silymarin alone and in combination in middle cerebral artery occlusion (MCAo) model of focal cerebral ischemia in rats.

Materials and methods: Male Wistar rats were pretreated with HEZJ (100, 250 and 500 mg/kg, p.o.) or silymarin (250 mg/kg, p.o.) for 3 days prior to induction of MCAo. Neurological deficit score, motor impairment and cerebral infarction were assessed 24 h following MCAo. HEZJ (250 mg/kg) co-administered with silymarin (250 mg/kg) for 3 days prior to induction of MCAo was also evaluated for above parameters and oxidative stress. Malondialdehyde (MDA), nitric oxide (NO) and superoxide dismutase (SOD) levels in the cortex, striatum and hippocampal brain regions were estimated 24 h post MCAo.

Results: Pretreatment with HEZJ and silymarin reduced the neurological deficit score, motor impairment and cerebral infarction volume. HEZJ and silymarin pretreatment also ameliorated the oxidative stress in different brain regions, which was evident from increased SOD levels, decreased MDA and NO levels as compared to MCAo control rats. Interestingly neuroprotective efficacy was potentiated by pretreatment with HEZJ and silymarin combination.

Conclusion: Pretreatment with HEZJ and silymarin combination was observed to have better neuroprotection mediated *via* amelioration of oxidative stress in the focal cerebral ischemia model.

1. Introduction

Stroke is the third leading cause of death and long-term disability after heart disease and cancer worldwide (Murray et al., 2012). The complex array of ischemic-reperfusion injury mainly involves glutamate excitotoxicity, calcium and sodium overload, activation of enzymes (proteases, phospholipases, endonucleases), oxidative stress, inflammation, necrotic and apoptotic cell death (Xing et al., 2012). Among these mechanisms oxidative stress due to excessive generation of reactive oxygen species (ROS) is a predominant contributor (Gandhi and Abramov, 2012). ROS has either direct deleterious effect on macromolecules such as lipids, proteins, nucleic acids or indirect effect through various signaling pathways such as release of excitatory amino acids (Chan, 2001).

Currently, thrombolytic drug recombinant tissue-plasminogen activator (rt-PA) is approved for the treatment of acute ischemic stroke. The use of rt-PA is restricted to administration within 3 h of stroke onset, however increased risk of hemorrhagic transformation, limits its clinical use (Donnan et al., 2008). Moreover, secondary prevention strategies such as aspirin, clopidogrel or warfarin for patients with atrial fibrillation, are also limited due to ceiling effect, risk of bleeding or mortality (Donnan et al., 2008).

Therapeutic interventions particularly of herbal origin are gaining attention due to their wider therapeutic window, minimal side effects and cost effectiveness. Indeed pre-administration of herbal drugs such as *Withania somnifera* extract and trans-resveratrol are reported to be beneficial in stroke models (Chaudhary et al., 2003; Sinha et al., 2002).

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Zizyphus jujuba Mill. fruit, commonly known as Chinese date, Korean date or Indian date is extensively used as an analgesic, *anti*convulsant, sedative and tranquilizer in Indian folk medicine and Asian traditional medicine (Chopra et al., 1956; Park et al., 2004). In Ayurvedic system of medicine root, bark, leaves, fruits and seeds of *Zizyphus jujuba* are documented for treatment of cough, headache, boils, diarrhea, pyresis, obesity, fatigueness, constipation, allergy, tuberculosis, hypercholesterolemia, aconite poisoning, wound, anxiety, blood, eye and bile production disorders. *Zizyphus jujuba* is widely used for the treatment of various malaises especially among Indian tribal communities. In Chattisgarh, the fruit is used for the treatment of fever, seeds for vomiting in combination with bar sprouts and sugar, roots in combination with cow's milk for dysentery and leaves in combination with cumin for urinary tract infections (Mahajan and Chopda, 2009). Tribal communities *Mahadeo Kolis*, *Dhangars* and *Ramoshi* in the Parinche valley, Pune district of Maharashtra state, administer the aqueous leaf extracts alongwith mashed mango kernels for diarrhea (Tetali et al., 2009). In the Satpuda forest region of Dhule and Jalgaon in Maharashtra, *Pawara*, *Bhil* and *Pardhi* tribes use *Zizyphus jujuba* for diarrhea, fever and blood purification (Jain et al., 2010). The *Artocerpus gomezianus* bark alongwith *Shorea robusta*, *Zizyphus jujuba* and *Cassia fistula* bark is used to cure headache, dizziness and tuberculosis by *Garo* tribes in North Garo hills, Meghalaya (Sharma et al., 2014). The *Warli*, *Katkari* and *Kokona* tribes in the Thane district, Maharashtra uses fruit and bark for cough, cleansing of mouth, improvement of digestion, while Oraon tribal community in Jharkhand uses in their traditional diet (Kurve et al., 2015; Ghosh-Jerath et al., 2015). *Zizyphus jujuba* is prescribed as a tonic, anticonvulsant, stomachic, anti-carcinogenic and analgesic in Chinese traditional medicine (Han and Park, 1986). The fruit extract is reported to improve spatial memory impairment associated with seizures, scopolamine, ethanol and global cerebral ischemia (Pahuja et al., 2011; Heo et al., 2003; Majid et al., 2011; Yoo et al., 2010).

Silymarin, a polyphenolic flavonoid isolated from fruits (dried seeds) of *Silybum marianum* L. Gaertn. (milk thistle) belongs to the family Asteraceae/Compositae. Its flavanolignan mixture includes silibinin, isosilibinin, silicristin and silidianin (Schuppan et al., 1999). Ethnopharmacologically, silymarin is well-documented for the treatment of ailments such as headache, melancholy, galactagogue, dyspepsia, eczema, abscesses, hepatobiliary diseases (such as jaundice, hepatitis, gallstones and cirrhosis), renal disorders, hypercholesterolemia, reduced immunity, lung ailments, migraine, motion sickness, psoriasis, skin and spleen disorders and skin cancer for more than 2000 years. This herbal drug is also indicated for food and seasonal allergies, digestive disorders, snake bites, insect stings, environmental toxins, *Amanita phalloides* mushroom poisoning and alcohol intoxication (Corchete, 2008; Braun and Cohen, 2010; Kren and Walterová, 2005; Wichtl, 2004, Hudaib et al., 2008). Silymarin is reported to have antioxidant, antifibrotic, anti-inflammatory, membrane stabilizing, immunomodulatory and liver regenerating properties (Pradhan and Girish, 2006). Silymarin has been reported to have antioxidant activity in various neurodegenerative disorders such as stroke, aging, parkinsonism depicting blood-brain-barrier permeability (Raza et al., 2011; Muley et al., 2012; Galhardi et al., 2009; Baluchnejadmojarad et al., 2010). Silymarin has been shown to exert preservation effect on delayed neuronal cell death in the rat hippocampus without consideration of the timing of silymarin administration (Hirayama et al., 2016). In the present study, the neuroprotective potential was investigated following pretreatment with hydroalcoholic extract of *Zizyphus jujuba* fruit (HEZJ), silymarin and their combination in MCAo model of stroke in rats. We hypothesized that HEZJ and silymarin combination will provide superior neuroprotection as compared to single treatment.

2. Material and methods

2.1. Animals

Albino male Wistar rats weighing 240–260 g were procured from the Central Animal Facility of All India Institute of Medical Sciences, New Delhi and group housed in polypropylene cages ($38 \times 23 \times 10$ cm) with not more than 4 animals per cage. The animals were maintained under standard laboratory conditions with natural dark-light cycle (14 ± 1 h light; 10 ± 1 h dark). They were allowed free access to standard dry rat diet (Aashirwad Industries, Chandigarh) and tap water *ad libitum*. All experimental procedures were reviewed and approved by the Institutional Animal Ethics Committee (591/IAEC/11).

2.2. Drugs and chemicals

The following materials were procured as: nylon filaments 3-0 ethicon (Johnson & Johnson, India), absorbable sutures 6-0 ethicon (Johnson & Johnson, India), 2,3,5-triphenyltetrazolium chloride (Sigma Chemical Co., USA), formaldehyde (Sigma Chemical Co., USA) and silymarin (Sigma Chemical Co., USA). All other reagents were of analytical grade and obtained from Central Drug House (P) Ltd. and Sisco Research Laboratories Pvt. Ltd., India.

2.3. Plant material

Zizyphus jujuba fruits purchased from commercial market at Khari Boali in New Delhi and was authenticated by Chief Botanist at the Department of Pharmacognosy, Hamdard University, Delhi (Voucher No. PRL/JH/08/13). Hydroalcoholic extraction was done at a GMP certified laboratory Sanat Products Limited, Sikandrabad according to the method as described earlier (Pahuja et al., 2011). Briefly, the coarsely powdered dried plant material was subjected to hot extraction for 3 h using 50% methanol (hydroalcoholic) as solvent. The filtrate was concentrated under vacuum at 40 °C with a rotavapor obtained from first, second, and third extraction. The semisolid hydro alcoholic extract dried in a vacuum tray dryer provided percentage yield 58.8% (w/w) in alcohol and 54.5% (w/w) in water.

2.4. Determination of betulinic acid in HEZJ by HPLC

The chromatographic analysis was performed using Shimadzu High Performance Liquid Chromatographic (HPLC) system, equipped with Photo diode array or UV detector at a wavelength of 205 nm. The analysis was carried using Hibar, prepacked column, LiChrospher 100, RP-18e (5 μm) (Merck) Phenomenex- Luna 5μ C-18(2), size: 250×4.60 mm with a flow rate of mobile phase at 1.5 ml/min. The mobile phase consisted of solvent A and solvent B (acetonitrile). Solvent A included 0.136 g of anhydrous potassium dihydrogen orthophosphate (KH_2PO_4) in 900 ml of HPLC grade water and 0.5 ml of orthophosphoric acid. The volume was made upto 1000 ml with water, filtered through 0.45 μ membrane and degased in a sonicator for 3 min. The gradient conditions of mobile phases used were solvent A (85): solvent B (15). The standard betulinic acid (0.2 mg/ml) was prepared in HPLC grade methanol. The HEZJ extract sample solution (500 mg) was prepared in 5 ml of methanol, boiled in water bath for 5 min at 70–80 °C. The solution was then cooled, sonicated for 10 min and again after cooling, volume was make up using HPLC grade methanol. The sample solution was shaken well and filtered through 0.2 μm membrane filter paper. The instrument was set up as per the chromatographic condition as prescribed above. The chromatogram in triplicate was acquired by injecting 20 μl of standard or sample. The mean area and the relative standard deviation (RSD)