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EFFECT OF B. MONNIERI LEAF EXTRACT TARGETED AT ADENOSINE RECEPTOR IN DIABETIC NEUROPATHIC PAIN

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ABSTRACT

Diabetic neuropathic pain is generally considered to be one of the most troublesome complications affecting diabetic patients and current therapy provides inadequate pain relief. In the present study, the neuroprotective effect of *Bacopa monnieri*, Linn. (Brahmi, BM) targeted at adenosine receptor was studied in a model of diabetic neuropathic pain. Diabetes was induced by streptozotocin (65 mg/kg, ip) in male Sprague Dawley rats and subjected to thermal (cold and hot) and chemical (formalin) stimuli. Diabetic rats developed hyperalgesia by the end of six weeks in thermal and chemical stimuli test. Ethanolic extract of *Bacopa monnieri* leaf (EEBM) (500 mg/kg, ip) produced significant reversal of responses to thermal and chemical stimuli in diabetic rats. The results indicate that EEBM is an effective analgesic in a model of diabetic neuropathy, and the protection produced by EEBM is via stimulation of adenosine A_1-receptors.

KEY WORDS

Adenosine receptor; Bacopa monnieri; Diabetes; Neuropathic pain.

INTRODUCTION

Bacopa monnieri (Linn.) belongs to the family Scrophulariaceae. It is a prostrate, juicy, succulent, glabrous annual herb rooting at the nodes with numerous ascending branches. Leaves are simple, opposite, decussate, sessile, ovate–oblong or spatulate, entire, fleshy, obscurely veined and punctate. Flowers are pale blue or whitish, axillary, solitary, arranged on long slender pedicels. Fruits are ovoid, acute, 2-celled, 2-valved capsules and tipped with style base. Seeds are minute and numerous (Warrier *et al.*, 1993). This plant are growing in grasslands occurring in aquatic sites, sand and wet soil occupying in the edges of freshwater or brackish pools and streams and lake beds. Distributed in the major part of the plains of India, Pakistan, Afghanistan, Nepal, Sri Lanka, subtropical US, tropical Asia, Africa and Australia (Russo and Borreelli 2005).

Neuropathic pain is generally considered to be one of the most troublesome complications affecting diabetic patients (Gilron and Coderre 2007, Clark and Lee 1995). Current therapy for painful neuropathy includes use of antidepressants, ion channel blockers, NMDA receptor antagonists, opioids, topical lidocaine and capsaicin (Gilron and Coderre 2007, Sindrup and Jensen1999). Pain relief with current therapy is often inadequate and associated with side effects (Gilron and Coderre 2007, Clark and Lee 1995, Arner and Meyerson 1988, Abuaisha *et al.*, 1998). Adenosine and adenosine receptor agonists have antinociceptive effect in animal models of acute (Bastia *et al.*, 2002, Curros-Criado and Herrero 2005, Malhotra *et al.*, 2000, Sawynok 1998), inflammatory (Maione *et al.*, 2007, Borghi *et al.*, 2002, Jarvis *et al.*, 2002, Poon and Sawynok 1998, Lavand'homme and Eisenach 1999) and nerve injury induced neuropathic pain (Lavand'homme and Eisenach 1999, Sjolund *et al.*, 1996). Systemic administration of adenosine and adenosine agonists produced analgesic action in patients with neuropathic pain (Gilron and Coderre

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2007, Segerdahl and Sollevi 1998). It has been reported that there is a significant reduction in the circulating and levels of adenosine in the cerebrospinal fluid in neuropathic pain patients (Guieu *et al.,* 1996). Adenosine is involved in the antinociceptive effect of intrathecal morphine following nerve injury (Sandner-Kiesling *et al.,* 2001). Amitriptyline, a tricyclic antidepressant, is effective in alleviating neuropathic pain conditions and this action is believed to be due to interaction with endogenous adenosine systems (Ulugol *et al.,* 2002, Esser and Sawynok 2000). Adenosine kinase inhibitors, which enhance endogenous levels of adenosine, have shown protection in neuropathic pain states (Zhu *et al.,* 2001, Lynch *et al.,* 1999). Above evidence indicate antinociceptive potential of adenosine. However, effect of Bacopa monnieri targeted at adenosine receptor in diabetic neuropathic pain has not yet been evaluated. Therefore, in the present study, effect of Bacopa monnieri targeted at adenosine receptor in diabetic neuropathic pain was investigated in diabetic neuropathic pain model.

Earlier workers have isolated a number of chemical compounds from Bacopa monnieri. Bacopa contains alkaloids such as Hydrocotyline, Brahmine and Herpestine (Dutta *et al.,* 1963). Glycoside such as Asiaticoside and Thanakunicide. Flavonoids such as Apigenin and Luteonin. Saponins such as D-mannitol, Acid A, Monnierin [$C_{51}H_{82}O_{21}$3H2O] Bacoside A [$C_{41}H_{68}O_{13}$4H2O] and Bacoside B [$C_{41}H_{68}O_{13}$5H2O]. Additional Phytochemicals such as Betulinic acid, Wogonin, Oroxindin, Betulic acid, Stigmastarol, beta-sitosterol, as well as numerous Bacosides and Bacopasaponins, and amino acids like alpha alanine, Aspartic acid, Glutamic acid, and Serine, and its esters, Heptacosane, Octacosane, Nonacosane, Triacontane, Hentriacontane, Dotriacontane, Nicotine (Schulte *et al.,* 1972), 3-formyl-4-hydroxy-2H-pyran ($C_6H_6O_3$), and its 7- glucoside. Brahamoside, Brahminoside, Brahmic acid, Isobrahmic acid, Vallerine, pectic acid, fatty acids, tannin, volatile oil, ascorbic acid, thanakunic acid and asiatic acid (Chatterji *et al.,* 1965, Basu *et al.,* 1967, Kulshreshtha and Rastogi 1973, Kulshershtha and Rastogi 1973, Kulshershtha and Rastogi 1974, Chandel *et al.,* 1977). Jujubacogenin and pseudojujubacogenin (Kawai and Shibata 1978). In a thorough review of the chemical composition of Brahmi, Russo and Borrelli (Russo and Borrelli 2005) point out that the first constituent identified was an alkaloid brahmine. Saponins are considered to be the major active constituents of the plant. Saponins are glycosides, a sugar unit attached to an aglycone portion (the sapogenin). The sapogenin portion describes the type of saponin either steroidal (4- ringed structure), or triterpenoid (5-ringed structure) (Mills and Bone 2000). The main active chemical constituents of Bacopa are the dammarane-type triterpenoid saponins[35] with jujubogenin and pseudojujubogenin as the aglycones (Saraswati *et al.,* 1996). The saponins consist of numerous subtypes designated as bacosides, bacopasides and bacopasaponins. Bacoside-A is considered the major active component, first identified by Chatterji et al., 1963, withbacoside- B being an optical isomer of bacoside-A (Singh *et al.,* 1998). Chemical structure of Bacoside-A, B and C are represented as 3-0-α-L-arabinopyranosyl- 20-0-α-L-arabinopyranosyl-jujubogenin, 3-0-[α-L-arabinopyranosyl (1-2)α-L-arabinopyranosy] pseudojujubogenin and 3-0-[β-D-glucopyranosyl (1-3){α-L-arabinofuranosyl (1-2)}) α-L-arabinopyranosy] pseudojujubogenin respectively (Saraswati *et al.,* 1996).

The leaf of B. monnieri was collected and dried in shade. Then it was powdered and leaf material was extracted with distilled water. The aqueous extract was discarded and the residual leaf material was extracted thrice with 90% ethanol. The residue obtained after removing the solvent was dried in vacuum and macerated with acetone to give a free-flowing powder. The extract of B. monnieri contained 40–50% bacoside estimated as Bacoside A. The estimation method involves acid hydrolysis of bacosides, which yields quantitatively a

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transformed aglycone–ebelin lactone which contained a conjugated triene system and was estimated by UV spectrophotometer at 278 nm (Pal *et al.,* 1998).

It is used in traditional Indian medicine, the Ayurveda, for the treatment of anxiety, and in improving intellect and memory for several centuries (Kishore and Singh 2005). In addition to memory boosting activity, it is also claimed to be useful in the treatment of cardiac, respiratory and neuropharmacological disorders like insomnia, insanity, depression, psychosis, epilepsy and stress[2] It was reported to possess anti-inflammatory, analgesic, antipyretic, sedative, free radical scavenging and anti-lipid peroxidative activities (Kishore and Singh 2005, Anbarasi *et al.,* 2005). The plant is reported to have shown barbiturate hypnosis potentiation effect. The plant is anticancerous and improves learning ability. It is used as a tranquilliser. The plant is astringent, bitter, sweet, cooling, laxative, intellect promoting, anodyne, carminative, digestive, antiinflammatory, anticonvulsant, depurative, cardiotonic, bronchodialator, diuretic, emmenagogue, sudorfic, febrifuge and tonic (Basu and Lamsal 1947, Rastogi *et al.,* 1994). The pharmacological properties of B. monnieriwere studied extensively and the activitieswere attributed mainly due to the presence of characteristic saponins called as bacosides (Singh and Dhawan 1997). In animal studies, both purified bacosides and extracts of bacopa standardized for bacosides have been found to enhance several aspects of mental function and learning ability (Singh and Dhawan 1997, Singh *et al.,* 1988, Singh and Dhawan 1982). Additional brain effects of bacopa demonstrated in animal research include reduction of both anxiety and depression (Bhattacharya and Ghosal 1998, Sairam *et al.,* 2002). Biochemically, these nervous-system effects have been attributed to an enhancement of the effects of the neurotran smitters acetylcholine (Stough *et al.,* 2001, Bhattacharya *et al.,* 2000) and possibly, serotonin or GABA (gamma aminobutyric acid) (Ganguly and Malhtora 1967, Dey and Datta 1966). Bacopa extracts also appear to have significant antioxidant activity in the brain (Bhattacharya *et al.,* 2000) and other effects that may help to protect brain cells (Russo *et al.,* 2003). Animal research has also reported that bacopa extracts can relax the muscles that control the blood vessels, the intestine, and the airways of the respiratory system (Dar and Channa 1997, Dar and Channa 1997, Channa *et al.,* 2003, Dar and Channa 1999) and can help both prevent and heal ulcers in the stomach (Sairam *et al.,* 2001). Traditional herbal references recommend 5 to 10 grams per day of the powdered herb. Human research has used 300 to 450 mg per day of an extract standardized to contain 55% bacosides. Bacopa appears to be well tolerated when taken in typical amounts (Singh and Dhawan 1997), although one double-blind study reported significantly more symptoms of dry mouth, nausea, and muscle fatigue in participants taking Bacopa (Stough *et al.,* 2001).

The herb has been described in Ayurvedic texts since around 800 BC and recorded as a treatment for a range of mental disorders in the 'Charaka Samhita' (Singh and Dhawan 1997), which, according to the literature, was written in the 6th century AD (Russo and Borrelli 2005). Ayurvedic medicine classifies Bacopa as belonging to a group of plant medicines known as medhya rasayana that improve mental health, intellect and memory (medhya) and promote longevity and rejuvenation (rasayana) (Singh and Singh 1980). Hence Bacopa shares its Sanskrit name, Brahmi, with another herbal nervous system restorative Centella asiatica. Learning ability in rats has been significantly enhanced by Bacopa extract as it facilitated acquisition, consolidation and retention of three newly learned behavioural responses at an oral dosage of 40 mg/kg three times daily (Singh and Dhawan 1982). In this study, effects on cognitive function were measured by foot shock motivated brightness discrimination reaction, active conditioned flight reaction (jump to avoid shock)

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and continuous avoidance response (shock avoidance by lever pulling) tests.

The antioxidant activity of Bacopa has been reported in a number of laboratory studies (Bhattacharya *et al.*, 2000, Russo *et al.*, 2003, Sairam *et al.*, 2001, Tripathi *et al.*, 1996, Sumathy *et al.*, 2002, Russo *et al.*, 2003, Negi *et al.*, 2000). Antioxidant effects of Bacopa in areas of the brain that are key memory areas such as hippocampus, frontal cortex and striatum have been documented by Bhattacharya et al 2000 in rat brain. Bacopa was shown to protect the brain (Sumathy *et al.*, 2002) and liver (Sumathy *et al.*, 2001), from morphine induced inhibition of antioxidant enzyme systems. Russo et al 2005 demonstrated a free radical scavenging activity which protected against cytotoxicity and DNA damage in human fibroblasts (Russo *et al.*, 2003). Further research by Russo et al 2003 also demonstrated that Bacopa significantly reduced oxidation and DNA damage in cultured rat astrocytes induced by a nitric oxide donor. Furthermore, Anbarasi et al 2005 demonstrated that isolated bacoside-A protected rat brain tissue from various parameters of oxidative stress caused by chronic cigarette smoke exposure. One of the foremost theories of brain ageing asserts that free radical damage results in both ageing-related changes in healthy brains (Trollor and Valenzuela 2001) and in neurodegenerative pathology, such as Alzheimer's Disease (Singh *et al.*, 2004). Good antioxidant status is associated with better memory performance in the aged (Perrig *et al.*, 1997) and antioxidant therapy has been targeted as a promising dementia strategy (Jorm 2002). Thus, the demonstrated antioxidant effects of Bacopa, particularly in brain tissue, support its potential as a therapy in neurodegenerative pathologies and age-related cognitive decline. Stress elicits a defensive response in living organisms. The defense response involves several mechanisms including stress gene expression, enhanced antioxidant protection, and enhanced toxin clearance. Bacopa has been shown to facilitate each of these adaptive resources by modulation of Hsp 70 expression, and enhancement of activity of both superoxide dismutase and cytochrome P450 enzymes in stressor exposed rat brain. Thus, Bacopa may facilitate the capacity of the brain to withstand stress, and help the brain to function under adverse conditions. These findings support the afore-mentioned medhya rasayana classification of Bacopa in ancient Ayurveda in that they imply a brain tonic and adaptogenic effect (adaptogenic meaning improved resistance to stress). This may indicate some similarities with Panax ginseng,which is considered to be amajor adaptogen and tonic, enhancing resistance to stress in numerous experimental situations as well as clinical trials (Mills and Bone 2000, Blumenthal ME 2003).

EXPERIMENTAL

Materials

All drugs were procured from Sigma, Aldrich India. They were prepared freshly and administered intraperitoneally by using a 26-gauge hypodermic needle. Streptozotocin was dissolved in citrate buffer (pH 4.4). Ethanolic extract of Bacopa monnieri (EEBM) was suspended in 10% Tween 20. 8-Cyclopentyl-1, 3-dipropylxanthine (DPCPX) and 3,7-dimethyl-l-propargylxanthine (DMPX) were dissolved in dimethylsulphoxide (DMSO) and distilled water respectively. Effects of EEBM on thermal (cold and hot immersion) and chemical (formalin) stimuli were carried out after six weeks of streptozotocin administration. Rats were pretreated (30 min) with different doses of EEBM (100, 200 and 500 mg/kg, ip). They were observed for a period of 2 h at the interval of 15, 30, 60 and 120 min in cold and hot immersion test. In case of chemical stimuli observations were made after 30 min of drug administration. To study the involvement of adenosine A_1 or A_2-receptor in adenosine effect, DPCPX (1 mg/kg, ip), an adenosine A_1-receptor antagonist or DMPX (1 mg/kg, ip, an adenosine A_{2A}- receptor antagonist was administered 15 min prior to administration of EEBM (500 mg/ kg).

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Control groups were also maintained with appropriate vehicles used for the different drugs.

Healthy male Sprague Dawley (140–160 g) rats were used for the study. They were procured from Animal House, University Department of Pharmaceutical Sciences, Utkal University, Vanivihar, Bhubaneswar, India. The animals were provided with the standard diet and water *ad libitum* and kept at standard conditions. All the animals were acclimatized for a minimum of one week prior to the study. The experimental protocol was approved by Institutional Animal Ethics Committee of University Department of Pharmaceutical Sciences, Utkal University, Vanivihar, Bhubaneswar, India.

Methods

Diabetes was developed by administration of streptozotocin (65 mg/kg, ip). Before and after injection of streptozotocin, plasma glucose levels were analysed using the glucose GOD-PAP span diagnostic kit method (Qualigen, Glaxo, India). Only those animals showing blood glucose > 250 mg/dl were included in the drug treatment study.

Cold and hot immersion tests were carried out according to the method described by Sharma et al., 2008. In the cold immersion test, the tail of the rat was immersed in cold water maintained at 10°C, while in the hot immersion test; the tail was immersed in water maintained at 45°C. In both tests, basal tail flick latency (withdrawal response of tail) or signs of struggle were observed. The cut off time was 15 s. Cold and hot immersion tests were carried out each week for a period of six weeks in normal and streptozotocin diabetic rats and changes in tail flick latency in both groups were compared. Effect of Ethanolic extract of Bacopa monnieri (EEBM) was studied after six weeks of streptozotocin administration.

The formalin test was carried out according to the method of Courteix et al 1993. Formalin challenge was done once in all the groups. Formalin (0.1 ml 10%) was administered to the dorsal surface of the left hind paw in both normal and diabetic rats. Each animal was then placed in a plexiglass chamber and observed for 15 min for duration of licking and duration of paw elevation. Changes in duration of paw licking and paw elevation were observed after first, fourth and sixth week of streptozotocin administration. The effect of EEBM was studied out after six weeks of streptozotocin administration.

Results were expressed as mean ± S.E.M. and analysed (SigmaStat) using student V test, repeated measures of analysis of variance (ANOVA) followed by post hoc comparison where appropriate. $P<0.05$ was considered statistical significance.

RESULTS

Streptozotocin (65 mg/kg, ip) induced diabetes in 80% of animals. Before administration of Streptozotocin plasma glucose levels were 76.46±0.30 mg/dl. Six weeks after Streptozotocin administration, blood glucose levels increased to 340.26±1.68 mg/dl. In normal rats, plasma glucose levels were 72.68±1.36 (0 weeks) and 78.48±0.56 mg/dl (after 6 weeks). Streptozotocin treatment significantly decreased rat body weight 152.66±0.64 g (0 week) to 108.86±1.84 g (after 6 weeks). However, in normal rats body weight was significantly increased from 148.83±1.86 g (0 weeks) to 268.25±1.60 g (after 6 weeks) (Table 1).

Table 1.
Blood glucose level in normal and Streptozotocin induced Diabetic Rat

Treatment		0 Week	After 6th Week
Solvent (Tween 20 + Water)	Plasma Glucose Level (mg/dl)	72.68±1.36	78.48±0.56*

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	Body wt. (g)	148.83±1.86	268.25±1.60*
Streptozotocin (65 mg/kg, ip)	Plasma Glucose Level (mg/dl)	76.46±0.30	340.26±1.68*
	Body wt. (g)	152.66±0.64	108.86±1.84*

All the values are expressed as mean ± S. E. M., N = 10-12, *p<0.05 as compared to vehicle treated group.

Cold immersion test

Cold immersion test: Diabetic animals showed thermal hyperalgesia as evidenced by a significant reduction (P<0.05) in the tail flick latency in 80% of the diabetic animals by the end of six weeks in comparison to normal animals in cold immersion test. However, only 60% and 70% of the diabetic rats showed significant changes in tail flick latency by the fourth and fifth week respectively. Tail flick latency was 13.42±0.36 s before streptozotocin treatment and this was reduced to 8.86±0.48 s (P<0.05) and 6.42±0.66 s (P<0.05) after fourth and sixth week post treatment, respectively. However in normal rats tail flick latency was 13.82±0.28 s, 13.29±0.86 s and 13.48±0.46 s after 0, 4th and 6th weeks, respectively (Table 2). In diabetic animals, EEBM (500 mg/kg) produced significant reversal of thermal hyperalgesia while EEBM 100 and 200 mg/kg did not produce any reversal (Table 3). This reversal was dose dependent. Maximum protection was observed 30 min following drug administration. The adenosine A_1- receptor antagonist DPCPX, at 1 mg/kg, completely reversed the protection offered by EEBM 500 mg/kg in diabetic rats. However, DMPX, an adenosine A_2-receptor antagonist, failed to reverse the protection offered by EEBM 500 mg/kg (Table 3).

Table 2.

Tail flick latency in Streptozotocin induced diabetic rat (conformation of hyperalgesia after induction of diabetes)

Treatment		0 Week	After 4th Week	After 6th Week
Solvent (Tween 20 + Water)		13.82±0.28 s	13.29±0.86 s	13.48±0.46* s
Streptozotocin (65 mg/kg, ip)	Cold Immersion Test	13.42±0.36 s	8.86±0.48 s	6.42±0.66* s
	Hot Immersion Test	13.26±0.38 s	8.26±0.34 s	6.45±0.29* s

All the values are expressed as mean ± S.E.M. N=10-12 *P<0.05 as compared to vehicle treated group.

Table 3.

Effect of ethanolic leaf extract of Bacopa monnieri on Streptozotocin induced diabetic rat (cold & hot immersion test)

Treatment		0 Week	After 4th Week	After 6th Week
Solvent (Tween 20 + Water)		13.82±0.28 s	13.29±0.86 s	13.48±0.46 s
EEBM100 mg/kg, ip	Cold Immersion Test	6.42±0.66 s	--	--
	Hot Immersion Test	6.45±0.29 s	--	--

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EEBM 200 mg/kg, ip	Cold Immersion Test	6.42±0.66 s	--	--
	Hot Immersion Test	6.45±0.29 s	--	--
EEBM 500 mg/kg, ip	Cold Immersion Test	6.42±0.66 s	9.88±0.42* s	11.94±0.52* s
	Hot Immersion Test	6.45±0.29 s	10.44±0.28 s	12.12±0.68* s
DPCPX (A_1 Antagonist) 1 mg/kg	Cold Immersion Test	--	--	5.26±0.12* s
	Hot Immersion Test	--	--	5.09±0.25* s
DMPX (A_2 Antagonist) 1 mg/kg	Cold Immersion Test	--	--	--
	Hot Immersion Test	--	--	--

All the values are expressed as mean ± S.E.M. N=6–8. *P<0.05 as compared to vehicle treated group. EEBM: Ethanolic extract of *Bacopa monnieri*, DPCPX: 8-Cyclopentyl-1, 3-dipropylxanthine, DMPX: 3,7-dimethyl-l-propargylxanthine.

Hot immersion test

Hot immersion test: Diabetic animals showed thermal hyperalgesia as evidenced by a significant reduction in the tail flick latency in 90% of diabetic animals by the end of six weeks as compared to normal animals. Tail flick latency was 13.26±0.38 s before streptozotocin treatment and this was reduced to 8.26±0.34 s (P<0.05) and 6.45±0.29 s (P<0.05} after fourth and sixth week post treatment, respectively. However in normal rats tail flick latency was 13.82±0.28 s, 13.29±0.86 s and 13.48±0.46 s after 0, 4th and 6th weeks, respectively (Table 2). In diabetic animals, EEBM (500 mg/kg) produced significant reversal of thermal hyperalgesia while EEBM 100 and 200 mg/kg did not produce any reversal (Table 3). This reversal was dose dependent. Maximum protection was observed 30 min following drug administration. The adenosine A_1- receptor antagonist DPCPX, at 1 mg/kg, completely reversed the protection offered by EEBM 500 mg/kg in diabetic rats. However, DMPX, an adenosine A_2-receptor antagonist, failed to reverse the protection offered by EEBM 500 mg/kg (Table 3).

Chemical stimuli

Chemical stimuli: Significant increase in durations of licking and paw elevation indicates the development of hyperalgesia in diabetic rats in comparison to normal rats. In streptozotocin induced rats, paw licking and paw elevation after six weeks was 86.24±1.36 s (P<0.05) and 92.84±1.56 s (P<0.05), respectively as compared to normal rats 34.12±0.84 s (paw licking) and 38.83±1.24 s (paw elevation). However in diabetic rats, paw licking and paw elevation after six weeks of EEBM (500 mg/kg) administration was 36.44±2.18 s (P<0.05) and 42.50±1.97 s (P<0.05), respectively as compared to normal rats 79.83±0.34 s (paw licking) and 88.83±0.78 s (paw elevation). EEBM (500 mg/kg) produced significant improvement in the parameters of pain in comparison to vehicle treated diabetic rats. DPCPX at 1 mg/kg completely reversed the protection offered by EEBM 500 mg/kg, while DMPX failed to reverse the protection offered by EEBM (Table 4).

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Table 4.

Effect of ethanolic leaf extract of Bacopa monnieri on Streptozotocin induced diabetic rat (chemical stimuli)

Treatment		0 Week Normal Value	After 6th Week of Streptozotocin administarion	After 6th Week of EEBM administration
Streptozotocin induced diabetic rat (65 mg/kg, ip)	Paw licking	34.12±0.84 s	86.24±1.36 s	--
	Paw elevation	38.83±1.24 s	92.84±1.56 s	--
EEBM (500 mg/kg, ip)	Paw licking	79.83±0.34 s	--	36.44±2.18* s
	Paw elevation	88.83±0.78 s	--	42.50±1.97* s

All the values are expressed as mean ± S.E.M. N=6–8. *P<0.05 as compared to vehicle treated group. EEBM: Ethanolic extract of *Bacopa monnieri*.

DISCUSSION

The present study confirms that streptozotocin-induced diabetes alters nociceptive thresholds and causes disturbances in responses to nociceptive and noxious stimuli. However, it requires several weeks to become evident. Alterations in pain thresholds were found to be progressive; the number of rats affected and the degree to which they were affected increased up to the sixth week of experimentation. These characteristics are close to the disturbances in pain sensation as observed in diabetic humans (Courteix *et al.*, 1993). Decrease in pain threshold was observed with mildly noxious stimulus. Water at 10°C failed to induce tail withdrawal in normal rats before the cut off time (15 s) showing that this temperature is mildly noxious. However, the diabetic rats behaved in a way as if this temperature was painful which indicates the development of allodynia. By the fourth week, a considerable number of animals showed tail withdrawal latencies of less than 15 s, but after six weeks the percentage of responders was 80%. This data with cold immersion is similar to that of Courteix et al.1993, who reported that diabetes induced thermal allodynic responses were present as early as 2 weeks post streptozotocin treatment in a subpopulation of rats, and the number of responders increased with time. There was also a similar increase in hyperalgesic activity in diabetic rats in comparison to normal rats when subjected to thermal (hot immersion) stimuli. The results are fully consistent with earlier reports in which streptozotocin diabetes produced a decrease in pain thresholds (Courteix *et al.*,1993, Forman *et al.*, 1986). Similar alterations in pain threshold were observed in alloxan diabetes model (Lee *et al.*, 1990). The mechanisms responsible for the decreases in pain threshold level in diabetic rats are not yet completely established. Alterations of receptor levels, hyperactivity of nociceptive fibres and injury to nerve fibres have been implicated (Burchiel *et al.*, 1985, Wuarin-Bierman *et al.*, 1987, Brown *et al.*, 1976). An exaggerated response to formalin induced pain behaviours of licking and elevation of the injected paw was observed in diabetic rats as compared to normal rats. This indicates hyperalgesia to chemical stimuli. About 80% of the animals kept their paw raised without ground contact for a much longer time than controls. In the present study, we investigated for the first time, effect of EEBM on thermal and chemical hyperalgesia in diabetic rats targeted at adenosine receptor. EEBM (500 mg/kg) produced significant reversal of hyperalgesia in diabetic animals. With formalin, the same dose of EEBM offered protection against licking and paw elevation behaviours. These results demonstrate the involvement of adenosinergic system in diabetic neuropathic pain. Results of the present

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study are further supported by a report in which adenosine administration showed improvement in motor nerve conduction and nerve blood flow in diabetic rats (Kumar *et al.*, 2005). Adenosine and directly acting adenosine receptor agonists have been shown to reduce nerve injury (Lavand'homme and Eisenach 1999, Gomes *et al.*, 1999, Karlsten and Gordh 1995) and carrageenan (Jarvis *et al.*, 2002) induced hyperalgesia in rats. Their administration also alleviates hyperalgesia and allodynia in human subjects (Karlsten and Gordh 1995, Eisenach *et al.*, 2002). Adenosine kinase inhibitors also have shown to have antinociceptive effect in neuropathic animal models of nociception (Zhu *et al.*, 2001). Collectively, these reports suggest a significant role of EEBM targeted at adenosine receptor in regulating neuropathic pain. EEBM stimulates the adenosine receptor subtype A_1 which is linked to a number of effectors namely, adenylate cyclase, ionositol phosphate, K^+ channel, Ca^{2+} channel and neurotransmitters release. Activation of adenosine A_1-receptors results in a decrease in adenylate cyclase activity leading to decrease in intracellular level of cyclic adenosine monophosphate, while activation of adenosine A_2-receptors stimulates adenyl cyclase activity (Fredholm *et al.*, 2001). In our study, the protective effect of EEBM (500 mg/kg) was reversed by DPCPX, an adenosine A_1-receptor antagonist but not by DMPX, an adenosine A_{2A}-receptor antagonist, indicating the involvement of adenosine A_1-receptors in alleviating diabetic neuropathic pain. Studies with adenosine receptor agonists and antagonists have demonstrated a pharmacological profile for spinal antinociception that is primarily adenosine A_1-receptor mediated (Sawynok J 1998). Specific activation of adenosine A_1-receptor reduced inflammation evoked responses of spinal cord dorsal horn neuron (Dickenson *et al.*, 2000). The exact mechanism of adenosine protection in neuropathic pain is unclear given the multitude of effectors system linked to adenosine receptors. Pre or postsynaptic mechanisms such as inhibition of excitatory amino acids and control of calcitonin gene related peptides, neuropeptide and substance P release (Mauborgne *et al.*, 2002, Sjolund *et al.*, 1997, Santicioli *et al.*, 1992) may contribute to their effect. Supraspinal (Herrick-Davis *et al.*, 1989) and peripheral mechanisms (Sawynok J 1998) may also involve in antinociceptive and anti-inflammatory action of adenosine. In addition, there is also evidence that adenosine agonists produce analgesia by interactions with neurotransmitters especially dopamine, norepinephrine and serotonin (Bastia *et al.*, 2002, Aran and Proudfit 1990, Sweeney *et al.*, 1988).

CONCLUSION

In summary, diabetes alters nociceptive thresholds indicating the development of diabetic neuropathic pain. EEBM showed significant effectiveness in a model of diabetic neuropathic pain and protection produced by adenosine was via stimulation of adenosine A_1-receptors. These findings suggest the potential of adenosinergic agents in diabetic neuropathic pain and may offer a therapeutic alternative to existing treatment. Further studies are required to understand the exact mechanism and its clinical implications

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