THE ROLE OF OPIOIDERGIC MECHANISMS IN THE ACTIVITY OF *BACOPA MONNIERI* EXTRACT AGAINST TONIC AND ACUTE PHASIC PAIN MODALITIES

Fazal Subhan[1], Muzaffar Abbas[1], Khalid Rauf[1], Mohammad Arfan[2], Robert D. E. Sewell[3], Gowhar Ali[1]

[1]Department of Pharmacy, University of Peshawar, Peshawar, Pakistan.
[2]Institute of Chemical Sciences, University of Peshawar, Peshawar, Pakistan
[3]Welsh School of Pharmacy, Cardiff University, Redwood Building, King Edward VII Ave., Cardiff, CF10 3NB, UK

[*]**Corresponding Author:**
Dr. Fazal Subhan, Department of Pharmacy, University of Peshawar, Pakistan
Email: fazal_subhan@upesh.edu.pk, Mob. No. +92301880596

Summary

A hydroethanolic extract of *Bacopa monnieri* (BM HE-ext) was examined in comparison with morphine and diclofenac for antinociceptive activity in the acetic acid induced abdominal constriction assay and the hot plate test in mice. Morphine and BM HE-ext produced dose-related activity which was naloxone-reversible in both tests. Diclofenac effects were dose dependent only in the abdominal constriction assay and they were not naloxone-reversible at a higher dose. The BM HE-ext antinociception was opioidergic in nature and most likely a manifestation of activity against both tonic and acute phasic pain modalities in each of these nociceptive paradigms.

Key words: *Bacopa monnieri,* Antinociceptive activity, Abdominal constriction test, Hot plate test, Naloxone, Tonic and phasic pain.

Introduction

Bacopa monnieri (family: Scrophulariaceae[1]) also known as Water hyssop or *Herpestis monniera*, is a perennial creeping, succulent herb found in marshy areas of the Indo-Pak subcontinent[2]. In India, It is commonly known as the medicinal plant "Brahmi" (Sanskrit), which has an ancient reputation as a memory vitalizer[3]. *Bacopa monniera* (*B. monnieri*) is highly valued in conditions affecting the central nervous system.

In traditional medicine, it is often prescribed for epilesy[4], insomnia, dementing disorders such as Alzheimer's disease[5] and as a memory enhancer[6]. It is also known to possess cardiotonic, antiinflammatory[7], antioxidant[8], anticancer, antipyretic, antistress[9], and anxiolytic properties[10]. *Bacopa monnieri* has also been reported to have antidepressant-like activity which is comparable to imipramine[11] and antidepressants have been used clinically for managing pain particularly of a neuropathic nature[12]. Antidepressants also yield antinociception[13], consequently, we have examined a hydroethanolic extract of *Bacopa monnieri* for antinociceptive activity in the abdominal constriction assay and hot plate test in mice as models for tonic (visceral) and acute phasic (supraspinal) pain[14]. We have also investigated the possibility of any opioidergic involvement against these pain modalities by testing for naloxone-reversibility.

Materials and Methods

Bacopa monnieri

Bacopa monnieri was collected from Ramli stream near Quaid-e-Azam University Islamabad, Pakistan and authenticated by Dr. Muhammad Ibrar, Professor of Botany University of Peshawar. A reference specimen was submitted to the herbarium of the Botany Department, University of Peshawar and a voucher specimen (029006/Bot. University of Peshawar) was used in the extraction.

Preparation of *Bacopa monnieri extract*

The aerial parts of the plant were separated from the roots, dried under shade, and coarsely ground. This material was then extracted with 70% aqueous-ethanol and concentrated on a rotary evaporator at 60 ^{0}C to the semisolid form (37.25% yield). The crude extract was completely solubilized in normal saline (0.9% sodium chloride) for use in the *in vivo* experiments.

Chemicals and Drugs

Ethanol was obtained from Khazana Sugar Mills Peshawar K.P.K. Pakistan. Diclofenac sodium was gratefully donated by Zinta Pharmaceutical Ptv, Peshawar, Pakistan. Morphine was secured through the Ministry of Health Pakistan from Punjab Drug House (PDH) Lahore, via proper legal channels. The opioid antagonist, naloxone was purchased from Sigma, USA. All drugs and extracts were dissolved in 0.9% sodium chloride.

Animals

Balb-C mice (18-22 g) of either sex and bred in the animal house of the Department of Pharmacy, University of Peshawar, were used in this study. Animals were housed in groups of eight per cage with sawdust bedding. Food and water were withdrawn two hours before testing and experiments were carried out during the

light phase between 9.00 a.m. and 3.00 p.m. strictly in accordance with procedures laid down under the Animal Scientific Procedure Act, UK (1986).

Acetic acid induced abdominal constriction assay

Visceral nociception was evaluated by the number of abdominal constrictions occurring in the 20 minute period following 1% acetic acid (10 mL/kg) intraperitoneal administration (i.p.) to Balb-C mice in groups of eight[15,16]. The hydroethanolic extract of *Bacopa monnieri* (40, 80, 160 mg/kg), diclofenac (12.5, 25, 50 mg/kg) or morphine (0.75, 1.5, 3 mg/kg) were administered i.p. 30 minutes before acetic acid challenge. In tests for antinociceptive antagonism, naloxone (0.5 mg/kg) was administered subcutaneously (s.c.) 5 minutes before nociceptive testing. All drugs were administered in a volume of 5 mL/kg i.p. or s.c.

The antinociceptive data were calculated and presented as "% protection" = (1- mean number of abdominal constrictions of drug treated group/mean number of abdominal constrictions of controls) x 100.

Hot plate test

Balb-C mice (n=8) were habituated to laboratory conditions for one hour before the start of each experiment. Animals were then subjected to pre-testing on the hot plate (Havard apparatus, USA) maintained at 54.0 ± 0.1^0C. They were placed on the hot-plate and the latency to response was measured in seconds[17,18]. The response end-point was signified by hindlimb flick, lick or jumping at which point animals were immediately removed from the thermal nociceptive stimulus in order to avoid any tissue damage or possibility of subsequent hypearalgesia. A cut-off time of 30 secs was imposed such that if they did not respond within this latency period then they were immediately removed from the hot plate stimulus. Thirty minutes after pre-testing, animals were administered drug treatment or saline i.p. and the response latency was determined 30 minutes post treatment. Where naloxone was employed as a pretreatment, it was administered s.c. 10 minutes before drug administration.

Antinociception was calculated using the following formula:

"% Antinociception" = (Test latency – control latency)/(Cut-off time – control latency) × 100

Statistical analysis

Results were analyzed by one-way analysis of variance (ANOVA) with post hoc Dunnetts test. Effects were considered significant at the $P < 0.05$ level.

Results

Protective action of morphine, diclofenac and *B. monnieri* hydroethanolic extract (BM HE-ext) in the abdominal constriction assay.

Saline treatment in control animals was found to be inactive against the occurrence of acetic acid induced abdominal constrictions in the test since the mean % protection value was calculated as only 5.0 ± 4.7. In comparison to saline control, morphine evoked significant ($P<0.01$) and characteristic dose-related antinociceptive responses in the assay and the dose-response relationship was both steep and linear over the dose range tested (0.75, 1.5, 3 mg/kg; i.p.) as shown in Fig. 1. Similarly, diclofenac produced significant protective activity in the test over the dose range 12.5 - 50 mg/kg but the resultant dose-response relationship lay to the right of that expressed by morphine. In addition, BM HE-ext also afforded significant protection against the visceral nociceptive stimulus producing a significant ($P<0.01$) graded protective response in the assay at all doses within the range 40-160 mg/kg such that the consequent dose-response relationship lay to the right of both morphine and diclofenac (Fig 1). The slope of the dose-response relationship for BM HE-ext was steep and comparable to that of morphine up to 80 mg/kg though there was a decline in its steepness between 80-160 mg/kg relative to morphine. Evaluation of extrapolated doses inducing 50% protection against abdominal constrictions revealed the following values: morphine (1.2 mg/kg), diclofenac (27.0 mg/kg) and BM HE-ext (61.0 mg/kg). Thus, the dose relationship for diclofenac and BM HE-ext were expressed respectively as 22.5 fold and 50.8 fold rightwards dose-response shifts compared to morphine (Fig 1.)

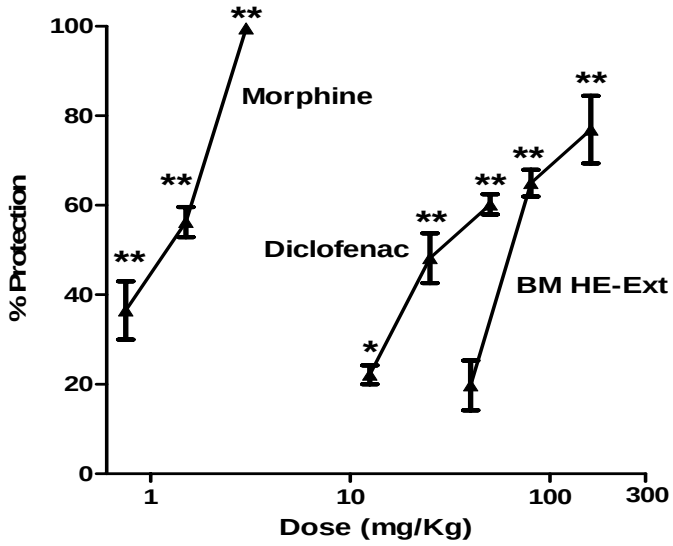

Fig. 1. Dose-response relationships for morphine, diclofenac and *B. monnieri* extract (BM HE-ext) expressed as % Protection (mean ± s.e.m) in the acetic acid abdominal constriction assay in mice (n=8). *P<0.05; **P<0.01; values are compared to saline control (ANOVA with Dunnett's post-hoc analysis).

Action of naloxone versus morphine, diclofenac and *B. monnieri* extract (BM HE-ext) in the abdominal constriction assay.

The selected dose of naloxone (0.5 mg/kg s.c.) used in the present investigation did not possess any significant protective activity (12.3 ± 5.5 %) by itself against the incidence of acetic acid-induced abdominal constrictions and this was analogous to the saline control response (Fig 2). The protection produced by the 3.0 mg/kg dose of morphine was extensively antagonized by naloxone (P<0.05) to a level which was not significantly different (P>0.05) from that observed in the naloxone plus saline control group or the saline control group. In contrast, the % protection imposed by diclofenac (50 mg/kg) was not antagonised by naloxone since there was no statistical difference in the occurrence of abdominal constrictions displayed by the diclofenac treated control animals and those receiving diclofenac plus naloxone (P<0.05) as shown in Fig 2.

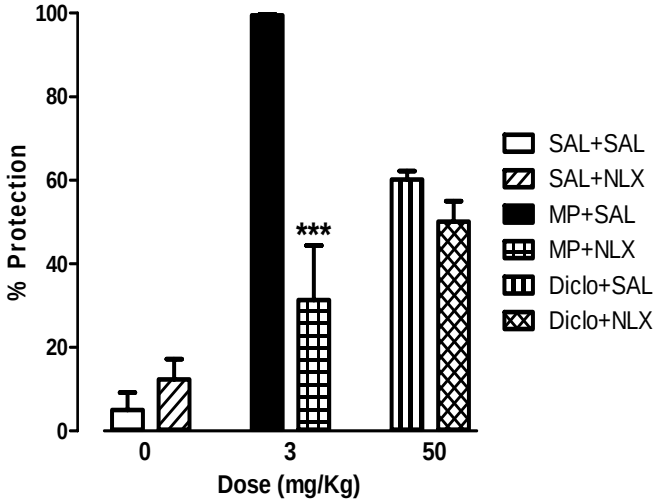

Fig. 2. Action of naloxone (0.5 mg/kg; NLX) or saline (SAL) on morphine (3.0 mg/kg, MP) and diclofenac (50 mg/kg; Diclo) induced % Protection (mean ± s.e.m) in the acetic acid abdominal constriction assay in mice (n = 8). ***P<0.01; values are compared to saline control (ANOVA with Dunnett's post-hoc analysis).

The dose-related protection against acetic acid induced abdominal constrictions generated by the BM HE-ext on the other hand was completely attenuated by

naloxone pretreatment and this achieved the greatest level of significance (P<0.001) in the group administered the highest doses of the extract (80 and 160 mg/kg) as depicted in Fig 3. In essence, the dose-response relationship to BM HE-ext was completely abolished by naloxone.

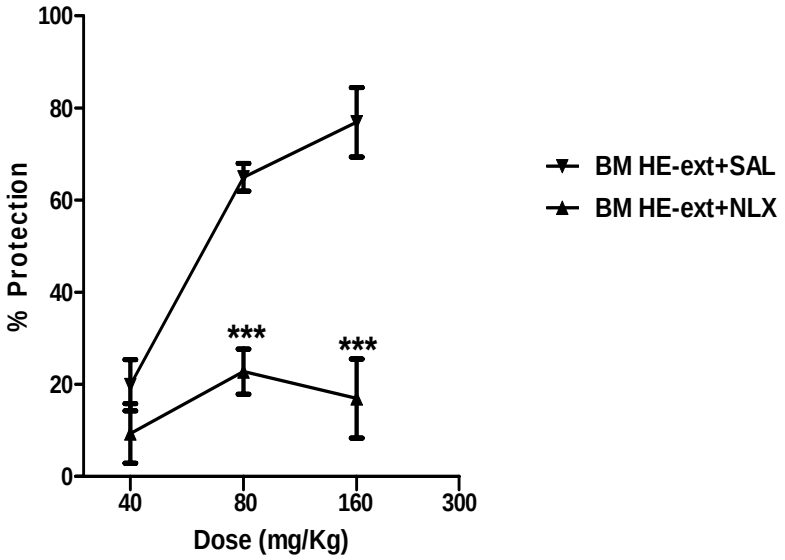

Fig. 3. Action of naloxone (0.5 mg/kg; NLX) or saline (SAL) on *B. monnieri* extract (BM HE-ext) induced dose related % Protection (mean ± s.e.m) in the acetic acid abdominal constriction assay in mice (n = 8).***P<0.001; values are compared to saline control (ANOVA with Dunnett's post-hoc analysis).

Antinociceptive activity of morphine, diclofenac and *B. monnieri* extract (BM HE-ext) in the hot plate test.

As shown in Fig. 4, morphine produced a significant (P<0.01) increase in the response latency to hot plate thermal stimulation measured 30 min after dosing (2.5, 5, 10 mg/kg, i.p.) in comparison with the saline vehicle control treated group. Although the hydroethanolic extract of *Bacopa monnieri* (BM HE-ext) did not yield any increase in hot plate response latency at lower doses of 40-60 mg/kg, it did produce a very significant (P<0.01) antinociceptive effect at the highest dose tested (80 mg/kg i.p.). Conversely, diclofenac (50 mg/kg, i.p.) was devoid of any detectable hot plate antinociceptive activity (Fig 4) even though it was protective in the abdominal constriction assay at the 50 mg/kg dose.

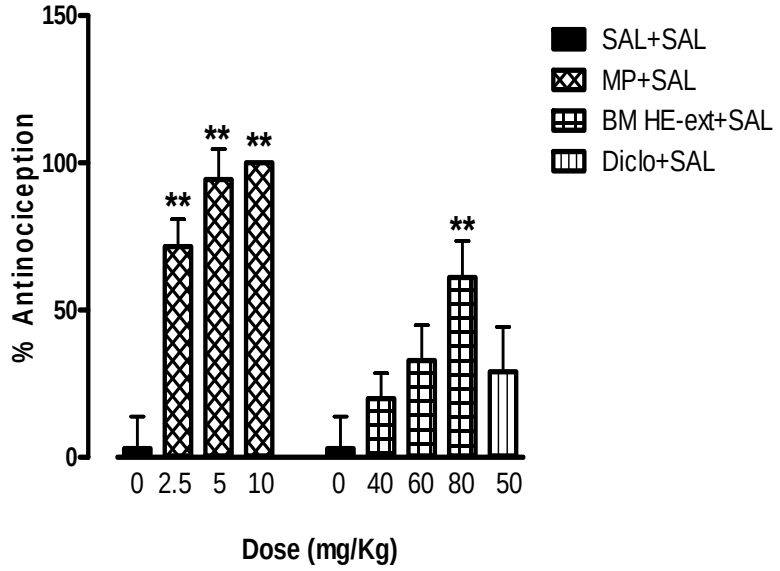

Fig. 4. Antinociceptive activity of morphine (2.5, 5.0 and 10.0 mg/kg; MP), *B. monnieri* extract (40, 60 or 80 mg/kg; BM HE-ext) or diclofenac (50 mg/kg; Diclo) in the hot plate test expressed as % Antinociception (mean ± s.e.m) in mice (n=8). **P< 0.01; values are compared to saline control (ANOVA with Dunnett's post-hoc analysis).

Action of naloxone versus morphine, and *B. monnieri* extract (BM HE-ext) in the hot plate test.

Administration of subcutaneous naloxone (1.0 mg/kg) did not produce any inherent change (P>0.05) in hot plate latency (2.3 ± 15.4 % antinociception) and this intrinsic lack of effect resembled that observed in saline vehicle administered controls (Fig 5). However, ten-minute pretreatment with naloxone significantly eradicated the mean group hot plate % antinociception not only to morphine (3.0 mg/kg, P<0.001) but also BM HE-ext (80 mg/kg, P<0.05). In both cases, the resultant downgraded hot plate response was no greater than that recorded in the saline vehicle treated group (Fig 5)

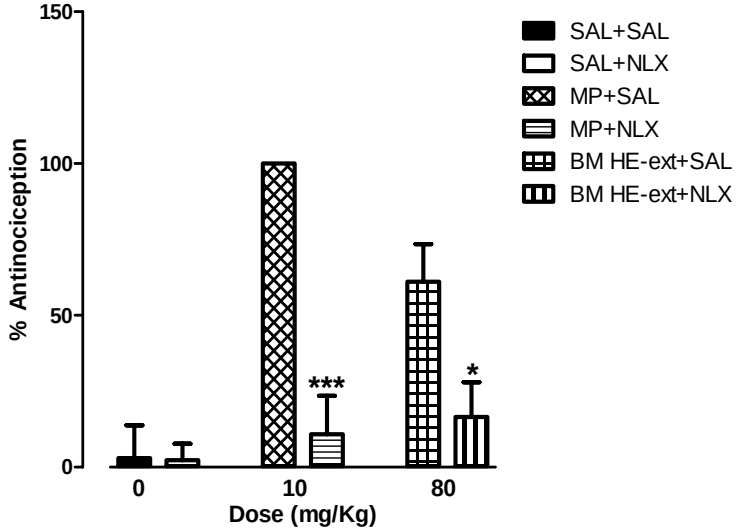

Fig.5. Action of naloxone (1.0 mg/kg; NLX) or saline (SAL) on the antinociceptive activity of morphine (10.0 mg/kg; MP) or *B. monnieri* extract (80 mg/kg; BM HE-ext) in the hot plate test expressed as % Antinociception (mean ± s.e.m) in mice (n=8).*P<0.05; ***P< 0.0001; values are compared to saline control (ANOVA with Dunnett's post-hoc analysis).

Discussion

The nociceptive response in the acetic acid induced abdominal constriction assay stems from the synthesis of prostaglandins[19,20] via the action of the constitutive enzyme cyclooxygenase-1 (COX-1) and its isoform COX-2 which produce hyperalgesia in sensory nerve endings and pain[20,21]. Induction of this mechanism through COX enzymes and stimulation of these sensory pathways in the mouse peritoneum incites a viscero-somatic reflex and the abdominal constrictions observed in response to an algogenic agent such as acetic acid[20,21]. The acetic acid induced abdominal constriction assay is sensitive to analgesics[22] and sensory afferents in the peritoneum carry $\alpha_{1/2}$-adrenoceptors, β-adrenoceptors and opioid receptors on their terminals[23]. When activated by appropriate agonists, these receptors depress the generation of pain impulses, in some instances there being an interaction between α-adrenoceptors and opioid receptors in the mouse peritoneum[13,15,16].

The abdominal constriction assay may implicate both central and peripheral mechanisms[24]. The response, however, is generally considered a viscerosomatic reflex and there is evidence of an involvement of peritoneovisceral chemoreceptors[25] particularly in the early phase of the nociceptive reaction. In a later nociceptive phase, there is a participation of inflammatory processes and in addition, neurons in the spinal cord dorsal horn become activated[26]. This type of long-duration visceral nociceptive stimulus has been categorised as a tonic pain modality[14]. In contrast, the nociceptive responses in the hot plate test are supraspinally integrated and this is usually considered as a suitable model for the determination of central pain mechanisms[27,28]. Consequently, the hot plate test has been classified as a supraspinal short-duration stimulus assay reflective of an acute phasic pain modality[14].

In this study, morphine, diclofenac and the hydroethanolic extract of *Bacopa monnieri* (BM HE-ext) all produced statistically significant antinociceptive effects against acetic acid challenge in the abdominal constriction assay in mice (Figs. 1, 2 and 3). In accord with this finding for BM HE-ext, one of its triterpenoid constituents known as bacosine has also been shown to possess analgesic activity[29].

Likewise, both morphine and BM HE-ext also exhibited antinociceptive activity in the hot plate test in mice (Fig. 4 and 5). The ability of BM HE-ext to reduce a thermally induced nociceptive response in this study suggests that there is a capacity of *B. monnieri* extract to inhibit non-inflammatory pain possibly through centrally mediated mechanisms on acute phasic pain which is modeled by the hot plate paradigm[14]. Furthermore, morphine, a standard centrally acting opioid analgesic, produced an analagous inhibitory effect on the thermal nociceptive response while diclofenac, a standard peripherally acting analgesic, failed to produce any significant antinociceptive effect in the hot plate test (Figs. 4 and 5) and this concurs with the findings of Stephens[30].

In order to probe the underlying antinociceptive mechanism of *B. monnieri* extract, its activity was examined in the presence of the non selective opioid receptor antagonist, naloxone in the hot plate and abdominal constriction tests. The antinociceptive activity of both morphine and BM HE-ext was antagonized by naloxone in the two tests. In contrast, the antinociceptive effect of diclofenac (80 mg/kg) was not antagonized by naloxone in the abdominal constriction test. However, at a dose of 25 mg/kg, diclofenac antinociception was attenuated by naloxone in this test (data not shown) indicating that at lower doses, diclofenac antinociception involves opioid receptors in the suppression of noxious visceral stimulation. This finding concerning diclofenac reversibility by naloxone is in an agreement with a previous report in rats for this anti-inflammatory agent[31].

In conclusion, BM HE-ext displayed dose-related antinociceptive responses in the abdominal constriction assay as well as the hot plate test and this is most likely to be the manifestation of activity against both tonic and acute phasic pain modalities in each of these animal paradigms[14]. The fact that BM HE-ext antinociception was naloxone reversible in both of these modalities also suggests that this action was opioid in nature[29] though it has yet to be established whether this opioidergic mechanism involves direct receptor activation or endogenous opioid release. Furthermore, it has been demonstrated that *B. monniera* extract reduces the *in vitro* effects of morphine withdrawal in guinea-pig ileum[32] which suggests that this plant extract may be useful in reducing the *in vivo* withdrawal symptoms induced by morphine.

Acknowledgements

We gratefully acknowledge the support of the Ministry of Health and Ministry of narcotic control, Pakistan for granting permission to acquire morphine for the study. We are also thankful to Punjab Drug House (PDH), Lahore for the gift of morphine.

References

1. Stewart RR. Flora of West Pakistan. Fakhri printing press, Karachi. 1972:646.

2. Nadkarni KM. Indian Materia Medica. Popular Prakashan Private, Bombay. 1976:624–625.

3. Anonymous. The wealth of India: Raw materials, Council of scientific and industrial research, New Delhi 1988, 2:2–3.

4. Mathew J, Paul J. Nandhu MS, Paulose CS. Bacopa monnieri and Bacoside - A for ameliorating epilepsy associated behavioral deficits. Fitoterapia 2010; 81:315-22.

5. Salil KB, Ashok K, Shibnath G. Effect of *Bacopa monniera* on animals models of Alzheimer's disease and perturbed central cholinergic markers. Molecular Aspects of Asian Medicine 2001; 1:21-32.

6. Roodenrys S, Booth D, Bulzomi S, et al. Chronic effects of Brahmi (*Bacopa monnieri*) on human memory. Neuropsychopharmacology 2002; 27:279-281.

7. Channa S, Dar A, Anjum S, Yaqoob M. Atta-ur Rahman. Anti-inflammatory activity of *Bacopa monniera* in rodents. Journal of Ethnopharmacology 2006; 104:286–289.

8. Tripathi YB, Chaurasia S, Tripathi E, Upadhyay A, Dubey GP. *Bacopa monneira* as an antioxidant: mechanism of action. Indian Journal of Experimental Biology 1996; 34:523-526.
9. Chowdhuri DK, Parmar D, Kakkar P, et al. Antistress effects of bacosides of *Bacopa monniera*: modulation Hsp70 expression, Superoxide dismutase and cytochrome P450 activity in rat brain. Phytotherapy Research 2002; 16:639-645.
10. Salil KB, Ghosal S. Anxiolytic activity of a standardized extract of *Bacopa monniera*: an experimental study. Phytomedicine 1998; 5:77-82.
11. Sairam K, Dorababu M, Goel RK, Bhattacharya SK. Antidepressant activity of standardized extract of *Bacopa monniera* in experimental models of depression in rats. Phytomedicine 2002; 9:207-211.
12. Saarto T, Wiffen PJ. Antidepressants for neuropathic pain. Cochrane Database of Systematic Reviews Issue 4. Art. No.: CD005454. DOI:10.1002/14651858. CD00 5454.pub2. 2007
13. Gray AM, Pache DM, Sewell RDE. Do α_2-adrenoceptors play an integral role in the antinociceptive mechanism of action of antidepressant compounds? European Journal of Pharmacology. 1999a; 378: 161-168.
14. Eaton M. Common animal models for spasticity and pain. Journal of Rehabilitation Research and Development 2003; 40:41-54.
15. Gray AM, Spencer PSJ, Sewell RDE. The involvement of the opioidergic system in the antinociceptive mechanism of action of antidepressant compounds. British Journal of Pharmacology 1998; 124:669-674.
16. Gray AM, Nevinson MJ, Sewell RDE. The involvement of opioidergic and noradrenergic mechanisms in nefopam antinociception. European Journal of Pharmacology. 1999b; 365:149-157.
17. Brochet D, Micó J-A, Martin P, Simon P. Antinociceptive activity of beta-adrenoceptor agonists in the hot plate test in mice. Psychopharmacology 1986; 88:527-528.
18. Grillet N, Pattyn A, Contet C, et al. Generation and characterization of *Rgs4* mutant mice. Molecular and Cellular Biology 2005; 25:4221–4228.
19. Berkenkopf JW, Weichmann BM. Production of prostaglandin in mice following intraperitoneal injection of acetic acid, phenyl benzoquinone and zymosan: Its role in the writhing response. Prostaglandins 1988; 36:693-709.
20. Matsumoto H, Naraba H, Ueno A, et al. Induction of cycloxygenase-2 causes an enhancement of writhing response in mice. European Journal of Pharmacology 1998; 352:47-52.
21. Ballou LR, Botting RM, Goorha S, Zhang J, Vane JR. Nociception in cyclooxygenase isozyme-deficient mice. Proceedings of National Academy of Science 2000; 97:10272-10276.

22. Bentley GA, Newton SH, Starr J. Evidence for an action of morphine and enkephalins on sensory nerves endings in the mouse peritoneum. British Journal of Pharmacology 1981; 73:325-332.
23. Bentley GA, Newton SH, Starr J. Studies on the antinociceptive action of α-agonist drugs and their interaction with opioid mechanisms. British Journal of Pharmacology 1983; 73:125-134.
24. Chan TF, Tsai HY, Tian-Shang W. Anti-inflammatory and analgesic activities from the roots of *Angelica pubescens*. Planta Medica 1995; 61:2-8.
25. Hammond DL. Inference of pain and its modulation from simple behaviors. In: Chapaman C.R., Loeser, J.D., eds. Issues in pain management: advances in pain research and therapy. Raven Press, New York. 1989:69–91.
26. Tjølsen A, Lund A, Hole K. Antinociceptive effect of paracetamol in rats is partly dependent on spinal serotonergic systems. European Journal of Pharmacology 1991; 193:193–201.
27. Pini LA, Vitale G, Ottani A, Sandrini M. Naloxone-reversible antinociception by paracetamol in the rat. Journal of Pharmacology and Experimental Therapeutics 1997; 280:934-940.
28. Hosseinzadeh H, Younesi HM. Antinociceptive and anti-inflammatory effects of *Crocus sativus* stigma and petal extracts in mice. BioMed Central Pharmacology 2002; 2:7.
29. Vohora SB, Khanna T, Athar M, Ahmad B. Analgesic activity of bacosine, a new triterpene isolated from *Bacopa monniera* Fitoterapia. 1997; 4:361-365.
30. Stephens R J. Evidence for a pharmacokinetic interaction between ibuprofen and meptazinol in the mouse. Journal of Pharmacology and Experimental Therapeutics 1984; 36:779-781.
31. Bjorrkman R, Hedner J, Hedner T, Henning M. Central, naloxone-reversible antinociception by diclofenac in the rat. Naunyn-Schmiedeberg's Archives of Pharmacology 1990; 342:171-176.
32. Sumathi T, Nayeem M, Balakrishna K, Veluchamy G, Devaraj SN. Alcoholic extract of '*Bacopa monniera*' reduces the in vitro effects of morphine withdrawal in guinea-pig ileum. Journal of Ethnopharmacology 2002; 82:75-81.