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Analgesic and Anti-inflammatory activity of *Andrographis paniculata* and Andrographolide in Diabetic Rodents

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Abstract

An analytically characterized extract of *Andrographis paniculata* leaves (AP) and isolated pure andrographolide were evaluated for their analgesic and anti-inflammatory activity in diabetic rodents. AP (100, 200 and 400 mg/kg/day, *p.o.*), or andrographolide (30, 60 and 120 mg/kg/day, *p.o.*) was administered for ten consecutive days. Pentazocine and indomethacin were used as standard analgesic and anti-inflammatory drugs, respectively. Diabetic control animals were demonstrated significant abnormal pain-associated behaviours, measured as hyperalgesia to painful stimuli in tail flick test, hot plate test and formalin-evoked pain test, and exaggerated inflammatory responses in carragennan-induced paw oedema and cotton pellet induced granuloma tests in comparison to nondiabetic control animals. AP and andrographolide treatments in diabetic animals demonstrated significant analgesic and anti-inflammatory activity in all these tests, and their maximal efficacies were always comparable to the standard drugs used. Taken together, these observations confirm that andrographolide is the major active constituent of *Andrographis paniculata,* and strongly suggest that anti-inflammatory and analgesic efficacies of AP are entirely due to the presence of high contents of andrographolide present in it.

Keywords: *Andrographis paniculata; Andrographolide; Hyperalgesia, Analgesic; Anti-inflammatory*

Introduction

Andrographis paniculata (Burm. F.) Wall. Ex Nees is a traditionally known medicinal plant of Acanthaceae family, and andrographolide is quantitatively the major bioactive secondary metabolite of the plant identified to date. Besides being well known as an Ayurvedic herb, *Andrographis paniculata* is also medicinally used in the traditionally known medical systems of China and Thailand. *Andrographis paniculata* is also known as Cheonshimryeon in Korea and Chuan Xin Lian in China [1,2]. Extracts of this plant parts and isolated andrographolide have been used to pharmacologically and experimentally verify its traditional usage for diabetes, rheumatoid arthritis, inflammation, cold, fever and diarrhea [3-7]. Amongst them the ones dealing with anticancer and anti-inflammatory activities of extracts rich in andrographolide, or of pure andrographolide, have attracted the most attention of modern drug discoverers [8,9]. Andrographolide diterpenoids and 2'-oxygenated flavonoids are common chemotaxonomic markers of the Andrographis genus to which *Andrographis paniculata* belongs [10,11].

Glucose toxicity on local site of spinal cord can contribute to the development of spinally mediated hyperalgesia and targeting on spinal sensory processing (spinal glutamatergic pathways) may assist development of novel therapeutic strategies for preventing and alleviating painful diabetic neuropathy [12,13]. Hyperglycemia is an important factor in pain hypersensitivity associated with diabetes and results in altered pain sensitivity. Appropriate blood glucose control can help relieve pain in long-term diabetes through indirect mechanisms [14]. Although during more recent years, many reports on anti-hyperglycemic and anti-inflammatory activity of *Andrographis paniculata* have appeared, yet little attention has been paid to their therapeutic potentials for combating hyperalgesia and exaggerated inflammatory responses accompanying diabetic conditions. Present study was designed to evaluate potential analgesic and anti-inflammatory activity of standardized extract of *Andrographis paniculata* and its isolated pure andrographolide in diabetic rodents.

Materials and Methods

Animals

Adult Charles Foster albino male rats (150±10g body weight) and Swiss albino male mice (20±5g body weight) were acquired from the Central Animal House of the Institute of Medical Sciences, Banaras Hindu University, Varanasi, India (Registration Number: 542/AB/CPCSEA, dated 22-01-2002). The animals were housed in groups of six in polypropylene cages at an ambient temperature of 25±1 °C and 45-55% relative humidity, with a 12:12h light/dark cycle. Except stated otherwise, they were always provided with commercial food pellets (Amrut Laboratory Animal Feed; Pranav Agro Industries Ltd., Sangali, India) and water *ad libitum* and were acclimatized to the laboratory environment for at least one week before using them for the experiments. Principles of laboratory animal care guidelines (NIH publication number 85-23, revised in 1985) were always followed. Prior approval from the Central Animal Ethical Committee of the University (CAECU) was taken for the study protocol used (Dean/11-12/CAEC/325, dated 30-11-2011).

Plant extract, andrographolide and other chemicals

Standardized hydro-methanolic *Andrographis paniculata* leaves extract (AP; KalmCold™, 32.20%, w/w andrographolide) and andrographolide (99.0% pure by HPLC) were generously supplied by Natural Remedies Pvt. Ltd., Bangalore, India. The plant leaves were collected in the month of March and identified as *Andrographis paniculata* (Burm. F.) Wall. Ex Nees by in-house botanist at R&D Centre of Natural Remedies Pvt. Ltd., Bangalore, India, and a voucher herbarium specimen (No: NR582) was kept in the R&D Centre of Natural Remedies Pvt. Ltd., Bangalore, India. Extraction procedure and analytical methods used for standardising AP and isolation of andrographolide were described in details elsewhere [4,15]. AP was analytically characterized to contained andrographolide (32.2%, w/w), isoandrographolide (0.5%, w/w), neoandrographolide 2.7%, w/w), andrograpanin (0.9%, w/w) and 14-deoxy-11,12 didehydroandrographolide 4.7%, w/w), and skullcapflavone I (0.06%, w/w) by HPLC [15]. Carboxymethylcellulose and agar (Central Drug House, New Delhi, India), Tween 80 (Sisco Research Lab., Mumbai, India) and other chemical and reagents used were from commercial sources.

Induction of type-2 diabetes

Type-2 diabetes was induced in overnight fasted animals as described elsewhere [16], by a single intra peritoneal (i.p.) injection of 65 mg/kg streptozotocin (STZ; HiMedia Mumbai, India), 15min after the i.p. administration of 120 mg/kg nicotinamide (SD Fine-Chemical Ltd., Mumbai, India). Animals were returned to their cages and provided normal food and 10% sucrose water to minimize hypoglycemic shock. The elevated glucose level in the blood confirmed hyperglycemia, quantified 72hour and 7th day after STZ injections. Animals with blood glucose levels higher than 250 mg/dl were used in all experiments as diabetic animal.

Animal grouping and drug administration

For each series of experiments, nine experimental groups consisting of 6 animals each were used, whereupon the animals were randomly allotted to different experimental groups as nondiabetic control (vehicle); diabetic control (vehicle); diabetic + AP 50 mg/kg/day; diabetic + AP 100 mg/kg/day; diabetic + AP 200 mg/kg/day; diabetic + andrographolide 30 mg/kg/day; diabetic + andrographolide 60 mg/kg/day; diabetic + andrographolide 120 mg/kg/day and diabetic + standard analgesic or anti-inflammatory drug mentioned below. The doses of AP and corresponding doses of andrographolide were selected based on previous study with the same extract [7,17,18].

Andrographis paniculata extract was suspended in 0.3% carboxymethylcellulose (CMC) for once daily per-oral administrations. Andrographolide was macerated with Tween 80 (0.2%) and suspended in 0.2% aqueous agar for daily administrations. Control group animals were similarly treated with vehicle only (0.3% CMC) only as negative control. For comparison purpose, indomethacin (5mg/kg, p.o) and pentazocine (30mg/kg, p.o) were used as standard anti-inflammatory and analgesic drug respectively (positive control).

Analgesic activity

Following animal models were used for evaluation of analgesic activity:

Tail flick latent period test in rats: The technique used was described elsewhere [19], using an analgesiometer. After 60min of the drug administration on day 10, each rat was placed in a rat holder, with its tail coming out through a slot in the lid. The tail was kept on the bridge of the analgesiometer, called jacket with an electrically heated nichrome wire underneath. The tail received radiant heat from the wire, heated by passing current of 6mA. Through the water jacket, cold water was continuously passed, so that the bridge did not get heated and tail could be conveniently placed over the bridge. The time taken for the withdrawal of the tail after switching on the current, was taken as the latent period, in sec, of 'tail flicking' response. This latent period was considered as the index of nociception. The cut-off time for determination of latent period was taken as 30 sec to avoid injury to the skin [20]. Three tail flick latencies were measured per rat at each time interval and the means of the tail-flick latencies was used for statistical analysis.

Hot plate reaction time in mice: Mice were screened by placing them on a hot plate maintained at 55±1 °C and recording the reaction time in seconds for forepaw licking or jumping [21]. Only mouse, which was reacted within 15sec and which was not show large variation when tested on four separate occasions, each 15min apart, was taken for the test. The time for forepaw licking or jumping on the heated plate of the analgesiometer maintains at 55 °C was taken as the reaction time. Reaction time of mice was noted on 60min after the drug administration on day 10.

Formalin test in rats: The efficacy for central analgesic activity of AP and andrographolide was evaluated in animal as described previously [22] with some modification. Briefly, rats were injected (s.c.) with 100µl of 12% formalin into the dorsal part of the right hind paw. After formalin injection, rats were placed individually in wire cages for observations. Pain reactions were continuously (for 5min periods) counted at 10, 20, 40, 60 and 120 min and scored according to a pain scale. Pain-related behaviour was quantified by counting the incidence of spontaneous flinches per minute of the formalin-injected paw. Analgesic response or protection was indicated if both paws were resting on the floor with no obvious favouring of the injected paw.

Anti-inflammatory activity

Following methods were used for evaluation of anti-inflammatory activity:

Carrageenan-induced pedal oedema in rats: The method described elsewhere was followed for pedal oedema test [23]. Rats were injected with 0.1ml of a 1% carrageenan solution in saline into the sub-plantar region of the left hind paw 60 min after 10th consecutive daily drug treatments. The paw was marked with ink at the level of the lateral malleolus and immersed in mercury up to this mark. The paw volume was measured before and 1, 2, 3, 4 and 6h after the injection of carrageenan by the mercury displacement method in plethysmography.

Percentage inhibition in pedal oedema was calculated as follow:

$$\%Inhibition = \frac{(A-B)}{A \times 100}$$

Where, A =difference in the paw volume (pedal oedema) of control group.

B =difference in the paw volume (pedal oedema) in the treatment group.

Cotton pellet induced granuloma in rats: Sub-acute inflammation was produced by cotton pellet induced granuloma in rats [23]. Sterile cotton (50±1mg) soaked in 0.2ml of distilled water containing penicillin (0.1mg) and streptomycin (0.13mg) was implanted subcutaneously bilaterally in axilla under the ether anaesthesia.

Diabetic animals were hyperglyemic at the time of cotton implantation. First treatment was started on after confirmation of diabetes (7th day of STZ injection) for ten consecutive days. Cotton pellets were implanted on day 3rd of treatment and animals were sacrificed on the 10th day of treatment. The granulation tissue with cotton pellets were dried at 60 °C overnight and then dry weight was taken. The weight of the cotton pellet before implantation was subtracted from the weight of the dried, dissected pellets. The dry weight of cotton pellet granuloma (in mg) for each animal was recorded.

Statistical Analysis

Mean±standard error of mean (SEM) was calculated for the observed values in each experimental group (n=6). Statistical analysis was performed by ordinary one way analysis of variance (ANOVA) followed by Student-Newman-Keuls multiple comparison test unless stated otherwise. GraphPad Prism-6 was used for statistical analysis (GraphPad Software Inc., San Diego, CA). P value less than 0.05 was always considered as statistically significant.

Results

Tail flick test in rats

The diabetic rats showed significant (p<0.05) decrease in tail flick reaction time compared to nondiabetic control rats on day 10. However, diabetic rats treated with 10 repeated daily dose of AP (100, 200 and 400mg/kg), or andrographolide (30, 60 and 120mg/kg) demonstrated significant (p<0.05) increase in tail flick reaction time (Figure 1). Qualitatively, the efficacies of AP and andrographolide were similar to standard drug pentazocine.

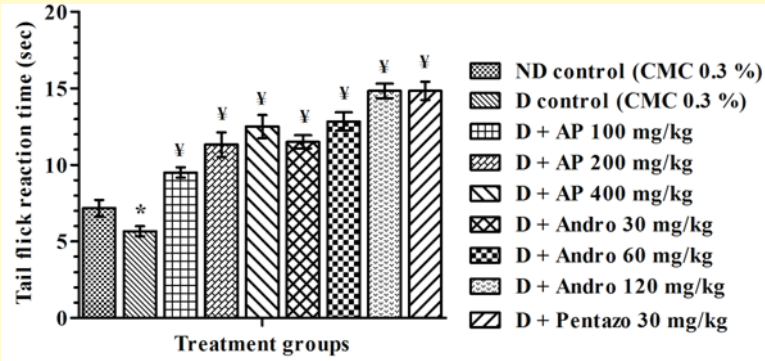

Figure 1: *Effect of Andrographis paniculata extract (AP) and andrographolide on tail flick reaction time in diabetic rats. *=p<0.05 vs. nondiabetic (ND) control; ¥=p<0.05 vs. diabetic (D) control. Andro= Andrographolide, Pentazo= Pentazocine.*

Hot plate reaction time in mice

The diabetic mice showed significant (p<0.05) decrease in reaction time on hot plate compared to nondiabetic control mice on day 10. However, diabetic mice treated with 10 repeated daily dose of AP (100, 200 and 400mg/kg), or andrographolide (30, 60 and 120 mg/kg) demonstrated significant (p<0.05) increase in reaction time (Figure 2). Qualitatively, the efficacies of AP and andrographolide were similar to standard drug pentazocine.

Formalin test in rats

The diabetic mice showed significant (p<0.05) increased number of spontaneous flinch per minute compared to nondiabetic control mice on day 10 at different time points.

However, diabetic mice treated with 10 repeated daily dose of AP (100, 200 and 400mg/kg), or andrographolide (30, 60 and 120mg/kg) demonstrated significant (p<0.05) decrease in number of spontaneous flinch per minute (Figure 3). Qualitatively, the efficacies of AP and andrographolide were similar to standard drug pentazocine.

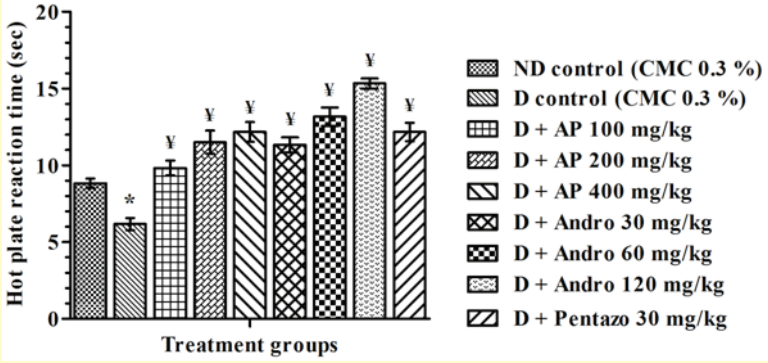

Figure 2: *Effect of Andrographis paniculata extract (AP) and andrographolide on hot plate reaction time in (B) diabetic mice. *=p<0.05 vs. nondiabetic (ND) control; ¥=p<0.05 vs. diabetic (D) control. Andro =Andrographolide, Pentazo =Pentazocine.*

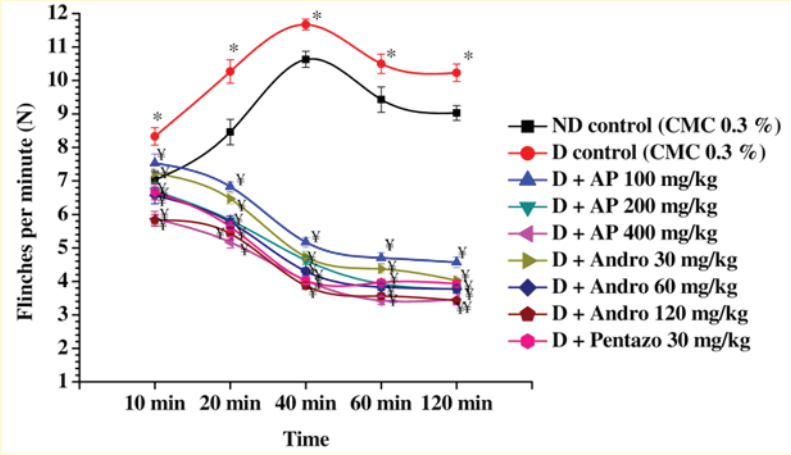

Figure 3: *Effect of Andrographis paniculata extract (AP) and andrographolide on incidence of spontaneous flinch in diabetic rats. *=p<0.05 vs. nondiabetic (ND) control; ¥=p<0.05 vs. diabetic (D) control (Two way ANOVA followed by Bonferroni post-tests). Andro =Andrographolide, Pentazo =Pentazocine.*

Carrageenan-induced pedal oedema in rats

The diabetic rats showed significant (p<0.05) increased pedal oedema volume compared to nondiabetic control rats on day 10. However, diabetic rats treated with 10 repeated daily dose of AP (100, 200 and 400mg/kg), or andrographolide (30, 60 and 120mg/kg) demonstrated significant (p<0.05) decreased pedal oedema volume (Figure 4A). Qualitatively, the efficacies of AP and andrographolide were similar to standard drug indomethacin. The percent inhibitions data of peal oedema test on day 10 in diabetic rats are shown in Figure 4B.

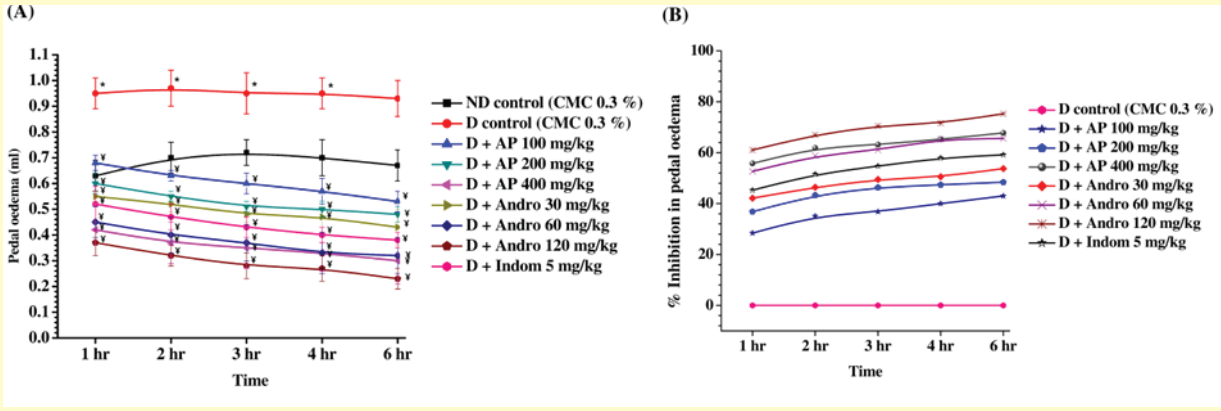

Figure 4: *Effect of Andrographis paniculata extract and Andrographolide on (A) pedal oedema, and (B) percentage inhibition of carrageenan-induced pedal oedema. *=p<0.05 vs. nondiabetic (ND) control; ¥=p<0.05 vs. diabetic (D) control (Two way ANOVA followed by Bonferroni post-tests). Andro= Andrographolide, Indom= Indomethacin. The percentage inhibition in diabetic control rats was taken as zero (no inhibition) for comparison with drugs treated groups.*

Cotton pellet-induced granuloma in rats

The diabetic rats showed significant (p<0.05) increase in cotton pellets-induced granuloma tissues weight compared to nondiabetic control rats. However, diabetic rats treated with AP (100, 200 and 400 mg/kg), or andrographolide (30, 60 and 120 mg/kg) demonstrated significant (p<0.05) decreased cotton pellets-induced granuloma tissues weight similar to standard drug indomethacin (Figure 5).

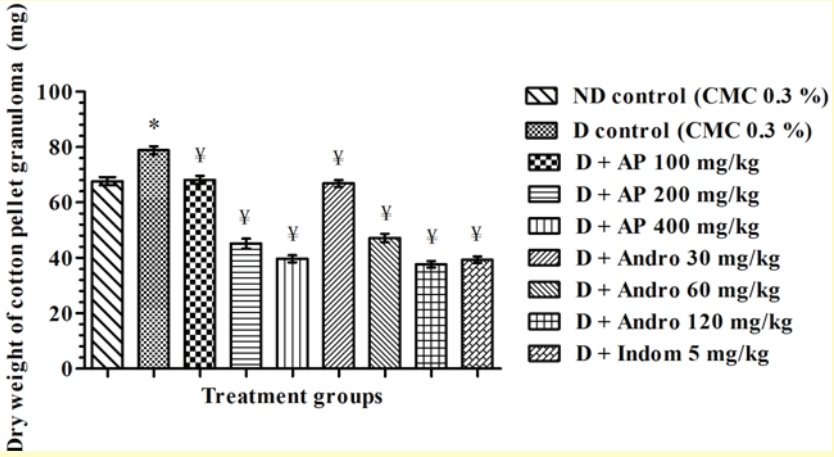

Figure 5: *Effect of Andrographis paniculata extract (AP) and andrographolide on cotton pellet-induced granuloma in diabetic rats. *=p<0.05 vs. nondiabetic (ND) control; ¥=p<0.05 vs. diabetic (D) control. Andro =Andrographolide, Indom =Indomethacin.*

Discussion

Central sensitization plays a pivotal role in the pathogenesis of pain hypersensitivity [24] and its development results from augmented spontaneous and burst discharges in primary sensory neurons in neuropathic pain [25]. Diabetes mellitus has become the most common cause of peripheral neuropathy and many diabetes patients suffer from chronic pain. Hyperglycemia results in pain hypersensitivity characterized by allodynia and hyperalgesia [26,27] probably by disrupting the functions of cell mitochondria and subsequent generation of reactive oxygen species [28] and oxidative stress [29]. Other possible target, activation of spinal microglia has been demonstrated in streptozotocin-treated animals, the most commonly used model of diabetes [30], and this activation can last 6 months [31]. Andrographolide having some inhibitory activity in microglial activation in mesencephalic neuron-glia cultures [32]. Therefore, targeting microglia and these receptors by antagonist or agonist may be considered as a novel approach to relieve neuropathy [33]. The diabetic rat model has been studied, but the literature data are conflicting, and analysis of the animal's behaviour in response to pain has often been incomplete. With the hot-plate test, Forman *et al*. [34] observed hyperalgesia in diabetic rats (age of diabetes: 8 weeks) as did Lee *et al*. [35] after 3 days of diabetes with the tail immersion test at 50 °C but other findings disagree with these results. Raz *et al*. [36] reported that diabetic rats did not develop hyperalgesia in the hot-plate test, even after 16 weeks of diabetes. Levine *et al.* [37] reported the same finding for mice, while Akunne and Soliman [38] found loss of pain sensitivity in rats subjected to the hot-plate test. With the paw-pressure test, Wuarin-Bierman *et al*. [39] reported hyperalgesia with focus on hyperactivity of nociceptive C-fibers in diabetic rats (duration of diabetes: 30 weeks). These discrepancy in findings can be avoided by correct study deign, preference in selection of rats over mice; use of pain tests with localised stimulus, use of noxious and non-noxious stimuli, longer test period after diabetes induction and selection of responder animals [30].

Animal models used in this study have been commonly used for identifying drug with analgesic and anti-inflammatory activities [40-42]. The observations described in this article confirmed that the diabetic rodents showed hyperalgesic conditions and exaggerated inflammatory response compared to nondiabetic rats. Interestingly analgesics-like effects were observed in both the tests and this effect of the AP or andrographolide were persistently observed in the tail flick test and hot plate test as well as decreases formalin-induced flinching behaviour in even three hours after its last dose similar to standard central analgesic drug used. All these models are well known for their predictive validity for identifying centrally acting analgesics. Therefore, it can be said that AP or andrographolide possess centrally acting analgesic-like activities. Efforts to clarify the major active constituents i.e. andrographolide and modes of actions involved in it analgesics-like efficacy in these models using diabetic animals could as well lead to novel pharmacological targets for pain therapy.

Qualitatively, the observed effects of repeated daily AP and andrographolide doses in the animal models used were quite analogous to those of the reference drugs used in our studies. In any case, it remains certain that inflammatory mechanisms involved in pellet granuloma, paw swelling/oedema, and pain behaviours are suppressed by repeated minimum dose of AP (100mg/kg) and andrographolide (30mg/kg). After this dose, significant effects of AP and andrographolide were observed in both the rodent models of inflammation, and such were also the cases in the tail flick and hot plate tests. Although the observed effects of the tested drugs in these tests increased after its lower dose, its dose effect relationships in the dose range tested were never very steep. These tests are widely used for identifying peripherally as well as central acting analgesics and anti-inflammatory agents, sedatives and other psychoactive agents are known to be effective in inhibiting the inflammation and pain. Since sedative and other behavioural effects of AP and andrographolide becomes apparent after its repeated daily doses [17,43], its observed effects could as well be due to its modulating effects on central nervous system functions.

Pain is a cardinal symptom of inflammatory swelling of almost all peripheral organs. Dose dependant anti-inflammatory effects of AP or andrographolide were apparent in both the models used. Moreover, the efficacies of andrographolide of corresponding doses were higher than the AP doses. It is now well recognized that oxidative processes and cytokines are intrinsically involved in the pathogenesis of inflammatory swelling.

Pain is a cardinal symptom of inflammatory swelling of almost all peripheral organs. Dose dependant anti-inflammatory effects of AP or andrographolide were apparent in both the models used. Moreover, the efficacies of andrographolide of corresponding doses were higher than the AP doses. It is now well recognized that oxidative processes and cytokines are intrinsically involved in the pathogenesis of inflammatory swelling. Repeated daily doses of AP or andrographolide have successfully augment anti-oxidative capacity and cytokine expression both in circulating blood and brain of stress rats [44]. Since these effects of the AP or andrographolide was accompanied with the increased cellular antioxidative capacity, it could as well be that its observed anti-inflammatory and analgesic activities might be the consequences of its effects on metabolic processes controlling cellular cytokine expression. Bioactive components of AP are known to possess analogous propertied are andrographolide and isoandrographolide [5] which are well recognized for their therapeutic potentials against diverse inflammatory pathologies [4,45]. However, it cannot be ignored that AP contains numerous other bioactive constituents with pharmacological activity profiles [1,2,46]. Therefore, it could as well be that the observed efficacy of AP might be due to modulating actions of diverse active components of the extract.

Observations reported in this article add further experimental evidences to the conviction that the broad spectrum of psychopharmacological activity profile of AP observed after its repeated daily doses is mainly due to its modulating, or inducing effects on peripheral and central mechanisms of inflammatory processes. Repeated daily doses of AP are necessary for observing its anxiolytic as well as antidepressant-like activities in nondiabetic and diabetic experimental models of rodents [17,18]. Moreover, its antidepressants-like activities were also apparent in mentally stressed nondiabetic animals [44]. Thus, it seems reasonable to assume that modulation of biological processes, or mechanisms, involved in anti-inflammatory effects of AP and andrographolide, leads to adaptive responses in the central control mechanisms involved in exaggerated depression and central stress responses. Such could indeed be the case is well supported by the postmodern concepts of psycho-neuro-immunology on comorbid mental health conditions [47-49].

Conclusion

These observations confirm that andrographolide is the major active constituent of *Andrographis paniculata,* and strongly suggest that anti-inflammatory and analgesic efficacies of AP are due to the presence of high contents of andrographolide present in it. Therefore, AP and andrographolide could be a starting point for discovering novel therapeutic leads and pharmacological targets urgently needed for combating inflammation-associated psychopathologies in diabetes.

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