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Evaluation of Ethanolic Leaf Extracts of MorusIndica and Asystasia Gangetica on Diabetic neuropathy in Alloxan induced Diabetic Mice

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ABSTRACT

Diabetes mellitus (DM) is a disorder recognized with increase of blood sugar level. Chronic hyperglycemia leads to the dysfunction of several organs especially eye, kidney, heart and blood vessels. DM eventually leads to the peripheral nervous system complications like diabetic neuropathy, in that especially neuropathic pain is the major presentation. The aim of this study was to investigate the effect of Asystasia gangetica and Morus indica in the treatment of diabetic neuropathy in alloxan induced diabetic mice. Diabetes was induced in the male albino mice weighing 20- 30 gm by intraperitoneal injection of alloxan. Four weeks after alloxan injection animals were divided into six groups as control (I), standard (II) and 4 experimental groups (III, IV, V, VI). Group I received normal saline and group II received intraperitoneal injection of standard diclofenac sodium (10mg/kg). The experimental groups III, IV and V, VI received oral different doses of ethanolic extract of *A.gangetica (*50,100 mg/kg) and *Morus indica (*100,200 mg/kg) respectively. Blood glucose and phytochemical analysis were performed by using standard kits. Phytochemical analysis reveals the presence of flavanoids and antioxidants. Neuropathic pain assessed by tail immersion, hot plate and electrical stimulation of tail methods. All the experimental groups shows significant increase in response time when compared to control groups. Overall, Asystasia gangetica and Morus indica exerted analgesic effect in neuropathic mice.

Keywords: Diabetic neuropathy, Neuropathic pain, Analgesic effect, Antioxidants

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INTRODUCTION

Diabetes is a multifaceted disease involving impaired insulin secretion and insulin resistance [(1)]. Worldwide projections suggest that more than 300 million people will contract diabetes by the year 2025 and the global cost of treating this disease and its complications could reach US$1 trillion annually[(2)]. The loss of glycemic control, associated with these defects results in long term complications which are both microvascular (eg; retinopathy, nephropathy and neuropathy) and macrovascular (eg; stroke, myocardial infarction and peripheral vascular disease [(3)]. Neuropathic pain is generally considered to be one of the most common complications that affect both types of diabetes equally [(4, 5, 6, 7)]. It is mostly characterized by pain which can occur spontaneously as a result of exposure to normally mildly painful stimuli, i.e., hyperalgesia [(8)]. Although estimates of diabetic neuropathy widely depending on the assessment criteria employed, as many as 50% diabetics have some degree of neuropathic pain[(9)]. A large number of neuroanatomical, neurophysiologic and neurochemical mechanisms are thought to contribute to the development and maintenance of diabetic neuropathic pain [(10, 11)]. Hyperglycemia clearly plays a key role in the development and progression of diabetic neuropathy as well as the other microvascular complications of diabetes. Understandably then investigations into the molecular and biochemical pathophysiology of diabetic neuropathy have focused on glucose metabolic pathways .Over the past 25 years animal experiments and invitro studies have identified biochemical pathways likely to be important in the development of diabetic complications and have led to possible approaches to treatment . All of these pathways are related to the metabolic and / or redox state of cell. Pathways which are mainly driven by metabolism are Glucose flux through the polyolpathway, the hexosamine, excess/inappropriate activation of protein kinase C isoforms and accumulation of advanced glycation end products. While each pathway may be injurious alone , collectively they cause an imbalance in the mitochondrial redox state of cell and lead to excessive formation of reactive oxygen species (ROS) [(12)]. Increased oxidative stress within the cell leads to activation of poly (ADP-ribose) polymerase (PARP) pathway, which regulates the expression of genes involved in promoting inflammatory reactions and neuronal dysfunction. Although neuronal loss or alteration of neurotransmitters have been reported to be responsible for the changed pain perception , the exact etiologic factors remains unexplored .Based on these mechanisms of injury , various prevention and treatment strategies have been already suggested and are under investigation[(13)]. Tight glycemic control has been shown to be effective slowing the progression of diabetic neuropathy [(14)]. Apart from glycemic control, a corresponding wide range of treatments have been employed to treat patients with

neuropathic pain, including several antioxidants such as vitamin E, melatonin, date extract, antiepileptic drugs, opioid analgesics, tricyclic antidepressants, selective serotonin reuptake inhibitor, N-methyl D-aspartate receptor antagonists, cholecystokinin receptor antagonists, adenosine, lipoicacid, cannabinoids, capsaicin, protein kinase C inhibitors, aldose reductase inhibitors, and VR-1 receptor modulators [11, 8]. The use of these compounds is limited by marginal efficacy and clinically significant adverse events which include liver dearrangement, somnolence, dizziness, nausea, constipation(antidepressants),dyspepsia, nausea (tramadol), a burning sensation at the site of application, coughing or sneezing, accidental irritation to other body parts, and rashes (topical agents) [8]. At present the only agents approved for the treatment of painful diabetic neuropathy are lidocaine patches 5%, duloxetine, gabapentin, and pregabalin [11]. In the clinical setting, despite the use of these agents, the successful therapy of diabetic neuropathy remains a challenge. Traditional plant medicines are used throughout the world for a range of diabetic presentations. Therefore, an investigation of such agents from traditional medicinal plants has become particularly important. India has a rich history of using various potent herbs and herbal components for treating diabetic complications. Many Indian plants have previously been investigated for their beneficial use in different types of diabetic complications [15, 16]. To date, there are many hundreds of herbs and traditional herbal formulas reported to have been used for the treatment of diabetes complications [17].Renewed interest has been observed in recent years on the multiple activities of natural molecule. *Morus indica* (Mulberry tree) of the family *Moraceae* has been widely cultivated in countries all over the world including temperate to tropical areas. Different parts of the plant are used as herbal medicine for blood serum glucose reduction, cholesterol and lipids levels reduction, antiphlogistic, diuretic and expectorant effects. Andallu and Varadacharyulu have reported antidiabetic activity of *M. indica* in streptozotocin induced diabetes in rats [18].

Asystasia gangetica of family *Acanthaceae*has been claimed for anti -asthmatic [19] ,antihelmentic and antidiabetic property [16]. Leaves are also widely used as food source, because it contains high amounts of proteins, amino acids, minerals and fibers [8]. Both plants have different category of flavanoids and antioxidants [16]**.** There are sporadic reports on the antinociceptive activity of certain flavonoids such as hydroxy ethyl rutoside [20] and gossypin [21]. Synergestic combinations of more than one formulation have been used many physicians for proper disease control .Therefore it will be of value to pharmacologically screen any such combinations [22] . Since some of these properties of the both plants may be useful in the treatment of diabetic neuropathy. Hence the present study

was an attempt to study the protective effect of Morus indica and Asystasia Gangetica individually against diabetic neuropathic pain in Alloxan induced diabetic mice.

MATERIALS AND METHOD

Drugs and Chemicals:

Alloxan monohydrate was purchased from Sigma-Aldrich, St. Louis, MO, USA. Glibenclamide was a gift from Cipla Ltd, Mumbai, India. All biochemical estimations were assayed by using kits from Span Diagnostics Ltd., Surat, India. All other biochemicals used in this experiment were purchased from Sigma-Aldrich, St. Louis, MO, USA. All chemicals were analytical grade.

Experimental animals:

Male albino mice (20g–30g) were used in the present study. The animals were housed under optimal laboratory conditions, maintained on a natural light and dark cycle and had free access to food and water ad libitum. Animals were acclimatized to laboratory conditions before the test. The experimental protocol was approved by the institutional animal ethical committee (1220/a/08/CPCSEA) of Annamacharya College of pharmacy.

Preparation of plant extract:

The leaves of *Morus indica* and *Asystasia gangetica* were collected from the Rayachoty, kadapa district.*(M.indica)* and Madhumalai hills of Coimbatore district *(A.gangetica*) respectively. Its botanical identity was authenticated by Dr. Yashodamma , professor of botany ,Sri venkataswara university ,Tirupathi ,A.P .The shade dried leaves were powdered to get a course granule. About 250 g of dried powder of both were extracted with 90% and 70% ethanol respectively by continuous hot percolation, using soxhlet apparatus. The resulted dark – brown extract was concentrated up to 100 ml on Rota vapour under reduced pressure. The concentrated crude extracts were lyophilized in to powder and used for the study.

Screening:

The alcoholic extracts obtained were subjected to preliminary phytochemical screening such as Shinoda test, Alkaline reagent test, Zinc chloride test to identify the chemical constituents. The methods of analysis employed were those described by Harbone and Trease [23, 24].

Experimental induction of diabetes in Mice:

The mice were injected intravenous with Alloxan monohydrate dissolved in sterile normal saline at a dose of 80 mg/kg body weight [25]. After 48 hrs of Alloxan injection diabetes was confirmed by measuring plasma glucose levels (PGLS) with GOD-POD diagnostic kit method .PGLS were also measured at 0^{th}, 7^{th}, 14^{th} ,21^{st} ,28^{th} day .The body weights were also monitored for the assessment of diabetes, about 90% of the Alloxan injected mice

developed diabetes and mice having PGLS more than 250 mg/dl [25] were used for the present study.

PHARMACOLOGICAL STUDIES:

Body Weight Measurement:

The diabetic induced mice were weighed before induction of diabetes and during the treatment also. The treatment was continued during analgesic studies. The animals were weighed on 0^{th},7^{th}, 14^{th}, $21^{st,}$ 28^{th} day of experiment.

Blood Glucose Monitor:

The FBG of the diabetic mice were monitored using GOD POD KIT during the antidiabetic neuropathic activity on 0^{th}, 7^{th}, 14^{th},21^{st}, 28^{th} day of experiment . Blood glucose was estimated by using glucose kit obtained from Span Diagnostics (table 1 & 2).

Table 1: Reagents used

Reagent no.	Reagent	Composition	Concentration
1.	Glucose Reagent	Phosphate buffer	200 mM/L
		Glucose Oxidase	≥ 15 KU/L
		Peroxidase	>3 KU/L
		4-AAP	0.3 mM/L
		Phenol	5 mM/L
2.	Glucose Standard	Dextrose preservative	100 mg/dL
3.	Glucose Standard	Dextrose preservative	400 mg/dL

Table 2: Assay parameters

SL.No.	Reaction type	End point
1.	Wave length	505 nm
2.	Optical path length	1 cm
3.	Temperature	37^{o} C
4.	Measurement	Against reagent blank
5.	Units	mg / dl

Analgesic activity:

1. Tail immersion method
2. Hot plate method
3. Electrical stimulation of tail

TREATMENT SCHEDULE

After a basal recording of nociceptive reaction at 4 th week of Alloxan injection, diabetic mice were randomly selected and divided into 6 animals in each group.

Group 1 -Diabetic control

Group 2 - Diabetic animal received i.p injection of standard diclofenac sodium (10mg/kg).

Group 3 -Diabetic animal received orally ethanolic extract of *A.gangetica* (AGLE) low dose (50mg/kg body weight).

Group 4-Diabetic animal received orally ethanolic extract of *A.gangetica* (AGLE)high dose (100mg/kg body weight).

Group 5-Diabetic animal received orally ethanolic extract of *Morus indica* (MILE)low dose (200mg/kg body weight) .

Group 6-Diabetic animal received orally ethanolic extract of *Morus indica* (MILE)high dose (400mg/kg body weight) .

Tail Immersion Method:

Painful reaction experimental animals can be produced by applying noxious stimuli such as [26] Thermal Method, Chemical Method, Physical Pressure. Young male albino mice are used, animals were divided into 6 groups of six animals each. They are placed into individual restraining cages leaving the tail hanging out freely. The animals are allowed to adapt to the cages for 30 min before testing. The lower 5 cm portion of the tail is marked. This part of the tail is immersed in a cup of freshly filled water of exactly 55^0C thin a few seconds the mice reacts by withdrawing the tail. The reaction time is recorded in 0.5s units by a stopwatch. After each determination the tail is carefully dried. The reaction time is determined after one hour oral administration of the test substance, the cut off time of the immersion is 15s, withdrawal time of untreated animals is between 1 and 5.5s.A withdrawal time of more than 6s therefore is regarded as a positive response [27].

Hot-plate test:

The hyperalgesic response on the hot-plate is considered to result from a combination of central and peripheral mechanisms [28]. In this test, animals were individually placed on a hot-plate (Eddy's hot-plate) with the temperature adjusted to 55 ± 1 ^{0}C. The latency to the first sign of paw licking or jump response to avoid the heat was taken as an index of the pain threshold, the cut-off time was 15 sec in order to avoid damage to the paw [29].

Electrical stimulation of the Tail:

Since the tail of mice is known to be sensitive to any stimulus, a method of electrical stimulation has been described as early as 1950 by Burn et al. The stimulus can be varied either by the duration of the electric shocker by an increase in the electric current. Male mice with a weight of 20 g are placed into special cages. A pair of alligator clips is attached to the tail whereby the positive electrode is placed at the proximal end of the tail. Rectangular wave pulses from a constant voltage stimulant or at an intensity of 40–50 V are applied. The frequency of the stimulation is 1 shock/s, and the pulse duration 2.5 ms. The normal response time range of the stimuli is 3–4 s. Following administration of the drug, the response time is registered at 15 min intervals until the reaction time returns to control levels [21].

Statistical analysis:

The data for various biochemical parameters were analyzed using analysis of variance (ANOVA) and the group means were compared by Turkey-Kramer Test (Graph-Pad V-3.06). Values were considered statistically significant when at $p < 0.05$.

RESULTS AND DISCUSSION

Phytochemical Screening:

Phytochemical screening of both the plant extracts revealed that the presence of flavonoids, alkaloids, glycoside, tannins, saponins, phytosterols (Table 3).

Table 3: Phytochemical screening

Sl.no.	Test	Mile	Agle
1	Shinoda test	+ve	+ve
2	Alkaline reagent test	+ve	+ve
3	Zinc hydrochloride test	+ve	+ve

Body weight measurement:

Body weight monitored during the time course of 0^{th}, 7th ,14^{th}, 21^{st}, 28^{th} day in alloxan induced diabetic mice . During this time there is markedly decrease in body weight was measured.

Blood glucose monitoring:

In animals treated with Alloxan (80 mg/kg, i.v) a significant increase in the serum glucose levels was observed on the 0^{th}, 7th, 14th, 21st, 28th day of alloxan induced diabetic mice (Table 4).

Table 4: Measurement of Serum glucose levels in mice

Group	Time period	Serum Glucose (mg/dl)(Mean ± S.E.M.)
Alloxan treated mice	0^{th} day	184.00±16.46
	7th day	218.70±16.56
	14^{th} day	197.80±16.26
	21^{st} day	219.60±16.85
	28^{th} day	222.30±18.44

Analgesic activity

Tail immersion method:

The ethanolic leaf extracts of *A.gangetica* & *M.indica* possesses analgesic property. The result of present study indicates effect which is in accordance with its ethnomedical use. Analgesic effect of the extracts was demonstrated in experimental models `using tail immersion method, an increase in reaction time is generally considered an important parameter of analgesic activity .Group –II treated with standard drug (diclofenac sodium-9mg/kg i.v) shows increased in reaction time when compared with diabetic control mice (Group-I).Group-III& Group-IV (*A.gangetica*) shows increased in reaction time when compared with diabetic control mice. Group-IV (100mg/kg) shows more analgesic activity

than low dose of Group-III (50mg/kg). Group-V& Group-VI Shows increased in reaction time when compared with diabetic control mice (Group-I).Group –VI (*M.indica*-400mg/kg) shows more analgesic activity than lower dose of *M.indica (*Group-V,200mg/kg) (Table 5) .

Table 5: Analgesic effect of ethanolic leaf extracts of *Morus indica* and *Asystasia gangetica* by Tail immersion test.

Group	Treatment	Response Time (Mean ± S.E.M.)
I	Diabetic control	1.833 ± 0.307
II	Standard (diclofenac sodium (10mg/kg)	8.5 ± 0.2236***
III	Eth. extract *A.gangetica* (50mg/kg,p.o)	4.5 ± 0.2236***
IV	Eth. Extract *A.gangetica* (100mg/kg,p.o)	5.5 ± 0.2236***
V	Eth. extract of *M.indica* (200mg/kg,p.o)	3.833 ± 0.3073***
VI	Eth. extract of *M.indica* (400mg/kg,p.o)	5.833 ± 0.3073***

One- way Analysis of Variance ANOVA: p value found to be 0.0001 is considered extremely significant.

The data were expressed as mean ± S.E.M.; Tukey Kramer multiple comparison test: ***p<0.001(Extracts vs control)**.**

Eddy's hot plate method:

The ethanolic leaf extracts of *A. gangetica* & *M.indica* possesses analgesic effect. The result of present study indicates effect which is in accordance with its ethnomedical use. Analgesic effect of the extracts was demonstrated in experimental models `using Eddy's hot plate method, an increase in reaction time is generally considered an important parameter of analgesic activity .Group –II treated with standard drug (diclofenac sodium-10mg/kg i.v) shows increased in reaction time when compared with diabetic control mice(Group-I).Group-III& Group-IV (*A.gangetica*) shows increased in reaction time when compared with diabetic control mice. Group-IV (100mg/kg) shows more analgesic activity than low dose of Group-III (50mg/kg). Group-V& Group-VI Shows increased in reaction time when compared with diabetic control mice(Group-I).Group –VI(*M.indica*-400mg/kg) shows more analgesic activity than lower dose of *M.indica (*Group-V,200mg/kg) (Table 6).

Table 6: Analgesic effect of ethanolic leaf extracts of *Morus indica* and *Asystasia gangetica* by Eddy's hot plate method.

Group	Treatment	Response Time (Mean ± S.E.M.)
I	Diabetic control	2.33 ± 0.2108
II	Standard (diclofenac sodium 10mg/kg)	12.83 ± 0.4014***
III	Eth. extract *A.gangetica* (50mg/kg, p.o)	4.33 ± 0.3333**
IV	Eth. extract *A .gangetica* (100mg/kg, p.o)	7.33 ± 0.3333***
V	Eth. extract of *M.indica* (200mg/kg, p.o)	6.66 ± 0.3333***
VI	Eth. extract of *M.indica* (400mg/kg,p.o)	10.33 ± 0.3333***

One- way Analysis of Variance ANOVA: p value found to be 0.0001 is considered extremely significant.

The data were expressed as mean ± S.E.M.; Tukey Kramer multiple comparison test: ***p<0.001, **p <0.01 (Extracts vs. control).

Electrical stimulation of tail:

The ethanolic leaf extracts of *A. gangetica* & *M.indica* possesses analgesic effect. The results of present study indicates analgesic effect which is in accordance with its ethnomedical use. Analgesic effect of the extracts was demonstrated in experimental models `using Electrical stimulation of tail, an increase in reaction time is generally considered an important parameter of analgesic activity. Group –II treated with standard drug (diclofenac sodium-9mg/kg i.v) shows increased in reaction time when compared with diabetic control mice (Group-I). Group-III & Group-IV (*A.gangetica*) shows increased in reaction time when compared with diabetic control mice. Group-IV (100mg/kg) shows more analgesic activity than low dose of Group-III (50mg/kg). Group-V& Group-VI Shows increased in reaction time when compared with diabetic control mice (Group-I).Group –VI (*M.indica*-400mg/kg) shows more analgesic activity than lower dose of *M. indica (*Group-V,200mg/kg) (Table 7).

Table 7: Analgesic effect of ethanolic leaf extracts of *Morus indica* and *Asystasia gangetica* by **Electrical stimulation of tail**.

Group	Treatment	Response Time (Mean ± S.E.M.)
I	Diabetic control	2.16 ± 0.15
II	Standard (diclofenac sodium 10mg/kg)	8.50 ± 0.21 **
III	Eth. Extract *A.gangetica* (50mg/kg,p.o)	4.60 ± 0.21 **
IV	Eth. Extract *A.gangetica* (100mg/kg,p.o)	7.20 ± 0.57 **
V	Eth. extract of *M.indica* (200mg/kg,p.o)	6.60 ± 0.42**
VI	Eth. extract of *M.indica* (400mg/kg,p.o)	7.80 ± 0.45 **

Result expressed as mean ± SEM from six observations; ** indicates P < 0.01 & *indicates P < 0.05

DISCUSSION:

Experimentally induced diabetes in animals has the advantage allowing the analysis of the biochemical, hormonal and morphological parameters that take place not only during the induction of a diabetic state but also after it has taken place and during its evolution to a severe insulin deficiency or even death. This strategy has great advantages but it has to be considered that none of the animal models with induced diabetes corresponds exactly to the human type-2 diabetes mellitus. Nonetheless they provide adequate models to investigate the pathogenic mechanism that leads to hyperglycemia and its consequences [(30)]. Alloxan became the first diabetogenic chemical agent when Dunn and Letchie accidentally produced islet-cell

necrosis in rabbits while researching the nephrotoxicity of uric acid derivatives. Alloxan is a specific toxin that destroys the pancreatic β cells, provoking a state of primary deficiency of insulin without affecting other islet types [31, 32]. Hence, Alloxan was selected to induce diabetes in the present study. Almost more than half of the diabetic patients suffer from different forms of neuropathy after passing 1 to 2 decades of their disease [33, 34]. Currently available drugs for treatment of Diabetic neuropathy have a number of limitations, such as adverse effects and high rate of secondary failure [33]. As there is a growing trend towards using natural remedies as adjuncts to conventional therapy, traditionally used plants might provide a useful source of new analgesic compounds [34]. Although *Asystasia gangetica* may be described as a medicinal plant used for various purposes, no scientific reports on diabetic neuropathy activity was described earlier. The present study demonstrates for the first time the analgesic properties of *Asystasia gangetica in diabetic neuropathy.* . The extracts of *Morus indica* have been reported to possess medicinal properties, including hypoglycemic, hypotensive and diuretic activities [18]. The hypoglycemic and antioxidant effect of mulberry leaves has been demonstrated using Alloxan induced diabetic animals [16].

One of the major mechanisms in the pathophysiology of neuropathy is a biochemical Polyol pathway, neurons and capillaries membranes are not insulin dependent in transporting glucose, great amount of glucose enters cells in diabetes. In neurons glucose is converted to sorbitol by aldose reductase enzyme and sorbitol accumulation increases the production of free radicals such as hydroxyl hydrogen peroxide superoxide that eventually leads to cell damage. Based on this mechanism of injury, different preventive and treatment approaches are suggested and are under research. One of them is the role of antioxidants in preventing neuropathy [36, 37]. Earlier studies reported the antioxidant property of *Asystasia gangetica* and *Morus indica* [16]. Pradeep et al., in their study on antioxidant effect of *Asystasia gangetica* and *Morus indica* have concluded that this medicine contains substances such as pancreatic catalase (CAT), pancreatic superoxide dismutase (SOD) and flavanoids. Therefore it seems that *Asystasia gangetica* and *Morus indica* with their antioxidant property could remove free radicals to some extent and caused improvement in diabetic neuropathy conditions. Diabetic neuropathy in its early stages associated with increase of nerve fibre activity and disorder of normal sensitivity of peripheral nervous system to injuries and painful stimulators resulted from diabetic hyperalgesia [35, 37]. However, after passing early stage the sensitivity of peripheral nerves decreases and caused various ranges of analgesia. According to obtained results in the present study *Asystasia gangetica* and *Morus indica* can exert positive effects in the treatment and decrease in the symptoms of diabetic neuropathy in male mice. In the present study response time to thermal stimuli and electrical stimuli

showed significant increase in the diabetic group than compared to the control group that is due to diabetic analgesia. In the present study ethanolic leaf extracts of *Asystasia gangetica* and *Morus indica* significantly reduce the pain associated with diabetic neuropathy.

CONCLUSION:

In preliminary phytochemical work, flavanoids present in the plants were examined. From the tabulated report it is clearly revealed that the antidiabetic neuropathic potential of *A. gangetica and M. indica*. This activity has been proved due to the presence of different constituents in the plants like flavanoids and antioxidants. Hence the present study provides a scientific evidence for the antidiabetic neuropathic potential of A .*gangetica* and *M. indica*. Further studies required to isolate bioactive compounds will give a path to identify potential lead compounds for developing safe and efficacious agents to treat diabetic neuropathy.

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