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Full Length Research Paper

Effects of indigenous medicinal plants of Bangladesh on blood glucose level and neuropathic pain in streptozotocin-induced diabetic rats

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We investigated the effect of freshly prepared aqueous extracts of *Psidium guajava*, *Momordica charantia*, *Coccinia indica* leaves and their combination on blood glucose level and neuropathic pain in hyperglycemic rats. Streptozotocin (STZ; 65 mg/kg, Iv) was injected to albino rats to induce diabetes. Oral administration of freshly prepared aqueous extracts of each of the leaves and their combination were given to STZ-induced diabetic rats until 8 weeks after the STZ injection at a dosage of 500 mg/kg bwt/day. Oral glucose tolerance test to follow the changes in blood glucose and von Frey test to evaluate the tactile allodynia were performed to investigate the antidiabetic effects. Treatment with these freshly prepared leaf aqueous extracts significantly reduced blood glucose in diabetic rats ($p < 0.001$). Blood glucose lowering effect of the combination treatment was significantly greater than individual treatments ($p < 0.01$). Oral glucose tolerance test also showed the improvement of glucose tolerance by each extracts ($p < 0.001$). These effects were significantly greater in the combination treatment group over the individual treatment groups ($p < 0.01$). STZ-induced loss of body weight was attenuated by the individual and their combination treatment ($p < 0.02$). Diabetic rats developed stable tactile allodynia measured by von Frey test. Treatment with these freshly prepared leaf aqueous extracts significantly improved tactile allodynia in diabetic rats ($p < 0.01$). Effect of the combination treatment on tactile allodynia was also significantly greater than individual treatments ($p < 0.05$). These data suggest beneficial effect of freshly prepared aqueous extracts of *P. guajava*, *M. charantia and C. indica* leaves on hyperglycemic and neuropathic pain. Their combination therapy works better against hyperglycemia and neuropathy of STZ-induced diabetes rats. Therefore, the use of these plants as antidiabetics in folkloric medicine is justified.

Key words: *Psidium guajava*, *Momordica charantia*, *Coccinia indica*, hyperglycemia, neuropathic pain, STZ-induced diabetic rats.

INTRODUCTION

Diabetes mellitus is a predominant public health concern that has grown steadily worldwide (Honeycutt et al., 2003; Said et al., 2008). Diabetes mellitus is caused by inherited and/or acquired deficiency in the production of insulin by the β-cells of pancreas, or by ineffectiveness of insulin produced. Diabetes mellitus currently affects more than 170 million people worldwide and is predicted to affect over 365 million people by the year 2030 (Wild et al., 2004). Several drugs to increase insulin sensitivity are currently being used. Recently, the search for appropriate hypoglycemic agents has been focused on plants used in traditional medicine (Rates, 2001). Medicinal plants are frequently considered to be less toxic and free from side effects than the synthetic ones. The world health organization has also recommended that this should be encouraged, especially in countries where conventional treatment of diabetes seems insufficient (Santhakumari et al., 2006).

Recent preliminary experimental studies in our

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laboratory and elsewhere have indicated that a number of indigenous medicinal plants of Bangladesh possess curative attributes and show promise as potential leads for the management and/or treatment of certain human and livestock diseases (Mostofa, 2007; Hoque, 2006; Khalid, 2003). In our search and ancient Ayurveda history for plants with potential for use as effective and safe remedies in the management of human diabetes, present study was designed primarily to investigate the effects of freshly prepared aqueous extracts of *Psidium guajava*, *Momordica charantia* and *Coccinia indica* leaves on blood glucose and diabetic neuropathy in Streptozotocin (STZ)-induced diabetic rats. *P. guajava*, commonly known as Guava is a native plant in tropical America and South East Asia (Shen et al., 2008). It is an evergreen. Different parts of this plant are used in traditional medicine for the treatment of various human ailments such as wounds, ulcers, bronchitis and diarrhoea (Gutierrez et al., 2008; ML, 1980). Many people in South East Asia boil *P. guajava* leaves in water and drink the extract as a folk medicine for diabetes. *M. charantia*, commonly referred to as bitterground or Korolla, is a climbing plant, cultivated throughout Southern Asia. Its fruits are very cheap and available throughout the year. Aqueous extract of *M. charantia* seeds lowered blood glucose level in STZ-induced diabetes in rats. Our previous study showed that *M. charantia* fruits extract also has antihyperglycemic effect in STZ-induced diabetic rats (Mostofa, 2007). *C. indica*, commonly known as Telakucha in Bangladesh. It is a climbing plant, cultivated throughout Southern Asia. Our previous study revealed that blood glucose level of STZ-induced diabetic rats was significantly reduced by its aqueous extract (Choudhury, 2006). However, combination use of freshly prepared aqueous extracts of these three plants in STZ-induced diabetic rats has not yet been studied. Therefore, the present study was designed to examine the effects of freshly prepared aqueous leaf extracts from the individual plants and their combination on blood glucose and diabetic neuropathy in STZ-induced diabetic rats.

MATERIALS AND METHODS

Animals

The experiment was conducted in the Department of Pharmacology, Bangladesh Agricultural University (BAU). Male adult cross-bred albino rats were procured from the animal research branch of the International Center for Diarrheal Disease and Research (ICDDR, Dhaka, Bangladesh). During the experimental period, rats were fed with normal laboratory chews and provided with drinking water *ad libitum*. Animals were acclimatized for a period of one week in the environment. The body weight was 200 to 250 g when the experiment started (average 234 ± 7 g).

Plant materials

Fresh *P. guajava* leaf, *M. charantia* leaf and *C. indica* leaf were collected from surrounding villages of Bangladesh Agricultural University (BAU) campus and the departmental medicinal garden. The plants were authenticated with the help of a taxonomist (Mr. Moniruzzman) in the Department of Botany, BAU, Mymensingh, Bangladesh. The specimens were stored in the departmental herbarium. Authenticated plant leaves were washed with distilled water and chopped into small pieces and pasted with pestle and mortar. The pasts of *P. guajava*, *M. charantia*, *C. indica* leaves and their combination (made at the rate of 33.33% w/w volume for each of the three pastes) were mixed with distilled water (1:3, w/v) to prepare the fresh leaf aqueous extracts. Freshly prepared leaf pasts were used orally at the dose of 500 mg/kg body weight daily for this experiment.

Induction of diabetes

A single dose of freshly prepared STZ (Sigma chemical Co., St. Louis, MO) in citrate buffer, pH 4.5, was immediately injected intravenously (65 mg/kg) through tail vein in a volume of 1 ml/kg body weight (Masiello et al., 1998). STZ injection rapidly produced the characteristic signs of diabetes, such as increased intake of water and food, frequent urination and increased blood glucose concentration. One week after the STZ injection, rats having more then 250 mg/dl random blood glucose levels and showing above mentioned characteristic signs of diabetes were selected for this experiment. A drop of blood samples were collected from the tip of the tail by needle puncture for blood glucose measurement on alternate weeks.

Experimental design

Animals were divided into 6 groups (n = 6 for each) and treated as follows: Group 1; was given citrate buffer and served as control (Con, without STZ). STZ-induced diabetic rats were divided in to five groups (Groups 2 - 6). Group 2; diabetic control (STZ). Group 3; STZ+ freshly prepared aqueous extract of *P. guajava* leaf at a dose of 500 mg/kg/day (STZ+ *P. guajava*). Group 4; STZ+ freshly prepared aqueous extract of *M. charantia* leaf at a dose of 500 mg/kg/day (STZ+ *M. charantia*). Group 5; STZ+ freshly prepared aqueous extract of *C. indica* leaf at a dose of 500 mg/kg/day (STZ+ *C. indica*) and Group 6; the combination of *P. guajava* leaf, *M. charantia* leaf and *C. indica* leaves at a dose of 500 mg/kg/day (STZ+Combination). Treatments by oral gavage daily were started one week after the single dose of STZ injection and continued for 7 weeks.

Determination of hyperglycemia preventive effect of combined aqueous plant leaf extracts on the diabetic rats, in addition to the above-mentioned groups, an additional experiment was performed with the following three groups; Group 7; same treatment as Group 1 (n = 5). Group 8; same treatment as Group 6, meaning that combination of *P. guajava*, *M. charantia* and *C. indica* leaves at a dose of 500 mg/kg/day (STZ+Combination) was started one week after the STZ injection (n = 5). Group 9; combination of *P. guajava*, *M. charantia* and *C. indica* leaves at a dose of 500 mg/kg/day (STZ+Combination-pretreatment) was started one week before the STZ injection (n = 6) and continued until 8 weeks after the STZ injection.

Oral glucose tolerance test (OGTT)

After 7 weeks treatment of the leaf aqueous extracts, oral glucose tolerance test was performed. Rats were fasted for 12-14 hrs before glucose was applied orally by gavage (2.0 g/kg); blood samples were collected from the tail by needle puncture before and at 30, 60, 90, 120 min after glucose treatment. Blood glucose level was determined by a commercial glucose kit based on the glucose oxidase method (Sigma Diagnostics, St. Louis, MO).

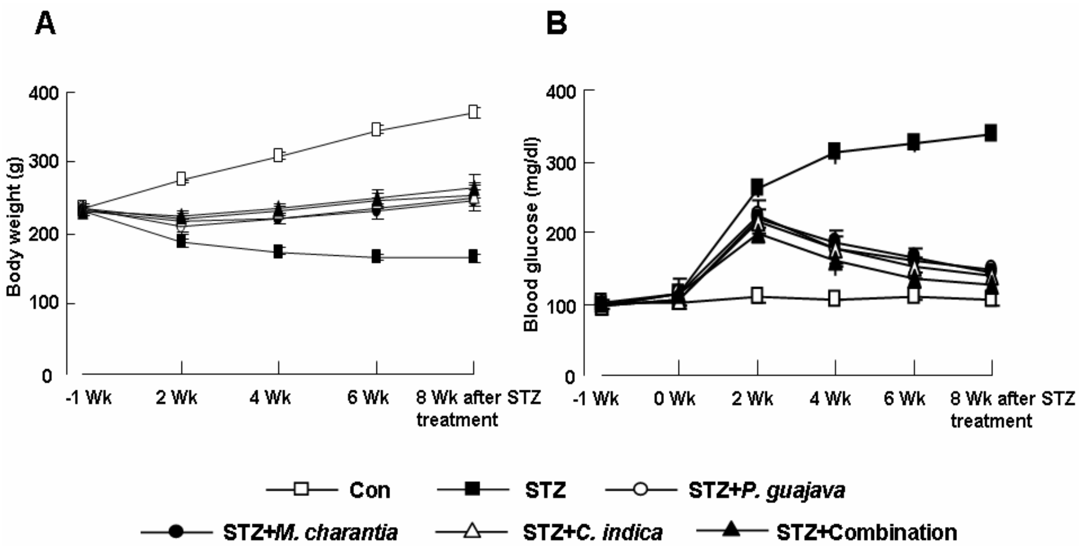

Figure 1. (A) Body weight in non diabetic, STZ-induced diabetic non-treated and treated rats during the 8 weeks after the STZ treatment. Freshly prepared leaf aqueous extracts treatment significantly improves STZ induced loss of body weight ($p < 0.02$, STZ vs. STZ+ *P. guajava*, STZ+ *M. charantia* , STZ+ *C. indica* and STZ+Combination). (B) Blood glucose levels in non diabetic, STZ-induced diabetic non-treated and treated rats during the 8 weeks after the STZ treatment. STZ-induced hyperglycemia is attenuated by the treatment of freshly prepared leaf aqueous extracts ($p < 0.001$, STZ vs. STZ+ *P. guajava*, STZ+ *M. charantia* and STZ+ *C. indica*) and their combination treatment shows better effect compared to single leaf extract treatments ($p < 0.05$, STZ+Combination vs. STZ+ *P. guajava*, STZ+ *M. charantia* and STZ+ *C. indica*). Values were calculated as mean ± SE for six rats in each group.

Tactile allodynia evaluation by von Frey filaments test

Calibrated von Frey filaments (Wei, 2003) were used for assessment of the development of tactile allodynia in the hind paws of diabetic rats (Groups 2 - 6) in comparison to non-diabetic rats (Con group) on alternate weeks. Von Frey filaments of varying tensile strengths (2 - 18 g) were applied in ascending order to the planter surface of the hind paw of the rat, until there was a brisk response of paw withdrawal. If the rat failed to withdraw its paw at the maximum force of 18 g, 18 g was recorded as having induced withdrawal response, because higher forces were not used in order not to induce tissue damage to the footpad of the rat.

Statistical analysis

All values are presented as the means ± SEM. Statistical comparisons of the differences were performed using one way analysis of variance for repeated measures combined with the Newman-Keuls post hoc test. P values below 0.05 were considered statistically significant.

RESULTS

Effect of freshly prepared leaf aqueous extracts of indigenous medicinal plant on body weight in diabetic rats

STZ-induced diabetic rats showed loss of body weight significantly ($p < 0.001$) at 2 week after the STZ treatment compared to Con rats, which further decreased at 4, 6 and 8 weeks after the STZ treatment (Figure 1A). At 8 week after the STZ treatment, average body weight of Con and STZ (Group 2) rats were 371 ± 6 and 164 ± 5 g, respectively ($p < 0.001$). On the other hand treatment with freshly prepared leaf aqueous extracts of indigenous medicinal plants in diabetic rats showed significant prevention of body weight loss ($p < 0.02$). At 8 week after the STZ treatment, average body weight of STZ+*P. guajava*, STZ+*M. charantia*, STZ+ *C. indica* and STZ+Combination groups were 250 ± 18, 245 ± 13, 255 ± 14 and 265 ± 16 g, respectively (Figure 1A).

Antihyperglycemic effect of freshly prepared leaf aqueous extracts of indigenous medicinal plant in diabetic rats

One week after STZ injection produces hyperglycemia in rats. For this experiment, we selected the rats which showed more then 250 mg/dl random blood sugar one week after the STZ injection. Random blood sugar was further increased at 2, 4, 6 and 8 weeks (Figure 1B) in STZ injected rats. At 8 week after the STZ treatment, average random blood sugar level of Con and STZ (Group 2) group were 105 ± 7 and 335 ± 12 mg/dl, respectively ($p < 0.0001$). Treatment with freshly prepared leaf aqueous extracts of indigenous medicinal plants in diabetic rats showed blood glucose lowering effect at 2 ($p < 0.001$) and also gradually lowered the elevated blood glucose level at 4, 6

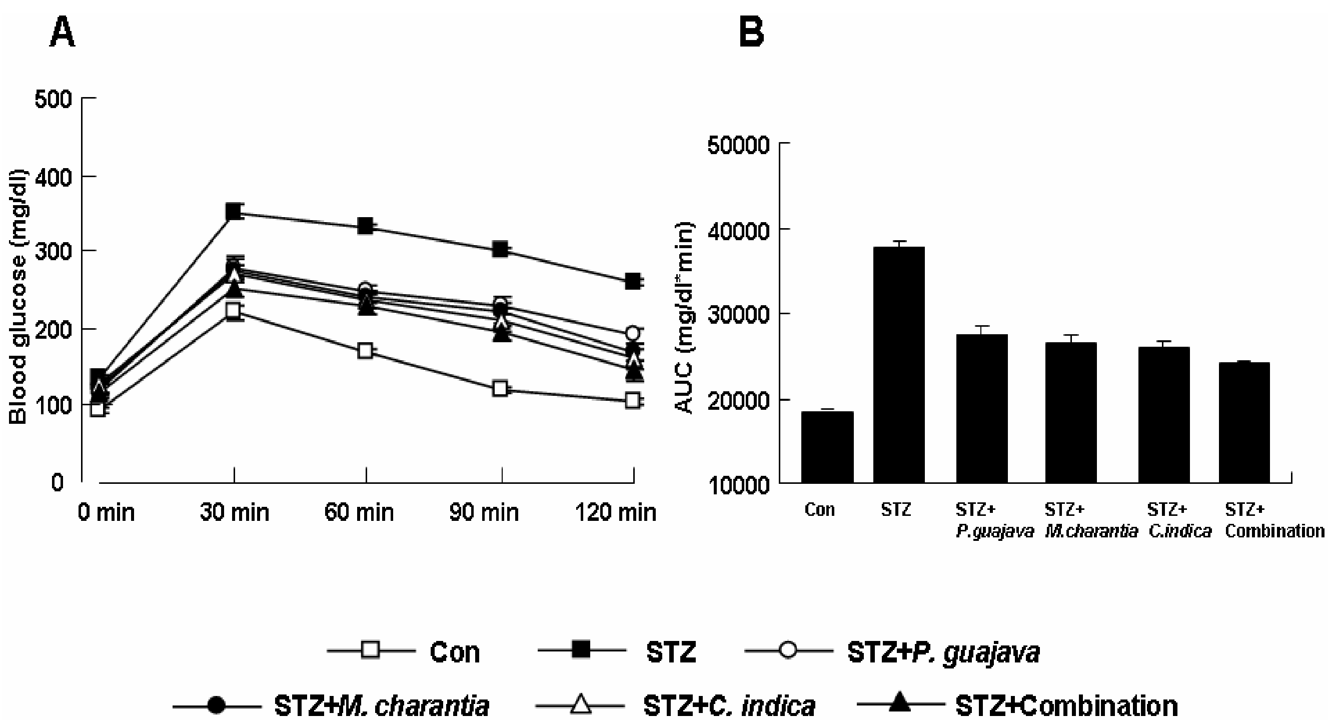

Figure 2. (A) Oral glucose tolerance test (OGTT) in non diabetic, STZ-induced diabetic non-treated and treated rats during the 8 weeks after the STZ treatment. STZ-induced diabetic rats shows higher blood glucose concentration than normal rats, which is markedly reduced by the treatment with freshly prepared leaf aqueous extracts; however, further reduction of blood glucose concentration is observed in combination treatment group compared to single leaf extract treatment group. (B) Area under curve (AUC) from OGTT blood glucose concentration in normal and STZ-induced diabetic rats. Higher AUC of STZ-induced diabetic rats is prevented by the treatment with freshly prepared leaf aqueous extracts ($p < 0.001$, STZ vs. STZ+ *P. guajava*, STZ+ *M. charantia*, STZ+ *C. indica* and STZ+Combination). Further lowered AUC is observed in combination treatment group compared to single leaf extract treatment group value ($p < 0.01$, STZ+Combination vs. STZ+ *P. guajava*, STZ+ *M. charantia* and STZ+ *C. indica*). Values are calculated as mean ± SE for six rats in each group.

and 8 weeks after the STZ treatment ($p < 0.001$). At 8 week after the STZ treatment, average blood glucose levels of STZ+*P. guajava*, STZ+*M. charantia*, STZ+ *C. indica* and STZ+Combination groups were 149 ± 15, 145 ± 16, 141 ± 14 and 128 ± 16 mg/dl, respectively.

We observed that the antihyperglycemic effect of STZ+Combination was greater than those of the three groups treated with individual extracts, which were also statistically significant ($p < 0.05$, STZ+Combination vs. STZ+ *P. guajava*, STZ+ *M. charantia* and STZ+ *C. indica*) (Figure 1B).

Effect of freshly prepared leaf aqueous extracts of indigenous medicinal plant on OGTT in diabetic rats

Freshly prepared leaf aqueous extracts of indigenous medicinal plant feeding in diabetic rats and oral glucose tolerance test were performed to evaluate the antihyperglycemic effect of *P. guajava*, *M. charantia*, *C. indica* leaves and their combination. Figure 2A shows blood glucose curve of six groups: Con, STZ non-treated and STZ rats treated with freshly prepared leaf aqueous extracts during OGTT. STZ-induced diabetic rats showed markedly higher glucose concentration curve compared with Con group. Figure 2B shows area under curve (AUC) calculated from OGTT blood glucose concentration, which is also higher in STZ-induced diabetic rats compared with Con rats value ($p < 0.001$). On the other hand freshly prepared leaf aqueous extracts of indigenous medicinal plant treated STZ rats (Groups 3 - 6) showed attenuation in their glucose concentration levels as well as their ACU, which are also statically significant ($p<0.001$, STZ vs. STZ+ *P. guajava*, STZ+ *M. charantia* and STZ+ *C. indica* and STZ+Combination). Furthermore, AUC of combination treatment group was significantly lower than that of individual treatment ($p < 0.01$, STZ+ Combination vs. STZ+ *P. guajava*, STZ+ *M. charantia* and STZ+ *C. indica*).

Effect of freshly prepared leaf aqueous extracts of indigenous medicinal plant on diabetic neuropathy

Von Frey testing values of different groups are shown in Figure 3. In Con rats, von Frey testing values are 13.4 ± 0.2 and 14.1 ± 0.1 g before and at the end of the experiment, respectively. Tactile allodynia developed in STZ (Group 2) rat after 2 week and gradually progressed.

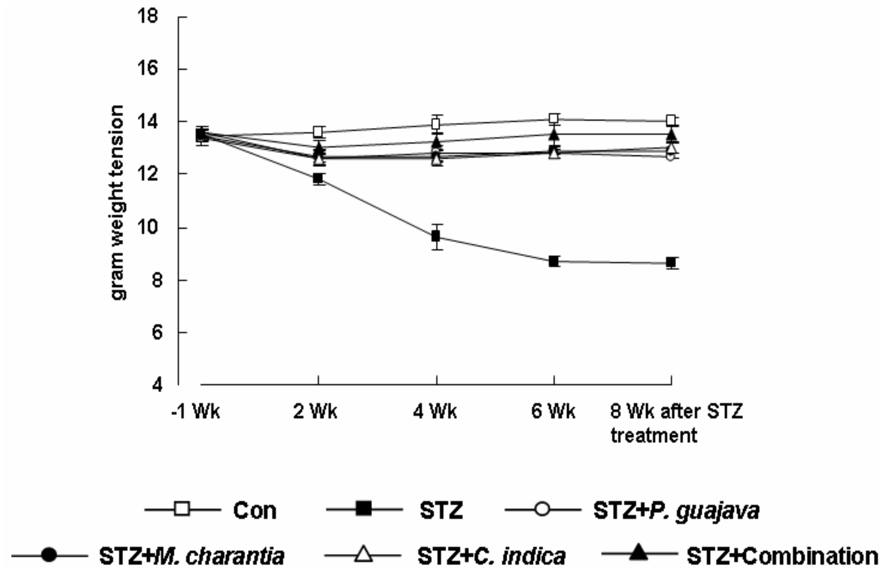

Figure 3. Von Frey test data in non diabetic, STZ-induced diabetic non-treated and treated rats during the 8 weeks after the STZ treatment. STZ-induced diabetic rats show tactile allodynia. Freshly prepared leaf aqueous extracts treatment significantly prevents the development of tactile allodynia in STZ-induced diabetic rat ($p < 0.01$, STZ vs. STZ+ *P. guajava*, STZ+ *M. charantia* , STZ+ *C. indica* and STZ+Combination). Further improvement of tactile allodynia is observed in combination therapy as compared to single therapies ($p < 0.05$, STZ+Combination vs. STZ+ *P. guajava*, STZ+ *M. charantia* and STZ+ *C. indica*). Values were calculated as mean ± SE for six rats in each group.

Table 1. Effects of freshly prepared leaf aqueous extracts of indigenous medicinal plant on blood glucose concentration (mg/dl) from additional experimental rats.

Groups	Treatment period (Weeks)				
	1 wk	0 wk	2 wk	4 wk	8 wk
Control	104 ± 4	106 ± 4	102 ± 6	109 ± 4	109 ± 4
STZ	99 ± 3	105 ± 4	289 ± 7α	320 ± 8α	346 ± 8α
STZ+Combination	100 ± 2	99 ± 5	260 ± 3β	162 ± 4β	128 ± 4β
STZ+Combination- pretreatment	106 ± 4	95 ± 7	222 ± 11β*	145 ± 12β	121 ± 9β

α $p < 0.001$ vs control group values and β $p < 0.001$ vs STZ group values. * $p < 0.01$ vs STZ+Combination.

In STZ (Group 2) rats von Frey testing values are 13.5 ± 0.2 and 8.3 ± 0.2 g before and at the end of the experiment, respectively.

Treatment with freshly prepared leaf aqueous extracts of indigenous medicinal plants and their combination significantly prevented the development of tactile allodynia in diabetic rats. At the end of experiment von Frey testing values of STZ, STZ+ *P. guajava*, STZ+ *M. charantia*, STZ+ *C. indica* and STZ+Combination are 8.3 ± 0.2 g, 12.7 ± 0.1, 12.9 ± 0.2, 13.0 ± 0.1 and 13.6 ± 0.3 g, respectively ($p < 0.01$, STZ vs. STZ+ *P. guajava*, STZ+ *M. charantia*, STZ+ *C. indica* and STZ+Combination). Interestingly, combination treatment significantly improved tactile allodynia compared to single leaf extract treatments ($p<0.05$, STZ+Combination vs. STZ+ *P. guajava*, STZ+ *M. charantia*).

Prevention of hyperglycemia by freshly prepared leaf aqueous extracts combination therapy

The combination therapy of fresh aqueous leaf extracts (at a dose of 500 mg/kg/day), started one week before the STZ injection (STZ+Combination pre-treatment group), showed significantly lower blood glucose level two week after the STZ injection compared to that of STZ + Combination (combination therapy started one week after the STZ injection, $p < 0.01$). Blood glucose level was further lowered during 4 and 8 weeks after STZ injection (Table 1).

DISCUSSION

Our data showed that freshly prepared *P. guajava*, *M. charantia* and *C. indica* leaves aqueous extracts individually or in combination attenuates hyperglycemia and diabetic neuropathy in STZ-induced diabetic rat model. We also showed that these combined therapy have more blood glucose lowering effects when treatment started one week before the STZ injection. Evidence from the literature suggests that some plants act like biguanides and do not affect the blood glucose basal state (Bailey et al., 1985; Hermann et al., 1994). In this study, it is possible that the combination therapy by using three plants extract could act by a similar mechanism. Because these plant either individually or in combination did not affect basal blood glucose level. However, hypoglycemic effects were more pronounced when combination therapy started one week before STZ injection.

In agreement with our present results, several investigators (Gutierrez et al., 2008; Lin, 1964; Shen et al., 2008) have reported that different parts of *P. guajava* including its leaf extract have an antihyperglycemic effect and also stimulate glucose utilization in liver tissues in STZ-induced diabetic rats. Tannins, flavonoids, pentacyclic triterpenoids, guiajaverin, quercentin and other chemical compounds present in the plant are speculated to account for the observed hypoglycemic and hypotensive effects of the leaf extract (Ojewole, 2005; Wang et al., 2005). *Mormordica* species have antioxidant, anti-inflammatory and antihypertensive properties (Ojewole et al., 2006; Van Wyk, 2004). Shibib et al. (1993) showed that ethanolic extracts of *M. charantia* (200 mg/kg) showed an antihyperglycemic and hypoglycemic effect in normal and STZ-induced diabetic rats. The authors suggest that these effects of *M. charantia* are mediated through inhibition of glucose- 6 -phosphatase besides fructose- 1, 6 -biphosphatase in the liver and stimulation of hepatic glucose-6-phosphate dehydrogenase activities. Two controlled short-term metabolic trails in patients with type 2 diabetes have reported the acute effects on blood glucose with *M. charantia* fruit juice, as well as subcutaneous vegetable insulin extract (Welihinda et al., 1986). We (Choudhury, 2006) and others (Pari and Venkateswaran, 2003; Venkateswaran and Pari, 2003) showed that *C. indica* leaf has antihyperglycemic and antioxidant effects in STZ-induced diabetes rats. Hossain and Rahman (Hossain and Rahman, 1992) examined the effect of *C. indica* leaf preparation on 48 h starved normal male rats and showed that the leaf extract depressed the activity of the enzyme glucose- 6 -phosphatase. However, there is no report published yet regarding the use of the combination of these three leaves and use of their freshly prepared aqueous extracts in diabetic rats.

A single dose administration of STZ was partially destroying to the pancreatic β-cells. Under this condition, insulin was secreted, but it may not be sufficient to regulate blood glucose levels and consequently, the rats became permanently diabetic (Cabrera et al., 2008; Fisher, 1985). In the present study, combination therapy produced a significant decrease in the blood glucose level compared with untreated diabetic rats. This antihyperglycemic effect was observed from the beginning of the treatment when combination therapy was started one week before STZ injection and hyperglycemia was further lower than that, when treatment was started one week after the STZ injection. Based on these data, it is possible that combination therapy may protect the pancreas or regenerate the damaged pancreatic cells in STZ-induced diabetic rat model, thereby maintaining blood insulin and blood glucose levels either by stimulation of insulin secretion from the remaining pancreatic β-cells and/or regenerating the damaged pancreatic β-cells. We speculate that these pancreas protective and/or regenerative effects may be due to their antioxidant properties. However, the actual mechanisms by which the combination therapy protects and regenerates the damaged pancreatic cells are still unclear. This remains to be discovered. Therefore, our future studies will focus on blood insulin level, histology of pancreas as well as oxidative stress.

The present study shows that the combination treatment with *P. guajava*, *M. charantia* and *C. indica* leaves freshly prepared aqueous extracts significantly prevented the development of tactile allodynia in diabetic rats. Hyperglycemia has been reported to result in increased polyol pathway activity, oxidative stress, advanced glycation end product formation, increased activation of protein kinase C, nerve hypoxia/ischemia (Pop-Busui et al., 2006) and all these pathways may contribute to the development of diabetic neuropathy. Based on our present experimental data, it is possible that single or combination therapy may prevent the STZ-induced diabetic neuropathic pain by their antihyperglycemic effect and/ or miscellaneous antioxidant compounds present in the extracts.

Conclusion

Combination of *P. guajava*, *M. charantia* and *C. indica* leaves freshly prepared aqueous extracts shows antihyperglycemic effect and attenuates neuropathy in STZ-induced diabetic rats. These findings lend pharmacological support to the suggested folkloric and ethnomedical user of these plants in managing and /or controlling of diabetes mellitus in rural communities of Bangladesh. Further pharmacological and biochemical investigations are underway to elucidate the exact mechanism of antidiabetic effects of combination of leaf extracts of these plants.

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