Extract of *Woodfordia fruticosa* flowers ameliorates hyperglycemia, oxidative stress and improves β-cell function in streptozotocin–nicotinamide induced diabetic rats

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**Abstract**

**Ethnopharmacological relevance:** The art of Ayurveda and the traditional healing system in India have reflected the ethnomedicinal importance of the plant *Woodfordia fruticosa* Kurtz, which demonstrates its vast usage in the Ayurvedic preparations as well as in the management of diabetes by the traditional healers.

**Aims of study:** The study aimed to ascertain the antidiabetic potential of *W. fruticosa* flower methanolic extract (WF) on Streptozotocin (STZ)–nicotinamide-induced diabetic rat model.

**Materials and methods:** Diabetes was induced in Sprague Dawley (SD) rats by STZ–nicotinamide and thereafter diabetic rats were treated with three different doses of WF (100, 200 and 400 mg/kg body weight) respectively and glibenclamide as a positive control. Biochemical parameters such as blood glucose, serum insulin and C-peptide levels were measured with oxidative stress markers. Furthermore, histology of liver and pancreas was carried out to evaluate glycogen content and β-cell structures. Moreover, immunohistochemistry and western blot analysis were performed on kidney and pancreas tissues to determine renal Bcl-2, pancreatic insulin and glucose transporter (GLUT-2, 4) protein expression in all the experimental groups.

**Results:** The acute toxicity study showed non-toxic nature of all the three doses of WF. Further, studies on diabetic rats exhibited anti-hyperglycemic effects by upregulating serum insulin and C-peptide levels. Similarly, WF shown to ameliorate oxidative stress by downregulating LPO levels and augmenting the antioxidant enzyme (ABTS). Furthermore, histopathological analysis demonstrate recovery in the structural degeneration of β-cells mass of pancreas tissue with increase in the liver glycogen content of the diabetic rats. Interestingly, protective nature of the extract was further revealed by the immunohistochemical study result which displayed upregulation in the insulin and renal Bcl-2 expression, the anti apoptosis protein. Moreover, western blot result have shown slight alteration in the GLUT-2 and GLUT-4 protein expression with the highest dose of WF treatment, that might have stimulated glucose uptake in the pancreas and played an important role in attenuating the blood glucose levels.

**Conclusion:** The overall study result have demonstrated the potential of WF in the management of diabetes and its related complications, thus warrants further investigation on its major compounds with in depth mechanistic studies at molecular level.

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1. **Introduction**

There is a growing research interest in the management of diabetes mellitus (DM) and its associated complications due to the unabated increase in the incidence of this metabolic syndrome. At present DM is one of the alarming global threat giving rise to complications such as retinopathy, neuropathy, heart attack and atherosclerotic vascular disease (Gandhi et al., 2014; Irudayaraj et al., 2012; Laakso, 1999). The impairment in carbohydrate, lipid
and protein metabolism and defects in insulin signaling leads to hyperglycemia, a known characteristic of DM, which is associated with oxidative stress by increasing the formation of reactive oxygen species (ROS) causing reduction in the antioxidant levels. Under the oxidative stress condition, an increased level of malondialdehyde (MDA), a highly toxic by-product is released by lipid peroxidation and ROS and consequently causing oxidative damage in the pancreas, liver and kidney (Evans, 2007).

According to WHO estimation, herbal medicinal plants have started to gain more attention in recent years for the management of diabetes owing to their less side effects compared to the synthetic pharmaceutical drugs which are more costly (Chavre et al., 2010). These ethnomedicinal plant species are known to exert anti-diabetic effect by ameliorating blood glucose levels and oxidative stress and improving the pancreatic expressions of insulin and glucose transporter proteins (Taha et al., 2014). The presence of glycosides, alkaloids, terpenoids, flavonoids, carotenoids, etc. account for the antidiabetic properties of the plants. As the search for more effective and less expensive anti-diabetic plant extract is still ongoing, there is a renewed research interest on the traditionally used antidiabetic plants. One such plant is Woodfordia fruticosa, the flower of which have been traditionally used in the Beed district of Maharashtra as anti-diabetic agent (Patil et al., 2011; Chavre et al., 2010; Das et al., 2007).

W. fruticosa Kurtz, the well-known plant belong to the family Lythraceae and located abundantly in the tropical and subtropical region of India growing upto an altitude of 1500 m and have been in use for a long time by the traditional practitioners of South East Asian countries. The flower fulfills huge demand of both domestic and international markets even though other parts of the plant also behold some medicinal values. The flowers which is specialized in the preparation of herbal medicines are brilliant red in color, pungent, acrid and it exerts uterine sedative and antihemorrhagic effect. The other therapeutic values of the dried flowers of W. fruticosa act against dysentery, sprue, bowel complaint, rheumatism, dysuria, hematuria, wounds, bleeding injuries, otorrhoea, leucorrhoea and dysmenorrhea. The flowers also contribute in the preparation of Ayurvedic fermented drugs named as ‘Aristhas’ and ‘Asavas’ and was made patented against diabetes and cancer conscious non-diabetics by Brindavanam et al. (2003). The anti-diabetic property of the plant was due to the phytochemicals such as tannins, flavonoids, anthraquinone glycosides and polyphenols present in flowers and leaves (Chavre et al., 2010; Das et al., 2007).

The immunomodulatory activity of the Ayurvedic drug ‘Nimba Arista’, possessing W. fruticosa flowers was demonstrated by Kroes et al. (1993). The presence of glycosides, quercetin and myricetin in WF attribute to the anti-inflammatory effect of the plant and it is validated by its ability to inhibit the two pivotal enzymes, lipooxygenase and cyclooxygenase of pelargonidin, which play dominant role in regulating the pathophysiology of inflammation.

Although various interesting pharmacological aspects have been identified in previous studies to reflect the antioxidant, anti-inflammatory, anti-microbial and anticancer properties of different parts of this plant, two recent studies demonstrated the anti-hyperglycemic potential of the methanolic and ethanolic extract of the flower on alloxan induced diabetic mice and STZ-induced diabetic rats (Bhatia and Khera, 2013; Verma et al., 2012). However, lack of scientific report on the anti-diabetic mechanism including the pancreatic expression of insulin and glucose transporters by the methanolic extract of W. fruticosa flower (WF) warrants further investigation. Keeping this in view, the present study attempts to understand the mechanism involved in anti-hyperglycemic activity of the flower methanolic extract, WF, associated with important biochemical parameters, oxidative stress markers, hepatic glycogen content, pancreatic β-cell mass, insulin and targeted GLUT-2 and 4 protein expressions and the renal Bcl2 protein analysis in STZ–nicotinamide induced diabetic rat model.

2. Materials and methods

2.1. Chemicals

Streptozotocin (STZ), Nicotinamide and Glibenclamide were purchased from Sigma-Aldrich (USA).

2.2. Collection of plant materials

Dried W. fruticosa (1 kg) flowers were procured from Amritum Bio-Botanica Herbs Research Laboratory Pvt. Ltd (Madhya Pradesh, India). The identification and authentication of W. fruticosa flower was carried out by the company’s Quality Control Department. Voucher specimen (PH-12W7) had been deposited with Department of Pharmacy, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia, 50603.

2.3. Extraction of W. fruticosa flower

The dried flowers of W. fruticosa (500 g) were coarsely powdered using grinder for the purpose of extraction with Soxhlet extractor. The powder was first extracted with 100% n-hexane using hot extraction at 43 °C with a Soxhlet extractor for 48 h. After completing the extraction with n-hexane, the obtained defatted residue was further extracted using 100% chloroform and lastly with 100% methanol. The solvents from each crude fraction were dried by rotary evaporator (BUCHI, R-215) under reduced pressure at a maximum temperature of 40 °C to get the extract of W. fruticosa flower. The final fraction was then stored at −20 °C until further use.

2.4. Phytochemical analysis of WF by LCMS/MS-QTOF

The chemical components in WF were analyzed through de-replication method based on MS published data (Alali and Tawaha, 2009). This strategy proved to be effective and useful in analyzing crude extracts. The analytical HPLC (Spark Holland Symbiosis PICO) system was coupled with a hybrid quadrupole-time of flight mass spectrometry (Applied Biosystems, Triple TOF 4600, CA) with an electrospray ionization (ESI) source. A hypersil ODS C18 column (4.6 mm i.d. × 100 mm, 3 μm particle diameter) was used for this analysis. Solid Phase Extraction (SPE) CEC18 cartridge (UCT, Bristol, PA) was used for sample clean-up. The mobile phase consisted of solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid). The HPLC method gradient and mass spectrometry parameters were followed by previous method for the determination of phenolic compounds with some modifications. Analyst software was used for data processing and acquisition.

2.5. Experimental animals

Healthy adult Sprague Dawley (SD) male rats were procured from the Experimental Animal House, Faculty of Medicine, University of Malaya. The rats weighing between 250 and 300 g were caged in polypropylene crates and preserved at normal pathogen free light controlled conditions, consisting of 12 h light/dark cycle at room temperature (25 ± 2 °C) with humidity level of 35–60% and provided with normal rat pellet diet with distilled water. The animal experiment was performed in accordance with the animal experimentation guidelines issued by the Experimental Animal House committee, Faculty of Medicine, University of Malaya.
2.6. Experimental design

The experimental rats were randomly assigned into six groups (n=6), containing of six rats in each group as follows:

**Group 1, Normal Control (NC):** Rats fed with distilled water.

**Group 2, Diabetic Control (DC):** STZ-nicotinamide induced diabetic rats.

**Group 3, WFa:** STZ-nicotinamide induced diabetic rats, treated with 100 mg/kg WF

**Group 4, Wfb:** STZ-nicotinamide induced diabetic rats, treated with 200 mg/kg WF

**Group 5, WFc:** STZ-nicotinamide induced diabetic rats, treated with 400 mg/kg WF

**Group 6, Positive Control (PC):** STZ-nicotinamide induced diabetic rats, treated with Glibenclamide (2.5 mg/kg).

The rats were orally administered once a day with WF (100, 200 and 400 mg/kg body weight) and Glibenclamide (2.5 mg/kg body weight) by dissolving in distilled water by intra-gastric tube. The animals were sacrificed, 24 h after 45 days of last treatment dose.

2.7. Acute toxicity study

Acute toxicity test was carried out according to the guidelines of the Organisation for Economic Co-Operation and Development (OECD) on normal rats with three different doses of WF. The rats were fasted overnight and the next morning fed with single dose of 500, 1000 and 3000 mg/kg body weight to three different groups respectively (n=6). All the rats were continually examined for 2 h to check any abnormalities in behavior of the animals and further continued to monitor and examine the rats for 24 and 72 h.

2.8. Diabetes induction by streptozotocin–nicotinamide

Type 2 diabetes mellitus was induced in overnight fasted rats by a single intraperitoneal injection of Streptozotocin at a dose of 45 mg/kg body weight. After 15 min, 120 mg/kg Nicotinamide was injected intraperitoneally. The glucose level of blood was measured from the tail vein after 72 h of STZ-nicotinamide injection and hyperglycaemic rats (blood glucose level > 16.7 mmol/L) were considered to be diabetic rats.

2.9. Collection of blood sample

The rats were moderately anaesthetised by combination of Ketamine 50 mg/kg and Xylazine 5 mg/kg at the end of 45 day of treatment and 3 ml of blood samples were collected from the inner canthus of the eye by using capillary tubes to collect the serum. For the preparation of serum, blood samples were collected in vacutainer tube and allowed to clot at ambient temperature for 15–30 min. After that, the clot was removed by centrifuging at 4000 rpm for 10 min in a refrigerated centrifuge and serum was collected and stored in −80 °C until further use.

2.10. Assessment of biochemical parameters

The fasting blood glucose levels and body weights of all the groups were measured on days 7, 21 and 45. Blood glucose level was determined by the portable glucometer (Accu-Check Nano Performa). Serum Insulin and C-peptide levels in all the treated, diabetic and normal groups were measured by Enzyme Linked Immunosorbent Assay (ELISA Kit, Item number 589501, Cayman, USA) and (Rat ELISA Kit K4757, Bio Vision Corporation, USA) following manufacturer’s protocol.

2.11. Antioxidant status

The Cayman Chemical Antioxidant Assay Kit determines the total antioxidant capacity (SOD, Catalase, GPx) by measuring the level of ABTS in the serum of diabetic, normal and treatment groups. However, the cumulative effect of all the antioxidant enzymes can be measured using ABTS. The ability of the extract in preventing the oxidation of ABTS (2, 2’-azino-di-[3-ethyl-benzthiazolone sulfonyl]) to ABTS by metmyoglobin was measured using the kit (Item number 709001) and was compared to that of Trolox, a water-soluble tocopherol analogue and was then quantified as molar Trolox equivalents. To determine the lipid peroxidation (LPO) levels, the hydroperoxides level in the serum of diabetic, normal and treatment groups were measured directly in the serum by utilizing the redox reactions with ferrous ions using LPO assay kits (Item number 705003). Both the assays were performed based on Cayman manufacturer instructions (Cayman Chemical, Ann Arbor, USA).

2.12. Histological study

At the end of experiment the liver, kidney and pancreas were dissected and washed with normal saline and preserved in 10% formalin for 7 days at room temperature. After that, the samples were washed and dehydrated with 70–100% of ethanol and cleansed with xylene, and inserted in paraffin blocks. The samples were then cut as thin as 5 μm pieces, with a revolving microtome. Pancreas sections were stained with hematoxylin and eosin dye; and the liver tissue sections were stained with Periodic acid Schiff (PAS) staining for 10 min (for demonstration of glycogen content), and then photomicrographs were obtained under light microscope.

2.13. Immunohistochemistry analysis

For the purpose of immunohistochemistry analysis, 5 μm sections of pancreas and kidney tissue blocks were placed on poly-L-lysine coated and the slide sections were immersed in target retrieval solution (DAKO, Lot 10069393) and heated in microwave oven at 98 °C for 20 min, and then cooled at room temperature. The sections of pancreas were incubated for 1 h in Insulin (Item no: ab46716, 1/50 dilution) primary antibody and anti-BcL-2 (Item no: ab7973, 1/100) antibody was used for kidney tissue incubation at room temperature. After that, three times rinsing was done with Dako Tris-buffered saline (TBS), the sections were incubated with biotinylated secondary antibody (LSAB system™2-HRP, Item no: 10069908) for 1 h at room temperature. Following TBS rinses, the sections were incubated for 30 min at room temperature with streptavidin–horseradish peroxidase conjugate, followed by a course of incubation in Diaminobenzidine (DAB) (DAKO, Item no: 10067468). Control immunohistochemistry reactions were performed to evaluate the specificity of the labels by omitting the primary antibody. Staining with hematoxylin was performed and used as a reference of the cytoarchitecture of the tissue.

2.14. Histomorphometric analysis

Positive signals of the target protein were determined by using a high resolution inverted microscope (Nikon Ti series). Furthermore, Image J software was used to evaluate the integral optical density (IOD) of immunohistochemistry stain. All the single pixels in the digital image signified the IOD of the target protein, which was more precise, according to the strength of stain and the labeled surface areas.
2.15. Western blot

The samples of rats’ pancreas were segregated with 4–20% sodium dodecyl sulfate (SDS-PAGE) gels to fill the polyvinylidene fluoride (PVDF) membranes with proteins. The membranes were blocked with 5% non-fat milk and as well as primary antibodies recognized as anti-glucose transporter GLUT-2 antibody (Item no: ab54460, 1/200) and anti-glucose transporter GLUT-4 antibody (Item no: ab33780, 1/1000). After that, the membranes were incubated at 4 °C overnight then cleansed and again incubated for 1 h with Horseradish peroxidase (HRP)-conjugated goat anti rabbit IgG. The quantities of particular bands were measured by densitometry by ImageJ 1.37 software (NIH, USA).

2.16. Statistical analysis

One way analysis of variance (ANOVA) was used followed by Tukey’s Multiple Comparison Test (Graph Pad version 5.0; Graph Pad Software Inc., San Diego, CA, USA) to analyze the effect of WF on all experimental rat groups. Experimental differences were considered statistically significant if \( P < 0.05 \).

3. Results

3.1. Extract yield

The extract yield of *W. fruticosa* flower was 2.92 g for hexane (WH), 3.44 g for chloroform (WC) and 151.65 g for methanol (WF). On the basis of preliminary screening of all the extracts, WF with the highest percentage yield possesses hypoglycemic effects on different biochemical assays (data not shown). Thus, the extract was chosen for further studies.

3.2. Acute toxicity study

The acute toxicity study represents the non-toxic nature of WF at all the three doses tested. The animals did not show any lethality and behavioral changes after 24 and 72 h of the treatment.

3.3. LCMS-QTOF analysis

The qualitative analysis of chemical compounds in WF were assessed by LCMS-QTOF method in which 4 flavonoid glycosides (Quercetin glucoside, Quercetin 3-O-(6'-galloyl)-β-D-galactopyranoside, Naringenin 7-glucoside, Kaempferol 3-O-glucoside), 3 flavonoid aglycones (Queretin, Naringenin, Kaempferol) and 2 phenolic acids (gallic acid, ellagic acid) were identified from WF based on mass fragmentation patterns and compared to the previous literature. In this analysis, identification of phenolic compounds and characterization of the compounds were achieved by the negative ion-mode ionization. All the compounds were identified and structurally elucidated in previous studies on WF. Fig. S1 shows the total ion chromatogram (TIC) of WF, which demonstrated the presence of polar compounds in the extract.

Quercetin 3-O-(6'-galloyl)-β-D-galactopyranoside was identified based on the fragment ions at m/z 615 [M–H]^– similarly identified Quercetin at m/z 300 [M–H]^– and Quercetin glucoside at m/z 463 [M–H]^–. The presence of a galloyl unit was indicated by the loss of 152 Da from the base peak at m/z 615 in Fig. S2. Mass spectra data of the fragment ions was compatible with previous literature. Likewise, the mass spectra of Naringenin and Naringenin 7-glucoside (Fig. S3), Kaempferol and Kaempferol 3-O-glucoside (Fig. S4), gallic acid (Fig. S5) and ellagic acid (Fig. S6) was shown.

3.4. Effect of WF on body weight

The effect of WF on body weight in normal and diabetic rats at week 1, 3 and 6 are shown in Fig. 1. The untreated type 2 diabetic rats exhibited significant reduction in body weight compared to that of the NC group \( (P < 0.05) \). Nevertheless, upon treatment with three different doses of WF, the diabetic rats demonstrated significant improvement in their body weight as compared to DC group \( (P < 0.05) \).

3.5. Effect of WF on blood glucose level

Fig. 2 illustrates the effect of PC and WF on blood glucose level of normal, diabetic and diabetic treated rats at week 1, 3 and 6. Monitoring of the rats’ fasting blood glucose levels for 45 days treatment period revealed significant reduction in the elevated blood glucose levels of diabetic rats treated with PC and WF compared to that of the DC group \( (P < 0.05) \). However, there was a higher reduction in the blood glucose level with WFc compared to the other two doses of WFa and Wfb in diabetic rats.

3.6. Effect of WF on insulin and C-peptide level

Fig. 3 demonstrates that the serum insulin and C-peptide levels in the untreated diabetic rats were markedly declined compared to
that of the NC group ($P < 0.05$). The WFa, WFB and WFc groups displayed significant increase in the serum insulin and C-peptide levels after the six-week treatment period as compared to DC group ($P < 0.05$). However, the diabetic rats treated with WFc group exhibited the highest increase in these parameters compared with those observed in the DC group and was quite similar to that in the PC group, suggesting a dose-dependent improvement in the glycemic control.

### 3.7. Effect of WF on antioxidant (ABTS) and LPO levels

Fig. 4 represents the effect of WF on oxidative stress in normal and diabetic rats. As demonstrated in Fig. 4A, the antioxidant (ABTS) level was significantly reduced in DC group against NC group ($P < 0.05$) and this effect was recovered by WF in a dose-dependent manner. PC and WFc showed higher antioxidant level when compared to the other lower doses of WF. On the other hand, DC group displayed a significant rise in the LPO level when
compared to that of NC group (Fig. 4B). In contrast, diabetic rats treated with PC and WFc group attained highest reduction in the LPO level compared to that of WFa and WFb groups, indicating the dose-dependent effect of WF.

### 3.8. Effect of WF on pancreas tissue

Fig. 5 demonstrates the impact of WF on pancreas structure in normal and diabetic rats. After 45 days of treatment, the pancreas section of rats were stained with hematoxylin and eosin as described in method section. Panel (A) showed a normal and round rat islet of Langerhans with surrounding of normal structure and normal exocrine acini tissues. Panel (B) which corresponds to the STZ–nicotinamide induced diabetic rats, showed severe β-cell damage and deformation with atrophic and vacuolated pancreatic islet. In addition, focal necrosis, congestion in central vein and infiltration of lymphocytes were examined. In Panel (C), the WFa treatment exerted minimal restoration of pancreatic endocrine cells and expansion of pancreatic islets, which showed a prominent hyperplastic islet. Panel (D) showed that WFb exhibited protective effects over the pancreatic islet, as evidenced by the absence of vacuolization. Although less degranulation was observed in the β-cells, the cell structure was disorganized with a reduced-sized islet and also, degeneration of some of the pancreatic acini was still observed. In Panel (E), the effect of WFc on diabetic rats has shown considerable reduction of lesion and normal β-cell structure. Panel (F) displays the protective role of Glibenclamide on the pancreas of diabetic rats against the destructive effect of STZ–nicotinamide.

### 3.9. Effect of WF on liver glycogen content

Fig. 6 illustrates the effect of three doses of WF extract on the pancreas of STZ–nicotinamide induced diabetic and normal rats after 45 days treatment. H & E staining of pancreas (400 x). A: NC; B: DC; C: WFa; D: WFb, E: WFc and F: PC.
has retained higher glycogen content reflecting its positive modulation on hepatic carbohydrate metabolism compared to that of DC group.

3.10. Effect of WF on Bcl-2 expression in kidney tissue

Fig. 7 reveals the effect of WF on Bcl-2 expression in normal and diabetic rats. According to Fig. 7(I), the expression level of Bcl-2 was found to be decreased in DC group against NC group \((P < 0.05)\) and this effect was reversed with three doses of WF after 45 days of treatment. This immunohistochemical observation was confirmed by histomorphometric study as shown in Fig. 7(II) which demonstrates that the recovery of Bcl-2 expression was dose dependent, as the highest Bcl-2 expression was achieved with the highest dose, which was WFc.

3.11. Effect of WF on insulin expression in pancreas tissue

Fig. 8 shows the changes in insulin expression level after treatment with WF and PC in diabetic rats. The reduced expression of insulin level in DC group was restored gradually with increasing dose of WF as illustrated in Fig. 8(I). The diabetic rats in WFc group showed higher level of insulin expression compared to that of WFa and WFb. These results were confirmed by histomorphometric study as displayed in Fig. 8(II). However, both the figures demonstrate significant enhancement of insulin expression level in diabetic rats treated with PC and three different doses of WF against DC group \((P < 0.05)\).

3.12. Effect of WF on GLUT-2 and GLUT-4 expression levels

The determination of GLUT-2 and GLUT-4 expression level in rat’s pancreas following WF treatment was presented in the western blot analysis in Fig. 9. The untreated diabetic rats exhibited significant reduction in the total amount of these two proteins as compared to that of the NC group \((P < 0.05)\). Interestingly, WF treatment led to slight alteration in the GLUT-2 and GLUT-4 proteins expression, reflecting the protective nature of WFc, among the tested doses (Fig. 9(II) a and b).

4. Discussion

In the present study, we have scientifically evaluated the
traditional claim for the use of *W. fruticosa* flower in the management of diabetes mellitus and its associated complications by its antioxidant and protective nature against the liver, pancreas and kidney of STZ-nicotinamide induced diabetic rats.

The acute toxicity study results demonstrated non-toxic nature of WF with no lethality at different dosages. Similarly, previous study have also shown that *W. fruticosa* flower extract was not toxic to mice and rats (Verma et al., 2012). Oral administration of

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**Fig. 7.** (I) Effect of WF on the kidney Bcl-2 expression level in STZ-nicotinamide induced diabetic and normal rats after 45 days treatment (400 ×). A: NC; B: DC; C: WFa; D: WFb; E: WFc and F: PC. (II) Bcl-2 expression level were quantified by Image J analysis software as mean ± SD and analyzed by one-way ANOVA (Tukey). *Indicates the significant difference when compared to the NC group (*P* < 0.05). $ Indicates the significant difference when compared to the DC group (*P* < 0.05).
three doses of WF (WFa, WFb and WFc) to diabetic rats for 45 consecutive days exhibited attenuation in the elevated blood glucose level and thus augmented serum insulin levels accompanied by rise in serum pro-insulin, C-peptide which serves as a linker between A and B chains of insulin (Al-Numair et al., 2009). The outcomes of our result is in agreement with the previous study that reported ethanolic extract of WF leaves was found to be effective in increasing plasma insulin in dexamethasone induced insulin resistance in mice (Bhujbal et al., 2012). The up-regulation in serum insulin and C-peptide levels could be due to the improvement in β-cell function which is indirectly/directly associated with the antioxidative and free radical scavenging properties of the extract as evidenced by a rise in antioxidant defense system, ABTS and reduction in LPO levels. The hyperglycemia mediated oxidative stress contributes to the accelerated formation of ROS by glycating and inactivating the antioxidant defense enzymes, SOD, catalase and GPx (Arunachalam and Parimelazhagan, 2013). In consistence to our results, (Verma et al., 2012; Singh and Kakkar, 2013) showed that catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase activities were

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**Fig. 8.** (I) Effect of WF on the pancreas insulin expression level in STZ-nicotinamide induced diabetic and normal rats after 45 days treatment (400 ×). A: NC; B: DC; C: WFa; D: WFb; E: WFc and F: PC. (II) Insulin expression level were quantified by Image J analysis software as mean ± SD and analyzed by one-way ANOVA (Tukey). * Indicates the significant difference when compared to the NC group (P < 0.05). $ Indicates the significant difference when compared to the DC group (P < 0.05).
improved and lipid peroxidation was declined in diabetic rats treated with WF.

Moreover, the degeneration of pancreatic \(\beta\)-cell caused by STZ-induced oxidative stress as shown in the histological analysis is associated with reduced level of insulin in the serum of diabetic rats. Diabetic rats treated with WF have shown regeneration of insulin-producing islet cells and upregulation of insulin expression in the pancreas. This restoration of \(\beta\)-cell mass and its structure by reducing the lesion and acini developed in the diabetic state could be due to the antioxidant nature of WF by scavenging the free radicals as determined in the immuno-histochemical study. Thus, the present investigation extends our postulation on the existing polyphenolic compounds in WF as identified by LCMS-QTOF that might have shown antidiabetic effects by improving the degenerated \(\beta\)-cell mass in the diabetic rats which is in agreement with the study results of Taha et al. (2014). The better regulation of hyperglycemic state may also support the hypothesis that WF directly modulates insulin release from remaining \(\beta\)-cells which eventually restores hepatic glycogen storage by potentiating glucose metabolism and/or peripheral glucose uptake.

Based on these findings, we determined the hepatic glycogen content in diabetic rats by PAS staining and observed that it was significantly restored in case of WF treated animals. The impaired capacity of liver to store glycogen is associated with reduced activity of glycogen synthase and enhanced glycogen phosphorylation due to lack of insulin in the diabetic state (Shirwaikar et al., 2004). The mechanism resulting hyperglycemia in diabetes mellitus was due to the excessive hepatic glycogenolysis and gluconeogenesis associated with decreased glucose uptake by the tissues (Qamar and Qureshi, 2013; Wang et al., 2010) reflecting the pivotal role of liver in maintaining the normal concentrations of blood glucose (Onkaramurthy et al., 2013). The hepatic damage as observed in the present histopathology analysis (Fig. 6) displays the deleterious effects of hyperglycemia-induced oxidative stress.

The existing flavonoids in WF, such as quercetin and quinic acid would have played important role in preventing hepatic damage in diabetic rats which is in line with the histological studies reported in our previous study and by other researchers where quercetin reduced the severity of hepatic injuries by increasing the antioxidant enzymes activity and by scavenging the free radicals (Arya et al., 2014; Bashir et al., 2014; Florence et al., 2014). The antioxidative protection rendered by WF may likewise functioned in safeguarding the hepatic system in the presence of elevated serum insulin level, a regulator of glycogen metabolism in mammals (Gandhi et al., 2014). Thus, diabetes reduces liver glycogen stores and insulin reverses this effect of hepatic damage independently and WF beside ameliorating hepatic glycogen content also plays a protective role against the hepatic complications in diabetes.

The correlation between insulin secretion and glucose uptake was further investigated by pancreatic protein determination as evidenced by upregulation in GLUT-2 and GLUT-4 expression in the WF treated diabetic rats. The improved glucose uptake in the insulin-signaling cascade is managed by glucose transporter (GLUT) proteins, GLUT-2 and GLUT-4 which translocates the
insulin-regulated glucose into the cells (Hajighaaliipur et al., 2015) and has impact on whole-body glucose homeostasis (Zhao et al., 2014). The availability of polyphenols in WF might have triggered glucose ingestion by modulating the glucose transporter proteins (Li et al., 2015). Interestingly, many researchers have identified the potential role of polyphenolic enriched herbal extracts in attenuating hyperglycemia mediated insulin resistance and thus modulated uptake of glucose by GLUT-2 and GLUT-4 expression in pancreas of the diabetic rats (Hajighaaliipur et al., 2015). The present result together with those of the previous study suggest that the ability of WF to improve cellular glucose uptake was associated with the upregulation of GLUT-2 and GLUT-4 expressions in the pancreas which was due to the restoration of β-cell mass (Bähr et al., 2012; Ong et al., 2011; Olson, 2012).

The impairment in renal function is one of the major complications associated with diabetes as caused by the inability of the kidney to clean the blood vessels due to the increased glucose and fatty acids accumulation in the blood (Zhang et al., 2014; Bernard et al., 1999). The diabetic kidney cells undergo apoptosis and widening of Bowman’s space as observed in previous studies and this severe pathological condition can be regulated by the anti-apoptotic nature of Bcl-2 protein which support the treatment of this severe pathological condition can be regulated by the anti-apoptotic nature of Bcl-2 protein which support the treatment of diabetes (Junhua et al., 2015; Arya et al., 2014). According to our renal immunohistochemical analysis, the Bcl-2 protein expression was found to be downregulated in the diabetic rats which was then restored with WF treatment. This effect of WF on the kidney proteins was probably due to the improved blood glucose caused by restoration of β-cell mass. It is the anti-apoptotic nature of Bcl-2 proteins which play vital role in β-cell physiology and apoptosis and hence cause increased survival of the cell (Gurzov and Eizirik, 2011). Interestingly according to (Kaedi et al., 2011) the olive leaf extract which contains high level of phenolic compounds have enhanced the expression level of Bcl-2 in diabetic rats. Research with polyphenolic compounds attenuated the diabetes-induced kidney damage by improving Bcl-2 expression. Hence, the presence of this apoptosis regulator within a cell is thought to be critical in the way the cell responds to apoptotic stimuli and determines cell death or survival (Gurzov and Eizirik, 2011; Zhang et al., 2004).

Our findings revealed the pivotal role of WF in protecting the pancreas, liver and kidney by restoring the antioxidant enzymes, pancreatic insulin, hepatic glycogen content and renal Bcl-2 with slight alteration in GLUT-2 and GLUT-4 protein expressions. Thus, we may suggest that the exhibiting antioxidant and anti-hyperglycemic properties of WF could be due to the combinatorial effect of the existing polyphenolic compounds.

5. Conclusion

The data from this study supports the traditional use of W. fruticosa flower in the management of diabetes. The outcomes of this study on STZ–nicotinamide induced diabetic rats indicates the protective nature of WF on the pancreas by increasing β-cell mass and the liver hepatic glycogen content. Altogether, the study result suggests the possible usage of WF in diabetes and its related complications, thus, requires further validation through in-depth study models at molecular level.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2015.08.057.

References


