

A Comparison of total phenolic content, free radical scavenging potential and anti-hyperglycemic condition from leaves extract of *Moringa stenopetala* and *Moringa oliefera*

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Abstract

Background: In Ethiopia *Moringa stenopetala* exists in the arid and semi-arid regions in the southern Rift valley and it is widely grown for its edible leaves and used traditionally for treating *Diabetes mellitus*.

Objective: The purpose of this study was to compare the total phenolic content, DPPH scavenging potential and anti-hyperglycemic effect in diabetic mice from leaves extracts of *M. stenopetala* and *M. oliefera*.

Method: Phenolic content in each *Moringa* sample was measured using the Folin Ciocalteu's reagent at an absorbance of 752 nm, whereas free radical scavenging potential was measured by *in vitro* by the DPPH assay. The anti-hyperglycemic effect of Fasting Blood Glucose level was assessed by administration of 300mg/kg of methanol water (80:20) extract and ethyl acetate fraction of the two *Moringa* leaves in Streptozotocin induced mice.

Results: The ethyl acetate fraction of *M. stenopetala* showed highest total phenolic content and strong free radical scavenging potential. The anti-hyperglycemic effect result showed that ethyl acetate fraction of *M. stenopetala* treatment resulted in significant reduction of fasting blood glucose level initially was 190 ± 3.4351 mg/dl and it reduced to 129 ± 1.8708 mg/dl and 109 ± 4.6368 mg/dl respectively after 14 and 21 days administration.

Conclusion: The results support the widely claimed use of *M. stenopetala* as potential antioxidant and anti-hyperglycemic effect to treat diabetes mellitus.

Key words: Total Phenolic Content, antioxidant, anti-hyperglycemic, *Moringa stenopetala* and *M. oliefera*

Introduction

The Family Moringaceae consists of a single genus with about 14 species indigenous to several countries. Among these *Moringa oliefera* is native to Northern India, Pakistan and Nepal. This tree also known as horseradish tree, drum stick tree, benzolive tree, *kelor*, *marango*, *mlonge*, *moonga*, *mulangay*, *nebeday* or *ben oil tree*. The other species *M. stenopetala* is native to South Ethiopia, Northern Kenya and Eastern Somalia. This tree is often named as African Moringa (Beyene 2005). In Ethiopia it exists in the arid and semi-arid regions in the Southern Rift valley. *M. stenopetala* widely grown for its edible leaves and used traditionally for treating diabetes mellitus and had already been explored (Makonnen 1997). It is known as *Haleko* and *Shelaqta* in Gamo and Konso (Mekonnen *et.al.* 1998).

All parts of the plant are utilized by human. It is rich in nutrients and has pharmacological properties as recognized by popular use and corroborated by the scientific community. Aqueous extract of *M. oliefera* leaves has significant hypoglycemic and anti-diabetic potential in a rat model (Dolly 2009). Dehydrated Drumstick leaves have positive impact on the lipid profile of hyperlipidemias (Vanisha 2009). It is also reported as anti-inflammatory, antimicrobial, antioxidant, anticancer, cardiovascular, anti-ulcer, hepatoprotective, diuretic, antiurolithiatic, and anthelmintic (Fozia 2012).

Different morphology of *M. oliefera* extracts such as leaf, pod and fruit has a potential in preventing Newcastle Disease virus, effective chemotherapy in renal carcinogenesis and strong reducing power and free radical scavenging capacity respectively (Didacus 2012; Paliwal 2011; Suaib 2011). *Moringa oliefera* is a significant source of β -carotene, ascorbic acid (Vit.C), α -tocopherol (vitamin E), iron (Ray-Yu Yang, 2006), potassium, calcium and protein (Suchada 2010). In *M. stenopetala* leaves, vitamins and minerals exist are present in significant concentration (Abuye *et.al.* 2003).

Increased intake of antioxidants may protect against chronic diseases including cancers, cardiovascular and cerebrovascular diseases (Prior 2002). A high content of γ -tocopherol has been found in practically the whole plant, ranging from 5.7 μ g/g to 27.8 μ g/g of dry mass and xanthins (neoxanthin 219mg/kg, violaxanthin 76.5mg/kg, zeaxanthin 19.4mg/kg) are also found (Paulo Michel Pinheiro 2008). Major polyphenols in *M. oliefera* leaf powder are quercetin glucosides, rutin, kaempferol glycosides and chlorogenic acids (Ndong 2007). Inhibition of α -amylase and α -glucosidases involved in the digestion and absorption of carbohydrates can decrease the postprandial increase of blood glucose level after a mixed carbohydrate diet. Flavonols and quercetin had the highest maltase, glucoamylase, and isomaltase inhibitory activities (Jo 2010).

Chlorogenic acid is an important intermediate in lignin biosynthesis. This compound, long known as an antioxidant, also slows the release of glucose into the bloodstream after a meal (Johnston et.al. 2003). It has also been demonstrated that flavonoids can act *per se* as insulin secretagogues or insulin mimetic (Brahmachari 2011). Thus, the present study was compare total phenolic content and free radical scavenging potential, blood glucose lowering effect of the hydroalcohol extract and ethyl acetate fraction of *M. stenopetala* and *M. oliefera* leaves.

Materials and methods

Collection of Plant material: Fresh leaves of *M. stenopetala* was collected from Melkasa Agricultural Research Center 150km east of Addis Ababa and *M. oliefera* was collected from Debrezeye Agricultural Research Center 47km east of Addis Ababa. Leave samples were identified and authenticated by a taxonomist in Ethiopia Agricultural Research Institute. The collected samples were washed well distilled water, dried under shade, crushed and powdered for extraction.

Standard drugs: Streptozotocin (STZ) (Sigma, USA) for induction of hyperglycemia and Glibenclamid (Glitisol, Cyprus) was used as a standard hypoglycemic drug.

Instrumentation: Rota vapor (Buchi R-500, Switzerland), lyophilizer (Labconco, USA), UV/Visible Spectrophotometer (Evolution 220, Thermo Scientific Germany) Sensocard glucometer (77 Electronic kit, Hungary) and GLAB active glucose test strip (77 Electronic kit, Hungary) were used in this study.

Chemicals and solvents: All the chemicals, reagents, and solvents used in the assay protocols were analytical grade methanol (Riedel-de Haen, Germany), petroleum ether (40°C-60°C, Fluka, Germany), ethyl acetate (Park Scientific, Nottingham UK), 1,1-diphenyl-2-picryl-hydrazyl (DPPH) (Sigma, USA), sodium hydroxide (Riedel-de Haen, Germany), ascorbic acid (Merck, Germany), Folin Ciocalteu's reagent (Sigma, USA) and sodium bicarbonate (Na_2CO_3) (Park Scientific, Nottingham UK).

Preparation of total hydroalcohol extracts of *M. stenopetala* and *M. oliefera*: Air dried powdered leaves of *M. Stenopetala* and *M. oliefera* (400g) were soaked in (80:20) methanol: water (Arnnok, 2012) for three days with occasional shaking. The extraction was repeated three times and filtered with Whatman No.1 filter paper and the filtrates combined. The combined filtrates were concentrated using a Rota Vapor. The aqueous residues were lyophilized and kept in desiccators for future use. The methanol-water

extract yielded 20.415 % (81.66g) for *M. stenopetala* and 18.753% (75.021g) for *M. oliefera*.

Solvent-solvent partitioning of the hydroalcoholic extracts: The procedure for solvent-solvent separation was adopted from (Samsam-sharjat 1992). Ten grams of the lyophilized hydroalcoholic extracts of the plant materials were dissolved in 100 ml of methanol and distilled and deionized water (80:20). The dissolved extracts were separated in a separator funnel with ethyl acetate (40-60°C), successively until the extracting solvents became colorless. In all cases of separation, 150 ml of solvents were used. After completing the separation process, the solvents were recovered by Rota Vapor. The separates were kept in the refrigerator for the next experiments.

Total Phenolic content determination: Phenolic content in each *Moringa* leaves powder sample was measured using the Folin Ciocalteu's reagent at an absorbance of 752 nm (Anderessa et al. 2013). The results are expressed as g Gallic Acid Equivalent. First, 2 mL Folin Ciocalteu's Reagent in 20 mL of water was prepared to form a stock solution of Folin Ciocalteu's reagent solution. Saturated solution of Na_2CO_3 (15%) was prepared by dissolving 7.5 g of Na_2CO_3 in 50 mL of water. About 100 mg of the powdered *M. stenopetala* and *M. oliefera* was placed in a 25 mL volumetric flask and 20 mL of 80% methanol in water (v/v) was added and sonicated for 25 minutes. After sonication, the flasks were filled to volume with water and 40 µL of the extract was transferred to a centrifuge tube with 900 µL of Folin Ciocalteu's reagent solution and set aside for five minutes. 400 µL of 15% Na_2CO_3 was added to the mixture, allowed to react for 45 minutes and measured at 752 nm. To develop the calibration curve, 6.0 mg of gallic acid was added into 25 mL of 60% methanol solution to provide the standard solutions. Six dilutions of concentrations ranging from 0.25 mg/mL to 0.0078 mg/mL and a blank were prepared.

$Y = 5.4135X - 0.007$ ($r^2 = 0.999$). 40 µL of each dilution was also used for the calibration curve.

DPPH (Diphenylpicrylhydrazine) assay: Free radical scavenging activity of methanol-water extract and the ethyl acetate fractionate of each sample was measured by the modified DPPH method (Manish 2011). DPPH scavenging activity was measured by the spectrophotometric method. Briefly: To 1 ml of various concentrations of extracts, 1 ml solution of DPPH (0.1 mM) was added. An equal amount of methanol and DPPH served as control. After 20 min of incubation in the dark, absorbance was recorded at 517 nm. The experiment was performed in triplicate and percentage inhibition calculated by the formula:

Scavenging % = $\frac{[(\text{Abs. of Blank} - \text{Abs. of Sample}) / \text{Abs. of Blank}] \times 100}{1}$

Pharmacological Evaluation Laboratory Animals: Laboratory bred Swiss albino mice, 6-8 weeks, weighing 25-30g were obtained from Ethiopian Health and Nutritional Research Institute, Addis Ababa. All animal procedures were in accordance with the standards set forth in guidelines for the care and use of experimental animals by committee for purpose of supervision of experiments on animals. All mice were fasted for 24 hour before diabetes was induced with STZ. The animals were allowed to acclimatize for 2 weeks before the experiment. The animals were housed in polypropylene cages inside a well-ventilated room. Each cage consists of not more than 5 rats. They were maintained under standard laboratory conditions and 12 hour light/dark cycle. They were fed a standard commercial pellet diet and water *ad libitum*. The diet consists of 71% carbohydrate, 18% protein, 7% fat, 4% salt mixture and adequate minerals and vitamins.

Induction of Diabetes Mellitus: Streptozotocin (STZ) was dissolved in cold 0.01 M citrate buffer, pH 4.5 and prepared freshly for immediate use. STZ injections were given intraperitoneally at 50mg/kg body weight. Blood glucose concentration was measured week from the day of STZ injection. The blood samples were collected from the tail vein once a week and blood glucose measured by glucose oxidase method.

Determination of anti-hyperglycemic effect of crude and fractionated extracts *M. stenopetala* and *M. oliefera* in STZ induced mice: STZ-induced diabetic mice fasted for 16 hours were selected and divided into six groups of five mice per group. Group 5 and 6 respectively served as positive and negative controls and received glibenclamide (0.66 mg/kg body weight) and distilled water (10 ml/kg body weight) daily for 21 days by oral by gavage. Group 1 to 4 were administered with 300 mg/kg body weight of hydroalcoholic and fractionated extracts of *M. stenopetala* and *M. oliefera* respectively, daily for 21 days by oral gavage. In all cases of pharmacologic evaluations, the extracts were dissolved in distilled. The blood samples were collected from the tail vein once a week at 0, 7, 14 and 21 day and the obtained blood glucose concentration determined by the glucose oxidase method.

Statistical analysis: The results are expressed as mean $\pm$ S.D. and all statistical difference between the treatment and the control were tested by one-way analysis of variance (ANOVA) followed by Duncan multiple test comparisons using SPSS version 16. The

difference in the mean values showing a p level of 0.05 or lower was considered to be statistically significant.

Results

Total phenolic content: The quantitative analysis of total phenolic content in crude extract and fraction of the leaves of *M. stenopetala* and *M. oliefera* revealed that the ethyl acetate fraction of *M. stenopetala* leaves containing highest amount of total phenolic content (283.8 μg GAE/ml) followed by crude methanol-water extract of *M. stenopetala* leaves (211.9 μg GAE/ml) whereas moderate amounts recorded in ethyl acetate fraction of *M. oliefera* leaves (93.56 μg GAE/ml) followed by methanol-water crude extract of *M. oliefera* leaves (58.7 μg GAE/ml) (Figure 1).

DPPH (Diphenylpicrylhydrazine) scavenging activities: By reduction of the stable radical DPPH to the yellow-colored diphenylpicrylhydrazine, the scavenging activity of the DPPH radical was tested. According to the experiment, *M. stenopetala* ethyl acetate fraction showed maximum activity of 96.22% followed by *M. stenopetala* methanol-water crude extract 94.14% whereas *M. oliefera* methanol-water crude extract showed 90.03% scavenging activity followed by *M. oliefera* ethyl acetate fraction 86.09% (Figure 2).

Ant-hyperglycemic effect of hydroalcoholic and fractionated leaf extracts of *M. stenopetala* and *M. oliefera* on fasting blood glucose level in STZ-induced mice: During the three weeks of extract treatment, fasting blood glucose level was measured once weekly. The results are summarized in Table 1 and Figure 3. Before induction of diabetes, there was no significant difference in fasting blood glucose (FBG) level among the treatment groups. FBG levels in the treatment groups showed no significant differences at 0 day of the administration ($p < 0.05$). After repeated oral administration of *M. stenopetala* ethyl acetate fraction and standard drug showed significant reduction in FBG level at the 7th day ($p < 0.05$). *M. stenopetala* methanol-water extract and *M. oliefera* ethyl acetate fraction showed similar reduction in FBG level at the 7th day ($p < 0.05$). *Moringa stenopetala* ethyl acetate fraction and *M. stenopetala* methanol-water crude extract showed significant reduction in FBG level at 14th and 21th day ($p < 0.05$). *Moringa oliefera* ethyl acetate fraction and *M. oliefera* methanol-water crude extract showed similar reduction in FBG level at 14th day ($p < 0.05$).

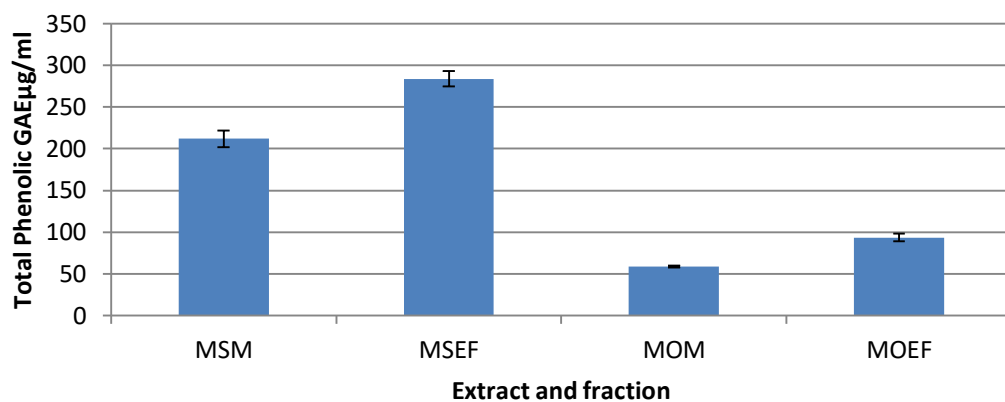


Figure 1: Total phenolic content GAE µg/ml

MSM-Moringa stenopetala Methanol-Water Extract,
MOM-Moringa oliefera Methanol-Water Extract,
SD-Standard Drug, DC-Diabetic Control

MSEF-Moringa stenopetala Ethyl Acetate Fraction
MOEF- Moringa oliefera Ethyl Acetate Fraction

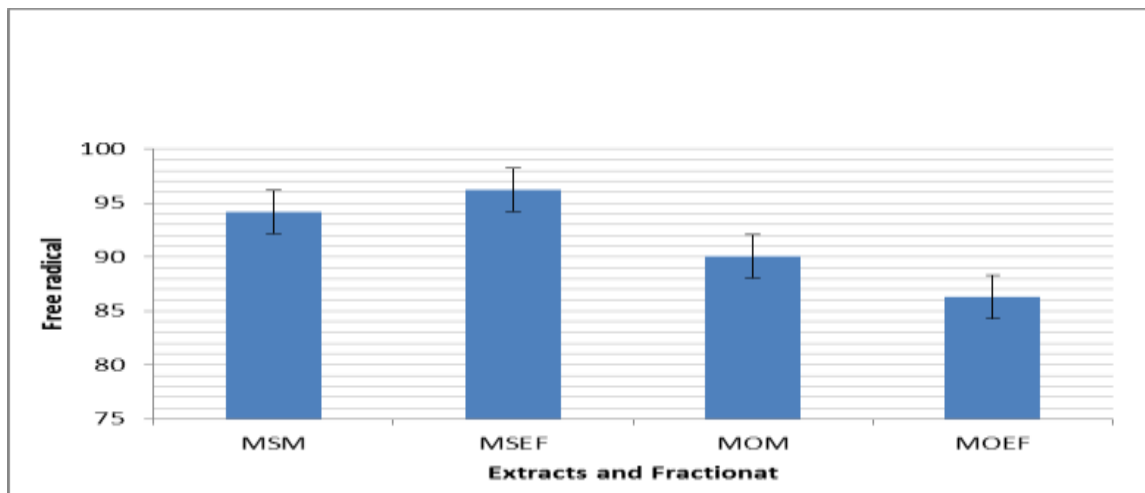


Figure 2: Free radical scavenging potential %

Table 1: Fasting Blood Glucose level (mg/dl)

Group	Treatment	0 day	7 day	14 day	21 day
I	MS M	198.6±6.3087	174.4±3.8471	147±5.1478**	117.2±10.7331***
II	MS F	190.6±3.4351	160.4±12.7397**	129±1.8708***	109±4.6368***
III	MO M	199.8±6.3796	182.6±8.1731	160.6±4.7223	129.8±10.3779***
IV	MO F	189.2±7.4297	173.4±6.2689	156.8±12.9884**	135±4.6368***
V	SD	200.8±6.8337	124.6±4.615***	135.8±8.6718***	115.6±7.4699***
VI	DC	183.8±5.4037	192.2±5.5857	217.2±4.037	253.2±1.304

= $p < 0.01$, *= $p < 0.001$ as compared to the control, Results are means $\pm$ SD of $n=5$

Dose of crude extracts and fraction = 300mg/kg, Dose of glibenclamide = 0.66mg/kg

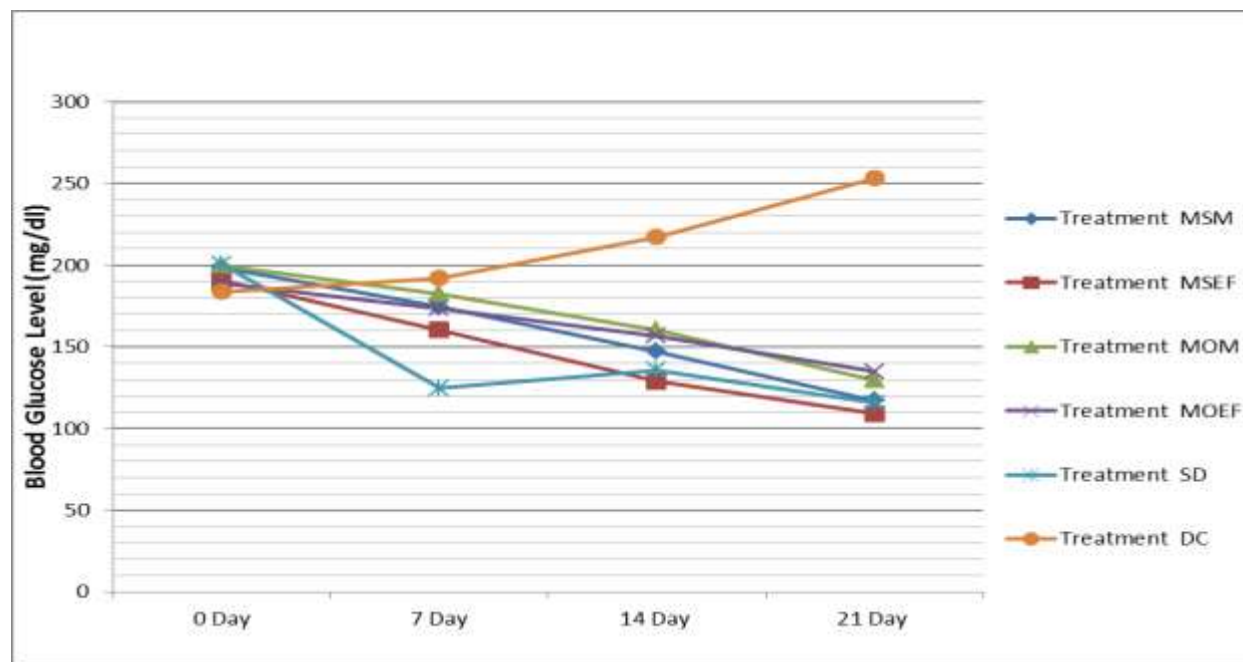


Figure 3: Antihyperglycemic effect produced by repeated dose oral administration of crude extracts and fraction of *M. stenopetala* and *M. oliefera*

MSM-Moringa stenopetala Methanol-Water Extract, MSEF-Moringa stenopetala Ethyl Acetate Fraction
 MOM-Moringa oliefera Methanol-Water Extract, MOEF- Moringa oliefera Ethyl Acetate Fraction
 SD-Standard Drug, DC-Diabetic Control

Discussion

Diabetes is a defect in the body's ability to convert glucose (sugar) to energy. It develops when the pancreas fails to produce sufficient quantities of insulin -Type 1 diabetes or the insulin produced is defective and cannot move glucose into the cells-Type 2 diabetes. This metabolic disorder is characterized by hyperglycemia (fasting blood glucose level greater than 126 mg/dl taken on at least two separate occasions) and disturbances of carbohydrate, protein and fat metabolisms. The effects of *Diabetes mellitus* include long term damage, dysfunction and failure of various organs (WHO 1999).

For hundreds of years, traditional healers have prescribed different parts of Moringa for treatment of skin diseases, respiratory illnesses, ear and dental infection, hypertension, diabetes, cancer treatment, and water purification, and they have promoted its use as a nutrient dense food source (Fozia 2012; Makonnen 1997; Suchada 2010). The previous works on hypoglycemic activity evaluation of the crude aqueous extract of *M. stenopetala* leaves (Makonnen 1997), hypoglycemic effect evaluation of the cured aqueous extract and n-butanol as well as chloroform

fractions of *M. stenopetala* leaves (Mussa 2008), effect of *M. oliefera* leaves aqueous extract therapy on hyperglycemic rats (Dolly *et al.* 2009) and anti-diabetic property of drumstick (*M. oliefera*) leaf tablets (Giridhari *et al.* 2011) confirmed the traditional claim. A comparative *in vivo* and *in vitro* analysis of anti-hyperglycemic effect and anti-oxidant activities for hydroalcoholic and fractionated extracts of *M. stenopetala* and *M. oliefera* leaves in the present study.

In this study, the anti-hyperglycemic effect was carried out in Streptozotocin induced diabetic mice. Streptozotocin (STZ, N-nitro derivative of glucosamine) is a naturally occurring, broad-spectrum antibiotic and cytotoxic chemical that is particularly toxic to the pancreatic, insulin producing beta cells in mammals. It has been widely used to induce DM in experimental animal models, allowing the investigation of hypoglycemic agents in treatment of diabetes. Streptozotocin injection consistently produces symptoms of DM including hyperglycemia, decreased insulin levels, polyuria, and weight loss. The dose of STZ required for inducing diabetes depends on the animal species, route of administration and nutritional status. In mice, doses vary between 50-

60 mg/kg by intraperitoneal route; clinical symptoms of diabetes are clearly seen within 2-4 days. (Abdu *et.al.* 2009).

The diabetic mice used in the present study were treated for 21 days with methanol-water crude extract and ethyl acetate fraction of *M. stenopetala* and *M. oliefera* leaves. The result showed that the ethyl acetate fraction of *M. stenopetala* administration produced significant hypoglycemia effect starting from the 14th day as compared with the standard drug. Hydroalcoholic crude extract of *M. stenopetala* and the standard drug caused similar significant reduction in blood glucose level at 21th day of administration. Hydroalcoholic and fractionated extracts of *M. oliefera* did not cause any significant change in blood sugar level as compared to the control.

Total phenolic content of the hydroalcoholic and fractionated extracts were performed using Folin-Ciocalteu's method that indicated *M. stenopetala* ethyl acetate fraction containing the highest amount followed by methanol-water crude extract. The exact antidiabetic mechanisms of actions of *Moringa* are unknown. However, it may be speculated that hydroalcoholic and fractionated extracts of *Moringa* leaf acts in a similar way as the standard antidiabetic drugs like glibenclamide. Glibenclamide is a second generation sulfonylurea derivative which acts by stimulating release of insulin from pancreatic-cells and by increasing sensitivity of peripheral tissue to insulin. Glibenclamide is not effective in pancreas ectomized animals or patient with no endogenous insulin (Habibuddin *et.al.* 2008).

Plants can have properties similar to known drugs like glibenclamide, which reduce blood sugar level in normoglycemic and hyperglycemic animals. Some other plants do not reduce sugar level in normal states of the animal like metformin (Stumvoll *et.al.* 1995). As the extracts of *Moringa* were observed to reduce sugar level in Streptozotocin induced diabetic mice similar to glibenclamide, the extracts perhaps have a mechanism of action similar to insulin secretagogues like glibenclamide.

In *in-vitro* antioxidant test, hydroalcoholic and fractionated extracts showed the presence of antioxidant components as visualized by DPPH reagent. Diabetogenic effect of Streptozotocin is due to generation of free radicals that can affect the normal functioning of β -cells of pancreas (Szudelske 2001). Hence, there might be relationship between the existing antioxidant and antihyperglycemic effect of extracts. As the antioxidants can normalize the β -cells of the pancreas and might renew cells to secrete efficient amount of insulin. However, this must be supported by further studies.

Conclusion

Based on the observation, the ethyl acetate fraction of *M. stenopetala* leaves maintain the fasting blood glucose level in Streptozotocin induced mice. The results support the potential antioxidant activity of *M. stenopetala* leaves followed by *M. oliefera* which adds one more positive attribute to its known pharmacological properties and hence its use in traditional system of medicine.

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