Research Article

Development and Characterization of A Novel Nanosuspension based Drug Delivery System of *Actinodaphne madraspatana* Bedd Plant Extract for Improving Oral Bioavailability and Prevention of Streptozotocin Induced Diabetes in Swiss Albino Rats

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ABSTRACT

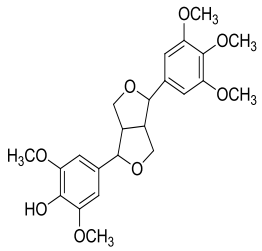
The aim of the present study was to formulate and evaluate the anti-diabetic effects of the ethanol extract of Actinodaphne madraspatana (AME) and its nanosuspension (AMN) on streptozotocin induced diabetes in Swiss Albino rats. The AMN was prepared by the high pressure homogenizer method and was characterized by particle size measurement, zeta potential analysis, morphology study, permeability study, and in-vivo anti-diabetic activity. The permeability study of AMN was evaluated using egg membrane and AME released was evaluated on in-vitro α-amylase inhibition method. Anti-diabetic activity of AME and AMN were studied on streptozotocin induced diabetic rats. The AMN showed the mean particle size and polydispersity index of 234.83±5.75 and 0.520±0.01nm correspondingly with zeta potential value of -2.76mV. The morphology of AMN showed particle size in the ranges of 70.1 to 135nm. The permeability study of the AMN showed the inhibition of alpha amylase activity with value of 69.67% at six hours compared to ethanol extract (54.42%). An oral dose of AME at 200 and 400mg/kg and AMN at 100 and 200mg/kg showed a significant anti-diabetic activity. Moreover, the results also indicated that an anti-diabetic effect of 100mg/kg of AMN was effectively better than 200mg/kg of AME. From the above studies, conclusion can be drawn that AME and AMN possess anti-diabetic activity at varying doses against streptozotocin induced diabetes in rats.

Keywords: Actinodaphne madraspatana, nanosuspension, bioavailability, in-vitro and in-vivo anti-diabetic activity.

INTRODUCTION

Nanotechnology can be an interesting field of research pertaining to formulation of nanosuspension in addition to their potential value in clinical drugs. The fast improvement of nanotechnology has opened the possibility of managing and influencing structures in the molecular levels as well as led to the development of fresh area architectures and components. Conventional biomedical applications integrate the usage of nanotechnology at an entire spectrum of areas. One of them, tissue engineering, biosensors, nanocomposites and intelligent systems are utilized in controlled release systems and implant design. Many nano oriented methods are now being planned to optimize the technological aspects of medications. The usage of these methods has drastically increased dissolution rates *in-vitro*, and bioavailability *in-vivo* of several drugs[1].

A.madraspatana is a medicinal herb belonging to the genus *Actinodaphne*, family of Lauraceae. It is commonly known as 'Ray Laurel' in English, 'Putta Thali' in Tamil, 'Irolimarom', 'Kovangutti' in Telugu, 'Mungali' in Malayalam[2]. It is a medium-sized evergreen Shrub, and tree. It is widely distributed on the Rock Hill slopes at higher elevations. They found in the places of Vishakhapatnam District, Aruku Valley, Talakona, Dharmagiri, Tirupathi[3-5]. The Leaves of the plant are used traditionally to cure diabetes, mania, wounds, and fickle minded behavior[6,7]. The plant contains various types of phenolic compounds *viz*. lignans such as (±)-syringaresinol (a), (±)-de-4'-O-methylmangnolin (b), and lyoniresinol (c)[8], flavonoids such as 5,7,8-trimethoxy flavones (d)[9], and quercetin-3-rhamnoside (e)[10]. As these types of phytoconstituents belong to the group of constituents with weak water solubility, their ingestion on oral administration might be limited. That's why; an effort has been made to increase their dissolution rates as well as bioavailability for AME by formulating its AMN.



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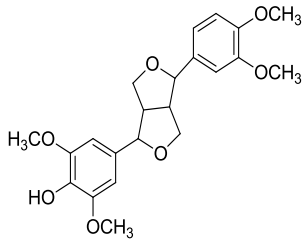

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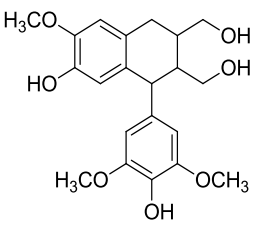

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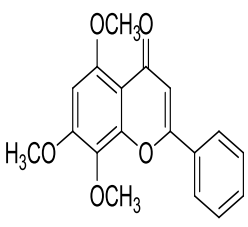

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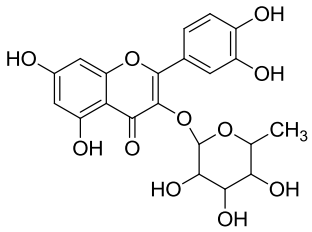

(e)

MATERIALS AND METHODS

Collection and authentication of plant material

The plant leaves of *A.madraspatana* were collected from Talakona forest, Tirupathi District and were authenticated by Dr. K. Madavachetty, S. V. University, Tirupati, Andhra Pradesh. A voucher specimen (ACD) has been kept in the Herbarium.

Drugs and chemicals

Streptozotocin (STZ) was purchased from sigma Aldrich Chemicals, Germany. Glucose, Citric acid and sodium citrate were purchased from M/S Hi media Ltd, Bombay. Glibenclamide was obtained as gift samples from Accent Pharma, Pondicherry, India. All other chemicals and reagents used in the study were of analytical grade.

Extraction procedure

About 100 gms of leaves pulverized powder of *A.madraspatana* was extracted with 250 ml of ethanol in a Soxhlet extractor for 2 to 3 days at a temperature between 60-65°C. The extract was filtered in hot condition, concentrated in vacuum under reduced pressure, and dried in desiccators.

Preparation of AMN

The AMN was prepared by high pressure homogenization method using different concentration of ethanol extract, polymer, binder, surfactant, and isopropyl alcohol. 2 gms of ethanol extract and isopropyl alcohol (50 ml) were taken in a beaker. At that same time, antisolvent was prepared taking 50ml water, 5 gms of poloxomer, 5 gms of PVP K30, and 0.8 ml of tween 80 in another one beaker. After the ethanol extract solution and anti-solvent were prepared, ethanol extract solution was slowly added drop wise with the syringe in to anti-solvent with constant stirring under magnetic stirrer with speed of 4000rpm until the ethanol extract solution was completely added in to anti-solvent and isopropyl alcohol completely evaporate from the mixture[11].

Characterization of AMN

Particle size and zeta potential analysis

The particle size distribution and zeta potential of AMN was measured by Malvern Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). The particle size distribution was expressed as mean diameter (dnm) and polydispersity index. All measurements were performed in triplicate.

Particle morphology

The particle morphology of AMN was observed using a scanning electron microscope (SEM; S-3700 N, Hitachi, Japan) and images were collected at voltage of 15 kV using a backscattered electron detector. Analysis was performed at 25°C ± 2°C.

Permeation study

The permeation study of prepared AMN was carried out using egg cell membrane by beaker method. The diffusion of the AMN through the egg membrane was analyzed in a specially designed diffusion tube with internal diameter of 2.25 cm. The egg cell was kept in concentrated Hydrochloric acid for 2 hrs and egg membrane was separated. The separated egg membrane was soaked in distilled water, 0.2% Sulphuric acid, 0.3% Sodium sulphate, and alcohol for 24 hrs. The required length of egg membrane was cut and attached to the bottom of the diffusion tube with glue. Weighed amount of AME (200mg) and AMN equivalent to specify the quantity of ethanol extract was added to the diffusion tube and tube was filled with 15 ml of Isopropyl alcohol:Phosphate buffer (20:80). This assembly (donor compartment) was placed in 500 ml beaker (receptor compartment) containing 200 ml of isopropyl alcohol:phosphate buffer pH 6.8 (20:80), and beaker was placed on thermostatically controlled magnetic stirrer set at 37±2 °C. The contents of the beaker were stirred with help of Teflon coated bead at 300 rpm for 6 hrs. The 5 ml samples were withdrawn at 1, 2, 3, 4, 5, and 6 hrs time intervals and replace with same amount of fresh Isopropyl alcohol:Phosphate buffer to maintain the sink conditions. The solutions were suitably diluted and the diluted solutions were tested for inhibition of α-amylase activity[12, 13].

In-vitro inhibition of α-amylase activity

In-vitro inhibition of α-amylase activity of AMN was studied by according to the method of Sangeetha with slight modification[14]. In brief, 200 µL of the sample solution from the receptor compartment was allowed to react with 400 µL of 1% α-amylase enzyme, and 200 µL of phosphate buffer (pH 6.9). After 20 minutes incubation, 200 µL of 1% starch solution was added. The same was performed for the controls where 400 µL of the enzyme was replaced by phosphate buffer. After 5 minutes incubation, 1.0 mL of dinitrosalicylic acid reagent was added to both control and test. They were kept in boiling water bath for 5 min. The absorbance was recorded at 540 nm against reagent blank using UV-Visible spectrophotometer and the percentage inhibition of α-amylase enzyme was calculated using the formula

$$\text{Inhibition (\%)} = \frac{\text{Control - Test}}{\text{Control}} \times 100$$

Experimental animals

The *in-vivo* anti-diabetic of activity of AMN was carried on Swiss Albino rats of both sexes, weighing between 180 to 250 gms. [15] The experiment was approved by Institutional Animal Ethical Committee and as per CPCSEA guidelines (Register number: 1558/PO/a/11/CPCSEA) under CPCSEA, India. The animals were housed in polypropylene cages in a cross ventilated animal house at controlled room temperature 22±10 °C with relative humidity of 60-70%. They were kept under standard conditions of 12:12 hours of light and dark cycle. The animals were fed with standard pellet diet (Kamadenu Enterprises, Bangalore) and water *ad libitum*. The animals were acclimatized to laboratory condition for seven days before commencement of the experiment.

Experimental induction of diabetes

The animals were fasted in minimum 12 hrs prior to the induction of diabetes. The rats were injected intraperitoneally with single doses of streptozotocin (60mg/kg) dissolved in freshly prepared 0.1M citrate buffer (pH 4.3). The rats were received a 2 ml of 5%w/v of glucose solution orally using orogastric tube to prevent mortality for 24 hrs due to initial hypoglycemia induced by streptozotocin. After 3 days of streptozotocin injection, fasting blood glucose level was estimated by using glucose oxidase-peroxidase reactive strips (One Touch, Life Scan India, Mumbai, India). Rats exhibiting fasting blood sugar level more than 250 mg/dl were considered as diabetic and taken to further study[16].

Evaluation of *in-vivo* anti-diabetic activity

The rats were randomized into seven groups (I-VII) consisting of six animals in each group after the induction of streptozotocin diabetes.[16] Group I served as normal control and rats were received orally with citrate buffer. Group II served as a diabetic control, and rats were received orally with citrate buffer. Group III rats were received orally with glibenclamide (4mg/kg/day). Group IV rats were received orally with AME (200 mg/kg/day). Group V rats were received orally with AME (400mg/kg/day). Group VI rats were received orally with AMN (100mg/kg/day). Group VII rats were received orally with AMN (200mg/kg/day). After administration, the blood was collected from the retro-orbital plexus of each rat under mild anesthesia on 0, 7, 14 and 21st day and serum glucose was estimated by using glucose oxidase-peroxidase reactive strips (One Touch, Life Scan India, Mumbai, India).

Histopathological study

At the end of study, the animals were sacrificed under anesthesia and pancreas was removed, cleaned, washed with ice-cold normal saline, and fixed overnight in 10% formalin solution. Pancreas sections were made by microtome, dehydrated in graduated ethanol (50-100%), cleared in xylene and embedded in paraffin. The pancreas sections (4-5 µm) were stained with haematoxylin and eosin dye and examined with a photomicroscope.

Statistical analysis

The data are expressed as the mean ±SEM. The statistical analysis was carried out using one way analysis of variance (ANOVA) followed by Dunnett's test for the multiple comparisons using prism Graph pad version 5.0. The p values less than 0.001 were considered statistically significant.

RESULTS

Characterization of AMN

Mean particle size and particle morphology

The mean particle size and polydispersity index for AMN were found to be 234.83±5.75nm and 0.520±0.01nm correspondingly [Figure 1].

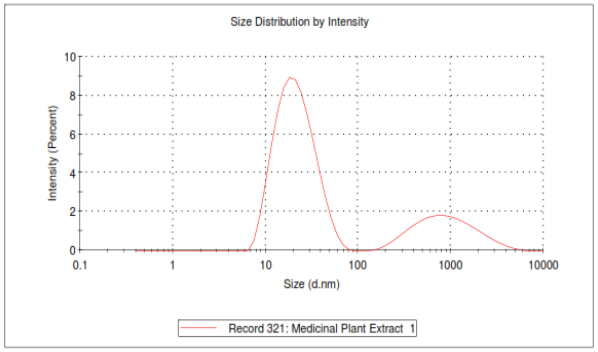

Figure 1: Typical laser scattering spectroscopy of AMN

Scanning electron microscopy had been done to analyze the surface morphology of nanoparticles from AMN [Figure 2].

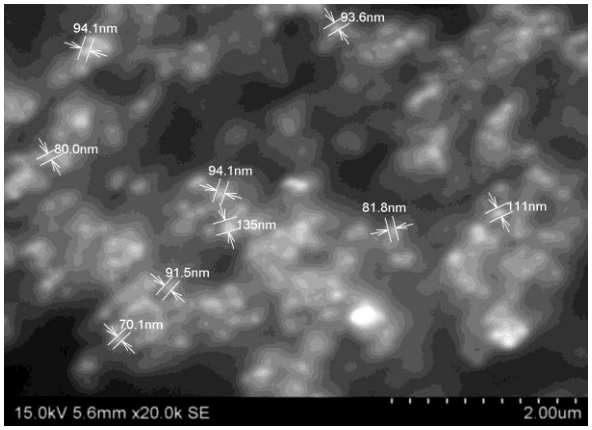

Figure 2: SEM images of AMN of ethanol extract

Zeta potential

Zeta potential evaluation had been carried out to analyze the surface properties of AMN as well as essential parameter for the prediction of stability of AMN. Zeta potential for the AMN was found to be -2.76 mV [Figure 3].

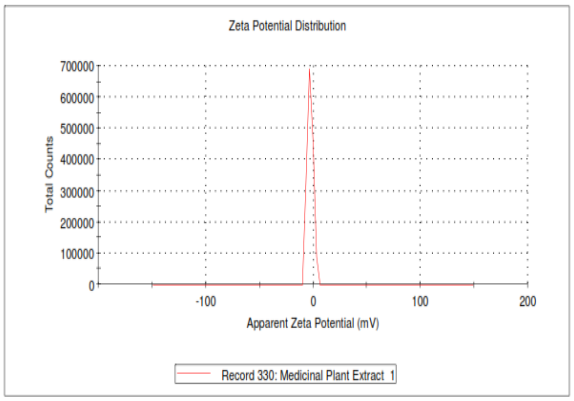

Figure 3: Zeta potential of AMN of ethanol extract

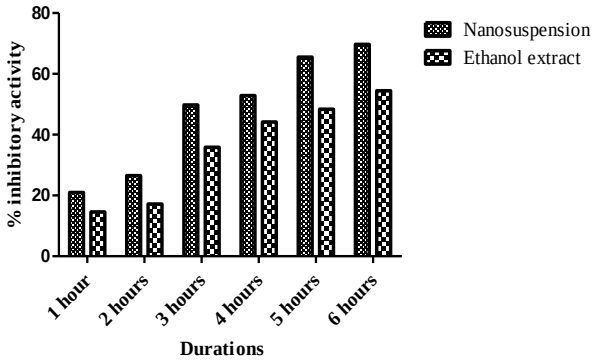

Figure 4: % inhibitory activity of AMN and AME on α-amylase

Permeation study and inhibition of α-amylase activity

The permeation study of prepared AMN was carried out using egg cell membrane by beaker method and nanosuspension released into the receptor compartment solution studied by inhibition of α-amylase assay. The inhibition activity of the released nanosuspension (equivalent to 200mg/5ml of AME) was compared with that of the inhibition activity of ethanol extract. The AMN inhibited 20.97%, 26.52%, 49.74%, 52.86%, 65.51%, and 69.67% of α-amylase at 1, 2, 3, 4, 5, and 6 hrs respectively. The AME inhibited 14.56%, 17.16%, 35.86%, 44.19%, 48.35%, and 54.42% of α-amylase at 1, 2, 3, 4, 5, and 6 hrs respectively as shown in Figure 4.

Evaluation of *in-vivo* anti-diabetic activity

The Table I reveals the blood glucose levels of normal, diabetic control, ethanol extract (200 and 400mg/kg), nanosuspension (100 and 200mg/kg) and standard drug glibenclamide (4mg/kg) treated rats. There was a significant (P<0.001) increase of glucose level in streptozotocin induced diabetic rats (Group II) as compared to normal control rats (Group I). Administration of ethanol extract (Groups III & IV) and nanosuspension (Group V& VI) of leaves of *A.madraspatana* and glibenclamide (Group VII) had a tendency to bring the blood glucose level significantly (P<0.001) towards the normal control rats.

Table 1: Anti-diabetic activity of AME and AMN on blood glucose level in streptozotocin induced diabetic rats

Groups	Blood glucose level in mg/dl			
	0 day	7th day	14th day	21st day
I	78.17±1.58	8.17±1.621	80.67±1.84	82.67±1.52
II	[a]304.67±1.98*	[a]303.17±2.73*	[a]307.83±2.26*	[a]301.33±2.87*
III	[b]300.50±2.17*	[b]269.50±2.17*	[b]201.33±2.14*	[b]167.50±1.61*
IV	[b]305.17±2.46*	[b]255.67±1.89*	[b]189.33±2.38*	[b]131.67±1.801*
V	[b]300.00±3.33*	[b]238.83±1.078*	[b]172.83±1.35*	[b]111.83±2.30*
VI	[b]303.00±2.463*	[b]226.33±1.61*	[b]154.50±1.23*	[b]100.33±1.28*
VII	[b]300.33±2.79*	[b]211.83±1.14*	[b]125.83±2.21*	[b]91.00±1.57*

All values are expressed as mean ± SEM for six animals; [a]p<0.001 compared to normal control; [b]p <0.001 compared to diabetic control; *statistically significant; and ns-non significant

Histopathological study of pancreas

Pancreases of group I (received vehicle alone) showed the normal architecture of pancreatic tissue [Figure 5(A)]. Pancreases of group II (received to streptozotocin) showed the extensive structural damages, congestion, and necrotic changes [Figure 5 (B)]. In groups IV and V, rats (received to AME) had improved histopathological changes as compared to the streptozotocin induced diabetic rats without treatment [Figure 5(D)]. In addition, groups VI and VII, rats (received to AMN) similarly ameliorated the histopathological damages [Figure 4(E)] from the streptozotocin induced diabetic in rats with nearly the same effectiveness as found with AME. Similar regenerative changes [Figure 5 (C)] were also found in pancreas of glibenclamide treated group.

DISCUSSION

Nanosuspensions are submicron colloidal dispersions of pure particles of drugs that are stabilized with surfactant and can be utilized for drugs which are poorly water-soluble. By reducing the particle size for the solid form of drugs, the dissolution rate is improved, thus dealing the many problems associated with poor oral bioavailability. The solid-state form of drugs provides approaches to problem of chemical stability, and the small particle size confers the improved physical stability with regards to sedimentation[17].

The polydispersity index is actually a measure of particle size distribution. Polydispersity index various from 0.0 to 1.0. The particle size was found to reduce with increase in

sonication. Particle size distribution is one of the very important physical characteristics of a nanosuspension used for oral drug delivery system and affects the bioavailability and biological activity. The small particle size in nanosuspension allows them to uniformly penetrate on the intestine. The small particle size stabilizes them against gravitational separation, and flocculation as well as small particle size increases the drug release, and absorption of the drug. In this way, nanosuspension improves the bioavailability of various oral formulations[18].

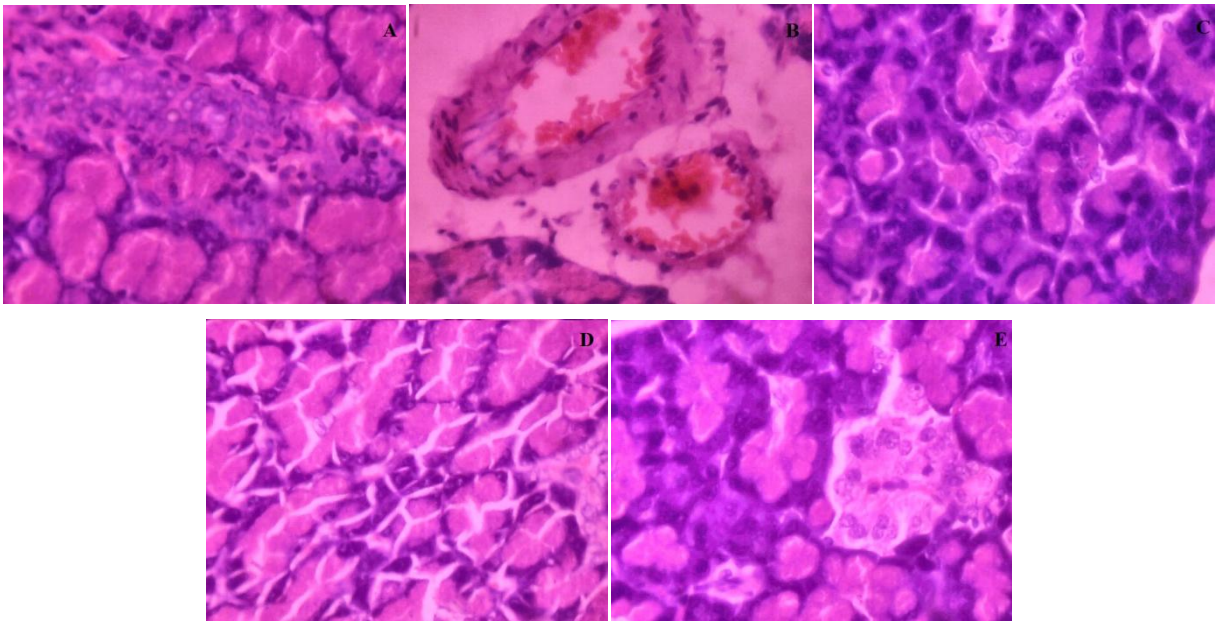

Figure 5: Histopathological pictures of pancreas for different animal groups. Normal control (A); Diabetic control (B); Glibenclamide treated (C); AME (400mg/kg) treated (D); AMN (200mg/kg) treated (E)

The Figures 2 revealed an irregular, asymmetrical morphology, and solid dense structure. The size of the particles resulted between 70.1 nm to 135 nm. Thus, poloxamer produced much better surface characteristics.

The zeta potential is a relative quantity of repulsive electrical potential difference at the suspension interface and the electro neutral area for the solution. It's determined by measuring the ionic migration of particles dispersed in a charged field. Whenever zeta potential is actually high, the repulsive forces exceed the attracting vander Waals forces, therefore particles are dispersed, and the system is deflocculated, while whenever zeta potential is low, the attractive forces exceed the repulsive forces, and the particles aggregate, leading to flocculation[19].

Poloxamer is a non-ionic wetting agent can be used as stabilizer that provides steric stabilization. So, negative zeta potential is actually attributed to AMN. Generally, zeta potential value of ±20 mV is adequate for the stability of nanosuspension stabilized by poloxamer that indicating the nanosuspension wouldn't suffer from instability issues[20].

Release and transport of AMN through an egg membrane is a helpful signal of how much amount of the extract is readily available for the absorption. Additionally, it can provide helpful information about how well the absorption process transmits the AMN through the intestine, so might supply helpful information about how well the AMN is absorbed through human intestine.

We assessed the *in-vitro* anti-diabetic activity for the studied nanosuspension released into the receptor compartment solution by inhibition of α-amylase assay. α-Amylase can be a key enzyme which catalyses the hydrolysis of 1,4-glucosidic linkages of starch into glucose, that can be easily available for the intestinal absorption. Destruction for this dietary starch causes enhanced postprandial hyperglycemia. Inhibition of α-amylase into the intestinal tract decreases the rate of digestion of starch and might be effective to managing the diabetes. The polyphenol compounds are responsible for the pharmacological effect of the extract, and have amphiphilic properties which facilitate their anti-diabetic properties. The presence of polyphenol compounds which could inhibit the α-amylase in the receptor solutions.

α-Amylase as a useful marker to identify the release of the AME as well as AMN and to evaluate the release in terms of anti-diabetic activity in the receptor solution. The *in-vitro* results of inhibitory activity of the AMN released through the artificial membrane towards the α-amylase confirms that sufficient polyphenolic compounds were released. Hence, this product could result in good penetration when taken to orally.

The *in-vivo* anti-diabetic activity of AME, and AMN were studied in streptozotocin induced diabetic rats. Diabetes was induced in 36 rats and they were divided into six groups such as group II, III, IV, V, VI, and VII. The group I acted as a normal control and group II acted as diabetic control. Group III treated with standard drug, glibenclamide at dose of 4mg/kg for 21 days. Group IV

and V were treated with AME at doses of 200mg/kg and 400 mg/kg respectively for 21 days. Group VI and VII were treated with AMN at doses of 100mg/kg and 200 mg/kg respectively for 21 days. The present study revealed that the administration of single dose (60mg/kg) of streptozotocin to rats significantly ($p<0.001$) increased the blood glucose level when compared to normal control rats. A dose dependent significant reduction ($p<0.001$) of glucose level was found in streptozotocin induced diabetic rats received with AME (200mg/kg and 400 mg/kg) and its AMN (100mg/kg and 200 mg/kg) of *A.madraspatana* when compared to diabetic control.

The variations in blood glucose level in normal and experimental groups were recorded. The blood glucose level in normal control rats was 76.5, 77.8, 81.6, and 82.3 on 0, 7, 14, and 21 days, respectively. In diabetic control rats, the blood glucose level was 304.67, 303.17, 307.83, and 301.33 on 0, 7, 14, and 21 days, respectively. The blood glucose level in AME treated rats was 300.50, 269.50, 201.33, and 167.50 at dose of 200mg/kg and 305.17, 255.67, 189.33, and 131.67 at dose of 400mg/kg on 0, 7, 14, and 21 days, respectively. The blood glucose level in AMN treated rats was 300.00, 238.83, 172.83, and 111.83 at dose of 100mg/kg and 303.00, 226.33, 154.50, and 100.33 at dose of 200mg/kg on 0, 7, 14, and 21 days, respectively. The anti-diabetic drug, glibenclamide treated rats showed the blood glucose level of 300.33, 211.83, 125.83, and 91.00 at doses of 4mg/kg on 0, 7, 14, and 21 days, respectively. During 21 days study, the AME and AMN produced a sustained significant ($P<0.001$) reduction of blood glucose levels in diabetic rats compared to diabetic control rats.

Diabetes induced by streptozotocin is the most commonly used animal model for the screening of anti-diabetic activity of extracts, and drugs. Streptozotocin is a selective β-cytotoxin, induces chemical diabetes in animals by damaging the insulin secreting β-cells of the Islets Langerhans of the pancreas, and the animals became permanently diabetic. It is well known that the anti-diabetic drug, glibenclamide directly act β-cells of the pancreas, and stimulating the β-cells of the Islets of Langerhans of pancreas to release more insulin. In this study, a significant increase in the blood glucose level was observed after administration of single dose of streptozotocin when compared to normal control rats received with vehicle alone that indicating the streptozotocin damages the insulin secreting β-cells of the Islets Langerhans of the pancreas[21,22,23]. However, the treatment with AME and AMN in streptozotocin induced diabetes rats decreased the serum glucose level compared to diabetic control that indicating the ethanol extract increases the secretion of insulin from damaged beta cells of the pancreas. The anti-diabetic effect of AMN at two doses was more than that of the AME at two doses. The reason for the elevated anti-diabetic activity of AMN may due to increase in bioavailability of the AME.

Further the anti-diabetic activity of AME and AMN were confirmed by histopathological study of pancreas. The histopathological study of pancreas showed the administration of streptozotocin to rats cause the destruction of β-cell, that were regenerated to rats treated with AME and AMN at both doses when compared to diabetic and normal control rats. The similar effects were observed to rats treated with glibenclamide.

CONCLUSION

The present study clearly shows that AMN from ethanol extract of leaves of *A.madraspatana* can be formulated successfully by high pressure homogenization method using PVP K30 as a carrier, and poloxomer as a stabilizer. In this process, the particle size of ethanol extract can be obtained in the nano size ranges, by adjusting the operations parameters such as stabilizer concentration, and the stirring rate. Permeation study in pH 6.8 phosphate buffer shows that AMN gives 23% alpha amylase inhibition within 30 minutes. In addition, we found that an oral dose of AMN that is two times less than the oral dose of AME could exhibit a similar anti-diabetic effect. Thus, we suggest that the AMN system can be applied to overcome other water poorly soluble herbal medicines and furthermore to decrease the treatment dosage.

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