



**DELIVERY OF AQUEOUS EXTRACT OF MISTLETOE AS SOLID LIPID NANOPARTICLES IMPROVES THE TIME TO ONSET OF ANTI-DIABETIC ACTION AND THE OVERALL SURVIVAL RATE**

**Nzekwe IT<sup>a</sup>, Ofoekii JN<sup>a</sup>, Agubata CO<sup>b</sup>, Mbah CC<sup>b</sup>, Okoye IE<sup>a</sup>**

<sup>a</sup>Department of Pharmaceutics and Pharmaceutical Technology, Nnamdi Azikiwe University, Awka, Nigeria

<sup>b</sup>Department of Pharmaceutical Technology & Industrial Pharmacy, University of Nigeria, Nsukka, Nigeria

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**ABSTRACT**

Mistletoe (*Loranthus micranthus*) is documented as a traditional treatment for diabetes. The present study was carried out to evaluate the anti-diabetic properties of solid lipid nanoparticles loaded with aqueous extracts of the leaves and stem of mistletoe on alloxan-induced diabetic mice. The plant parts were harvested and dried, then extracted by infusion. Phytochemical tests were conducted on the extract followed by formulation into solid lipid nanoparticles by the method of hot pre-emulsion and subsequently homogenization and dilution in ice-cold water. The extract-loaded nanoparticles were evaluated for size, thermal stability, drug loading, and *in vivo* hypoglycaemic activity (with determination of test animal percentage survival). The phytochemical tests revealed the presence of alkaloids, tannins, flavonoids and glycosides. The solid-lipid nanoparticles (SLN) loaded with aqueous mistletoe exhibited significant blood glucose lowering ( $p < 0.01$ ) after 24 h and this activity was superior to that obtained with metformin (500 mg/70 kg) or the extract (16 mg/70 kg or 500 mg/70 kg). The blood glucose level returned to normal by the 7<sup>th</sup> day following the induction of diabetes and administration of the extract at 16 mg/70kg. Formulation of the aqueous extract of mistletoe as solid lipid nanoparticles improves the time to onset of action and also percentage survival rate.

**KEYWORDS:** *Loranthus micranthus*, diabetes, nanoparticles, lipid, delivery, mistletoe

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**INTRODUCTION**

Diabetes is a multifactorial disease characterized by hyperglycemia and glucosuria [1], as well as polyphagia, polyuria and weight loss. It has been associated with increase in the concentration of reactive oxygen (ROS), which seems to play a role modified to different extents by environmental factors [2, 3]. Hyperglycaemia results from excessive production of glucose, or reduced utilization of glucose, but it also associated with abnormalities in the endocrine regulation [4].

The most prevalent form of medication is the use of hypoglycemic agents and/or insulin therapy. Due to the increasing importance of traditional medicines due to pharmaco-economic implications [5], especially with chronic disease management, remedies traditionally used in diabetes management in folk medicine are receiving increasing research attention [6]. These medicines also have low risk potential [7], having been safely used as food or medical remedy for ages. Mistletoes are semi-parasitic plants of family *Loranthaceae* or *Santalaceae*. African mistletoes belong to the former while European mistletoes

**\*Corresponding author:** [it.nzekwe@unizik.edu.ng](mailto:it.nzekwe@unizik.edu.ng); +2348066555469  
[ajopred.com](http://ajopred.com)

belong to the latter. However, all mistletoes have been highly useful in ethnomedicine for the treatment of a broad spectrum of diseases, including epilepsy, headache, infertility, hypertension, diabetes, hypertension, and hepatitis [8, 9]. The antidiabetic activity of mistletoe has been shown to be dependent on the plant where it was grown [10] and on the harvest season [11]. The variation in activity with host plant has been shown to be due to variations in chemical constituents [9, 12-14]. Mistletoe has also been shown to relieve polydipsia, hyperphagia and weight loss without affecting blood glucose level or insulin concentrations [15], and to evoke a stimulation of insulin secretion from clonal pancreatic  $\beta$  cells [16]. Formulation approaches apply nanotechnology in the preparation of nanosized carriers containing the active pharmaceutical ingredient (API) [17], with the aim of improving their *in vivo* performance. Nanodelivery can be used to increase the safety margins of medicines by permitting use at low doses, or by enabling targeting. Over the years, solid lipid nanoparticles, have gained increasing attention due to low toxicity [18, 19], ease of fabrication and scalability [19, 20]. Unlike liposomes, they are very stable, being composed of lipid which is in the solid phase at room temperature. They are capable of penetrating the layered anatomical barriers encountered in the peroral delivery of systemic-acting drugs, and can help achieve high drug levels where gastric or intestinal degradation may lead to low bioavailability [21, 22]. In the application of nanocarriers in clinical medicine, however, often encountered limitations include high cost of polymers and safety concerns [23].

In this study, an aqueous extract of Eastern Nigeria mistletoe (*L. micranthus*) has been incorporated into a solid lipid nanoparticle (SLN) carrier formulated from stearic acid and polysorbate 80. Polysorbate 80 is safe and well tolerated [24]. The aim was to improve the anti-diabetic activity of the plant extract and to improve on its safety profile.

## MATERIALS AND METHODS

### Materials

Stearic acid and Tween® 80 were purchased from (Sigma Aldrich, USA). Metformin was kindly donated by Michelle Laboratories, Enugu, Nigeria. All other materials were used as procured from the manufacturers without further purification.

### Plant collection and identification

The leaves and stem of the mistletoe plant were collected from Nnokwa, Anambra State, Nigeria, in late April, 2014. The plant was identified by Mr. P.O. Ugwuozor, herbarium curator in Department of Botany, Nnamdi Azikiwe University, Awka, Anambra state, Nigeria. The plant was rinsed under tap water, and dried under a shade for six weeks and then milled into fine powder and stored at room temperature in an air-tight jar.

### Preparation of aqueous plant extract

The aqueous extract was prepared by infusion as described by Gray & Flatt [16] with minor modifications. The powdered material (25 g) was placed in 1L of boiling distilled water, then removed from the heat source and allowed to infuse for 15 min. The suspension was filtered with muslin cloth and then with filter paper (Whatmann no. 1). The filtrate was lyophilized and stored at 4 °C until required for use.

### Phytochemical evaluation

The aqueous extract was tested for the presence of alkaloids, saponins, tannins, flavonoids, terpenoids, steroids, and glycosides using standard methods, as described by Edoja *et al.* [25].

### Formulation of SLNs loaded with lyophilised mistletoe extract

The hot homogenization method [26] was used for preparing the SLN. The preparation followed a 2: 1 surfactant-to-lipid ratio optimized in a previous study (27). Five grams (5 g) of stearic acid was heated on a hotplate to just above its melting point after which 0.5 g of the lyophilized mistletoe extract was gently dispersed into, and mixed with the molten lipid. Distilled water was added to the surfactant (10 g) in a beaker and the resulting mixture was brought to the same temperature as the lipid. The surfactant-water mixture was added to the mistletoe dispersion while stirring continuously. The resulting mixture (adjusted to 100 g with distilled water) was homogenized using a homogenizer at 1000 rpm for 15 min to form a hot pre-emulsion which was added drop-wise into 500 ml of ice-cold distilled water with continuous stirring. During homogenization, the temperature of the system was kept above the melting point of the lipid by gentle heating, using a hot-plate. Then the

dispersion was filtered using Whatmann no.1 filter paper, and the residue was dried in a glass desiccator containing fused calcium chloride.

### Particle size and zeta potential analyses

The particle size and size distribution of the formulation as well as the zeta potential values were measured at 25 °C by dynamic light scattering method (Zetasizer, Malvern Instruments version 7.01).

### Differential scanning calorimetry

This was conducted using a differential scanning calorimeter (DSC 204 F1 Phoenix, Netzsch, 6.240.10, Germany). A 1.0 mg sample of the SLN was carefully weighed and sealed in aluminium pan with a similar empty pan serving as control. The machine was calibrated with indium and purged with nitrogen gas. Heating of the sample was carried out at the rate of 10 °C/min from 26 °C to 400 °C under nitrogen flow rate of 20 ml/min, followed by cooling back to 30 °C at the same rate.

### Drug loading capacity

The drug loading capacity was determined using the direct method. Forty milligram (40 mg) of the dried residue collected after filtration was weighed and dispersed in water to a total volume of 100 ml. The mixture was stirred at a constant rate for 2 h and filtered. The amount of the aqueous mistletoe extract was determined by measuring the absorbance of the filtrate at 227 nm using a UV-Vis spectrophotometer. The absorbance value was related with a calibration curve obtained at the same wavelength.

$$\text{Loading Capacity (\%)} = \frac{\text{amount of drug in filtrate} \times 100}{\text{Amount of SLN analyzed}}$$

### In vivo hypoglycaemic activity study

The animal experiments were carried out in accordance with instructions for care and use of animals in research, including free access to water and food. A total of 30 albino rats (5 rats per group) were used for this study. The study involved two stages - induction of diabetes and hypoglycaemic activity testing. Diabetes was induced in the rats using alloxan. The rats were injected intra-peritoneally with 190 mg/kg of alloxan monohydrate in a single dose. The fasting blood glucose (FBG) level of each rat was taken before injecting the rat. Diabetes was

confirmed by a FBG level  $\geq 9$  mmol/L. Treatment of diabetes commenced about 48 h after alloxan administration. The animals were grouped into six groups (A, B, C, D, E and F) with five rats in each group. Group A rats were given distilled water. Group B rats were given pure metformin at a dose equivalent to the normal adult dose of (500 mg/70 kg). This group served as the positive control. Groups E and F rats were given the mistletoe extract at doses of 16 mg/70kg and 500 mg/70kg respectively. Group C rats were given the nanoparticle formulation at a dose of 500 mg/70kg, and group D rats were given stearic acid at a dose of 500 mg/70kg. The blood samples were collected from the albino rats by cutting the tip of their tail under aseptic conditions. The glucose concentration was measured after 1 h, 3 h and 24 h and also on the 3<sup>rd</sup> and 7<sup>th</sup> days following the administration of alloxan. The concentration of glucose in the blood of the rats was determined with the One Touch glucometer using strip method [28] according to manufacturer's instructions. Percentage survival rate was also calculated.

### Statistical analyses

Statistical analysis was done using Microsoft excel 2007. Statistical significance was tested and p values less than 0.05 were considered significant.

## RESULTS AND DISCUSSION

As shown in Table 1, the extract revealed the presence of glycosides, as well as phenolic compounds such as flavonoids and tannins, and also alkaloids. These are in agreement with the constituents identified by Nazaruk and Orlikowski [29], though variations are known to occur, depending on manufacturing process, time of collection or host plant [30]. In this case, terpenoids are absent. The powerful antioxidant activity of mistletoe [31] has been attributed to these flavonoids and other compounds. This can help reduce the concentration of reactive oxygen, activity, which can help reduce reactive oxygen species, as previously discussed. In order to navigate the anatomic barriers imposed by mucus, cell surface charges, M-cells and enterocytes, size restriction is important. The nanoparticles had average particle size of 895.8 d.nm (with peaks at 742.8, 153.7 and 5560 nm) and polydispersity index (PDI) of 0.65, which is within recommended limits. Particle size distribution is an important attribute

which can affect stability and also particle uptake and distribution in the systemic environment.

**Table 1: Levels of significance of various phytochemical constituents in aqueous extract of mistletoe**

Phytochemical constituent	Indicator	Level of Significance
Flavonoids	Yellow colour in the ammoniacal layer	+
Glycosides	A brick-red precipitate at the bottom of the test tube	++
Tannins	A cream precipitate	+
Alkaloids	With Wagner's reagent; a reddish-brown colour With Hager's reagent; a yellow colour	+
Terpenoids	A reddish-brown interface	-
Steroids	A change from violet to blue or green	-
Saponins	Frothing which remains on standing	-

Key:

- : Absent
- +: Slightly present
- ++: Moderately present

The main advantage of the method of hot pre-emulsion followed by low-pressure homogenization and dilution is the ability to produce drug-loaded nanocarriers of sizes less than 1 micron. This is suitable for production of suitably sized small particles in low-facility laboratories obtained in many third world countries. As we reported in a preliminary study using Tween 20 [27], the same lipid-surfactant ratio had been used to produce batches of nanoparticles of hydrodynamic diameters of less than 500 (d.nm). Low size would be an advantage for improved nanoparticle kinetics and activity, but it has been demonstrated that in diabetic rats, the uptake of fairly large particles (2 microns) increases 100-fold, in comparison to normal rats [32, 33].

It has been postulated that molecular weight cut offs for translocation through mucus and M-cells are in the range of 400 nm and 500 nm [34, 35], but this is likely

to be modified by disease state. Moreover, polymer coatings can improve particle mobility and translocation in situations where the sizes of uncoated drugs do not improve particle kinetics. Surface charge measurement was not feasible in this case but slightly negative values of zeta potential (-0.3 mV) were obtained for solid lipid nanoparticles prepared from stearic acid using the same method [36].

Drug loading was calculated as 3.2%. The high linearity factor ( $R^2 = 0.99$ ) of the calibration curve ( $y = 0.078x + 0.090$ ) indicated that the extract can be comfortably determined by ultraviolet absorption of components, most probably flavonoids. The loading capacity in this case may be limited by the low solubility of the aqueous extract in the lipid melt. Blending of triglycerides or fatty acids with dissimilar chain lengths, especially odd and even number chains is expected to increase the level of imperfection, decrease crystallinity and improve loading. Time-dependent crystallization may lead to drug extrusion from the voids or imperfections, if insoluble drugs are employed [37-40].

The thermograms of the formulation, stearic acid and erythromycin drug are presented in Fig. 1-4. Being a liquid, the thermogram of Tween 80 (not shown) does not have any peaks in this range.

The thermograph of stearic acid alone (2) shows a definite endothermic peak at about 64 °C, corresponding closely to its melting peak temperature. Melting peaks of 61.8°C [27], 69.8°C [41], melting point of 56.7°C and corresponding solid to liquid transition temperature of 59.43°C [42], had been reported for stearic acid. The thermograph of the extract (1) had no definite transition peak temperature probably due to the complex nature. The thermograph of the formulation (3) shows a diffuse endothermic transition around same temperature as that of stearic acid lipid carrier, indicating a positive physical interaction and amorphous nature after materials loading. The extract was amorphously distributed in the stearic acid carrier matrix. Increase in amorphous nature encourages loading of particles in the matrix [43], and confers increased solubility and propensity for enhanced bioavailability.

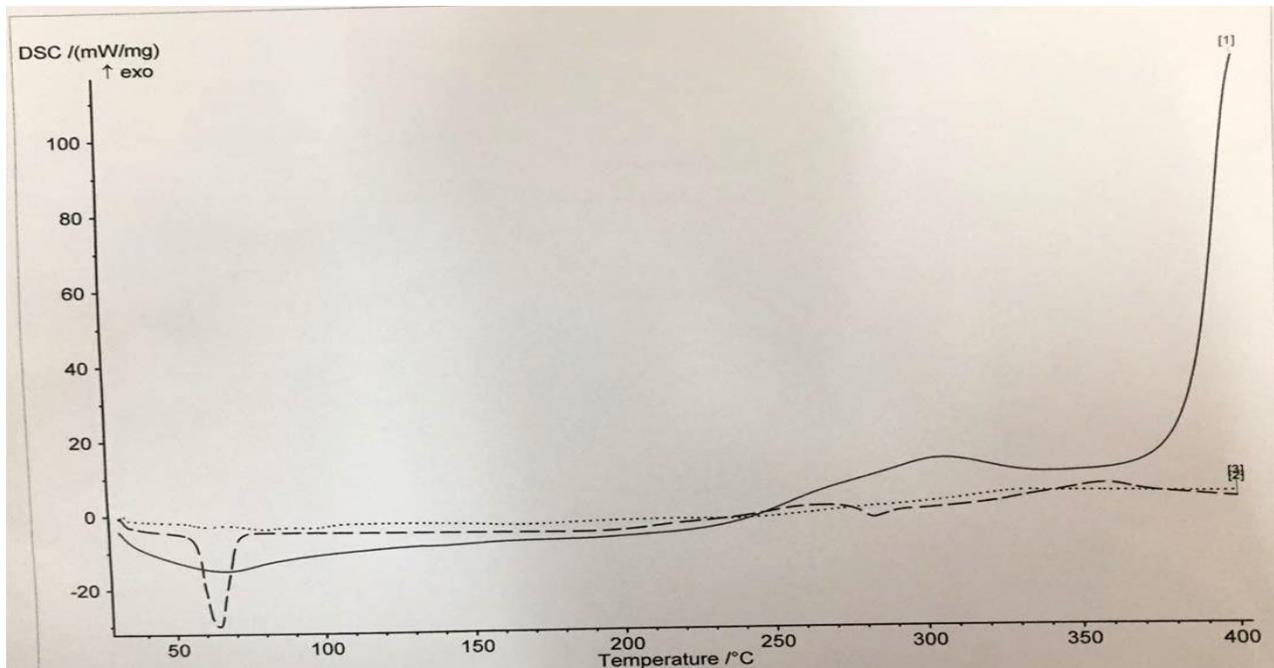


Figure 1: Superimposed DSC thermographs of stearic acid, mistletoe extract and the solid lipid nanoparticle. [1] extract; [2] stearic acid; [3] solid lipid nanoparticle

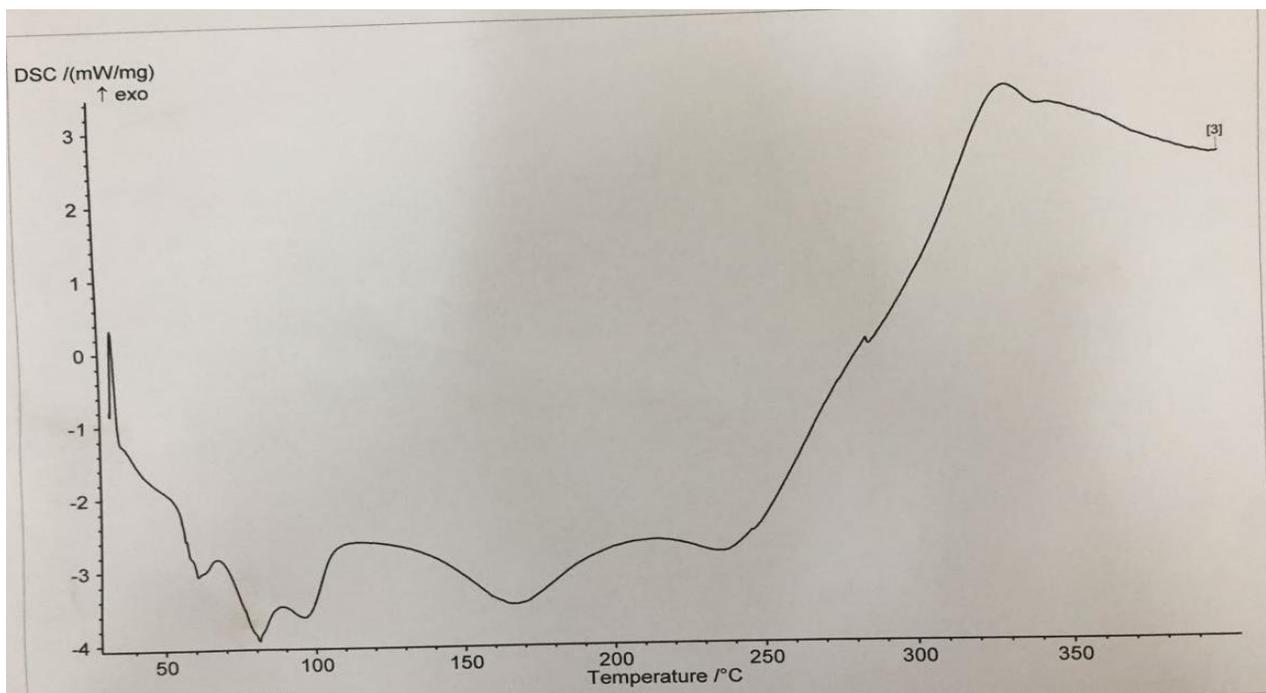


Figure 2: DSC thermograph of solid lipid nanoparticle containing stearic acid, Tween 80 and mistletoe extract

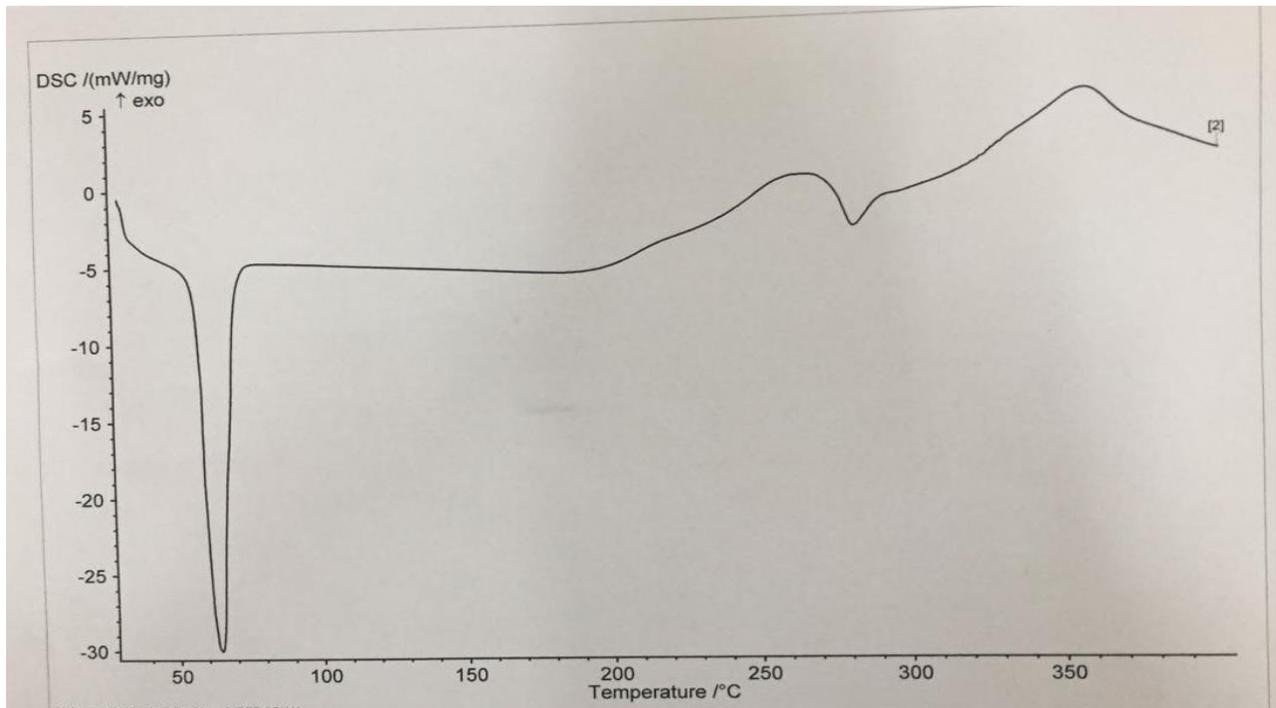


Figure 3: DSC thermograph of stearic acid

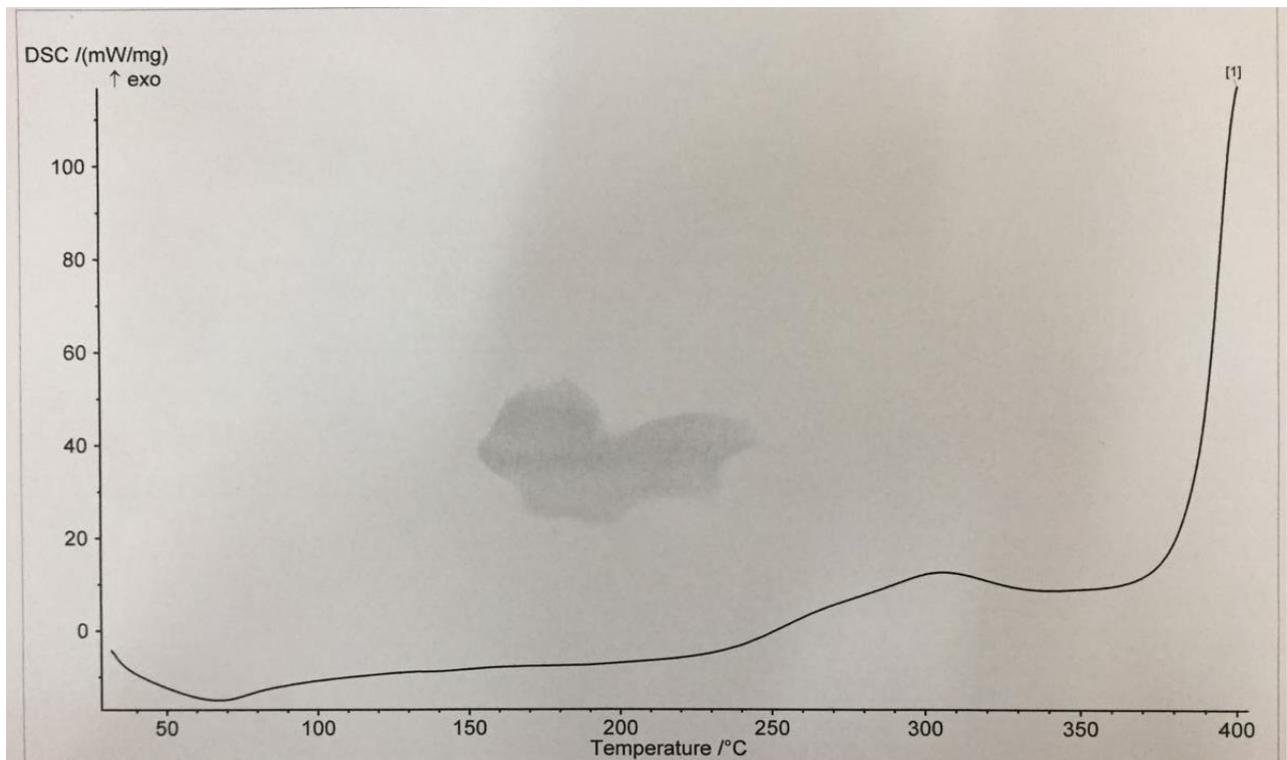
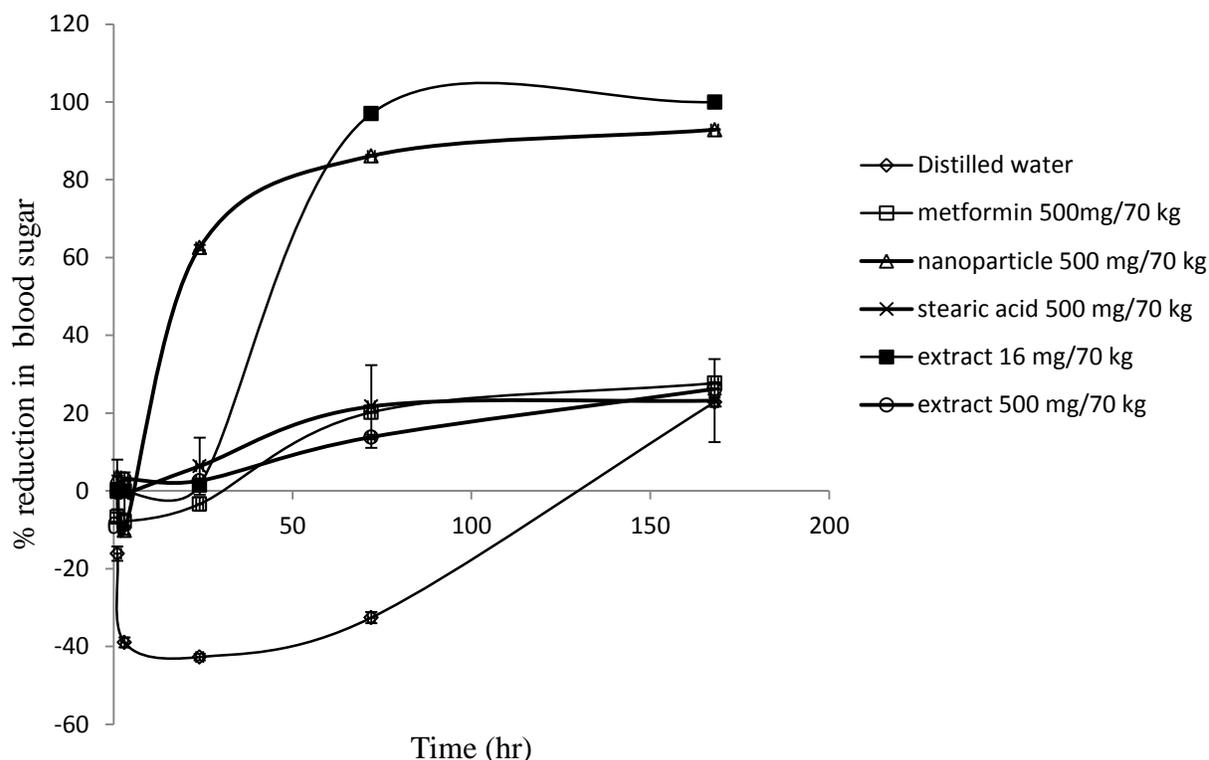


Figure 4: DSC thermograph of extract

The percentage reductions in blood glucose are presented in Figure 5. Group C showed the fastest rate of onset of activity. From statistical standpoint, only C achieved a level of reduction considered extremely significant ( $p < 0.01$ ) as by 24 h post-administration, which was maintained till the end of the study (day 7). In comparison, for metformin, the reduction in blood glucose was significant ( $p < 0.05$ ) by 72 h post-administration and extremely significant ( $p < 0.01$ ) by the 7<sup>th</sup> day. Use of the extract alone resulted in reduction in time to onset of action and also percentage survival rate. Increasing the dose of

the extract (alone) from 16 mg/70 kg (Group E) to 500 mg/70 kg (Group F) did not result in any significant improvement in time of onset of activity, percentage reduction, or percentage survival rate. This means that use of higher doses of the extract may be unnecessary, considering that the extracts may contain some toxic lectins whose effect would be most prominent in high dose [44, 45]. Though the hypoglycaemic effect agrees with the findings of



**Figure 5: Percentage glucose reduction with time following treatment with preparations of aqueous extract of mistletoe**

Osadebe *et al.* [46], dose-dependent hypoglycaemic effect is less obvious in this case. Both extracts and SLN demonstrated superior activity to metformin, a common anti-diabetic drug used in type 2 diabetes. The explanation attempted here is that the extract may exert other effects on insulin production or utilization in a different manner from metformin. As noted previously, mistletoe may possess insulin-stimulating activity [16]. The percentage survival rate for the group administered with the nanoparticles is the same (80 %) with that for the metformin-treated

group, both being higher than the survival rates for the groups treated with either dose of the extracts alone (Table 2). The percentage survival rates observed here for groups A (distilled water) and D (stearic acid) were of the same value of 40 %, which fairly agrees with the 37 % mortality rate quoted for use of alloxan in induction of diabetes without treatment [47].

**Table 2: Percentage survival rates of diabetic animals treated with preparations of aqueous extract of mistletoe**

Group label	Composition of drug administered	Percentage survival (%)
A	Distilled water	40
B	Metformin 500 mg/kg	80
C	Extract-loaded nanoparticle 500 mg/kg (equivalent to 16 mg/kg pure of extract alone)	80
D	Extract 500 mg/kg	40
E	Extract 16 mg/kg	60
F	Stearic acid 500 mg/kg	60

*Each group consisted of 5 rats and diabetes was induced with alloxan monohydrate at a single intraperitoneal injection dose of 190mg/kg*

## CONCLUSION

In conclusion, no differences were observed between the two doses (16 mg/70 kg and 500 mg/70 kg) of the aqueous extract administered, either in time to onset or action or in percentage survival rate or percentage lowering of glucose level. In comparison, 500 mg/kg of nanoparticles (containing just 16 mg or 3.2 % of extract) was able to achieve a faster onset of action than all other preparations. In addition, it achieved a very high survival rate (80 %) equivalent only to that of metformin. Therefore SLN delivery of aqueous extract of mistletoe is a very attractive option for enhancement of its anti-diabetic activity.

## CONFLICT OF INTEREST

Authors declared no conflict of interests in the conduct and reporting of this study

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