Original Research Article

Annona stenophylla biosynthesised Zinc Oxide nanoparticles (ZnONPs), structural and activity characterisation

ABSTRACT

Biosynthesis of functional nanomaterials from metallic salts using polyphenolic components of plant extracts provides an eco-friendly, easily scalable one pot fabrication of novel biotherapeutics. Among the nanomaterials that can be obtained through green synthesis, Zinc Oxide nanoparticles (ZnONPs) have shown considerable potential in the management of type 2 diabetes mellitus which is a global scourge affecting more than 50% of the adult population at some point in their lives. *Anonna stenophylla (A. stenophylla)*, a native Southern African dwarf tree has been widely used in traditional medicine as a remedy for diabetes. Its use is supported by numerous scientific studies that have validated its hypoglycaemic effects *in vitro*. Thus, the main objective of this work was to investigate the phytoconstituents of *A. stenophylla* and their capacity to mediate in the biosynthesis of ZnONPs as bio reduction, capping and stabilising agents. The nanostructures obtained were characterised by Transmission electron microscopy (TEM), UV -Vis spectrometry and Dynamic light scattering (DLS). The acute oral toxicity of the lyophilised root extract was also determined in a rat model. The participation of polyphenols in the synthesis was confirmed by spectral absorbances around 270nm. The presence of the biogenic ZnONPs was confirmed by UV-Vis spectra showing absorbance around 370nm. The synthesized nanostructures appeared mostly spherical in morphology, and the particle sizes were in the range of 40 to 60nm. No toxicity was observed at 2000mg/kg. The findings demonstrate the feasibility of fabricating ZnONPs from the polyphenolic constituents of the *A. stenophylla* root extract. Safe biogenic ZnONPs were successfully synthesized from *A. stenophylla* and could potentially offer an opportunity to combine anti-diabetic metallic oxides and polyphenolics in novel, effective and eco-friendly dosage forms.

Key words: *Anonna stenophylla*, Zinc Oxide Nanoparticles, Diabetes, polyphenols, biosynthesis, hypoglycaemic.

UNDER PEER REVIEW

1 INTRODUCTION

1.1 Annona stenophylla

Annona is a genus of the *Annonaceae* family, which was first catalogued by the researcher de Jussieu as far back as 1789[1]. The broad *Annonaceae* family of tropical and subtropical plants has more than 120 genera with *Annona* being the most studied genus due to its proliferative edible fruits and the reported medicinal properties by different cultures[2]. In Southern Africa, Zimbabwe in particular, the wildly growing medicinal and edible fruit tree, *Annona stenophylla (A. stenophylla)* is the most prevalent representative of the genus (Figure 1). It i*s a* dwarf rhizomatous shrub with an average height of less than 1 meter. The miniature fruit tree may grow from one simple stem or branched stems. It is characterised by hairy red brown branchlets which are swamped with alternate equally hairy, broad leaves[3]. The plant flowers in the rainy season with cream-yellow to brown hairy petals which culminate into edible orange-yellow ripe fruit with black shiny seeds[3]. Metabolomic studies of the plant have reported the isolation of a wide catalogue of medicinally promising phytochemical components, including poly-phenolic compounds, acetogenins, alkaloids and flavonoids from almost all the plant parts including the seeds and leaves. Studies by various scholars have demonstrated biological activities, including antifungal, antioxidant, anticancer, and antibacterial properties[3].

A. stenophylla has diverse applications in ethno-pharmacology, including the treatment of non-communicable diseases such as diabetes and carcinomas. Its anti-hyperglycemic potential has been investigated by many scholars. Studies by Chagonda and confirmed by Taderera in Zimbabwe attributed the anti-diabetic activity to the presence of unique bioactive polyphenolic compounds, flavonoids and alkaloids in the root bark extract [4,5]. *A. stenophylla* was shown to regulate glucose metabolism and reduce hyperglycemic effects. The hypoglycemic benefits of these phytochemicals can be achieved by strengthening pancreatic beta-cell activity, boosting glucose absorption, and increasing insulin sensitivity. The extract increases GLUT4 mRNA and translocation potentially acting via the PI-3-K pathway, a common glucose transporter, leading to increased glucose uptake[5]. The use of *A. stenophylla* potentially offers a cure for diabetes, as opposed to conventional treatments which merely manage diabetes, and has potential to be incorporated into anti-diabetic treatment regimens [4, 6].

Figure 1: Dwarf A. stenophylla tree with fruit

1.2 Polyphenols and their effects on diabetes management

Plant-based diets contain a broad and diverse class of phytochemicals known as polyphenols, which include phenolic acids, proanthocyanidins, flavonoids, and resveratrol. These substances have been studied for their potential benefit in diabetes, including their anti-inflammatory, antioxidant, and insulin-sensitizing qualities[7]. Studies on animals and humans have demonstrated that food and beverages that are high in polyphenols have been proven to increase insulin sensitivity and increase insulin secretion while also reducing postprandial and fasting hyperglycemia[7]. Reduced intestinal absorption of glucose, inhibition of the digestion of carbohydrates, increased pancreatic β-cells secretion of insulin, altered

UNDER PEER REVIEW

liver glucose release, activation of insulin receptors and glucose uptake in insulin-sensitive tissues, modulation of intracellular signaling pathways, and gene expression are some of the potential mechanisms[8]. It has also been reported that certain polyphenols cause pancreatic beta-cells to secrete more insulin, which could lead to better glycemic control[8]. Additionally, they have potent antioxidant qualities that can aid in lowering inflammation and oxidative stress, two factors linked to the onset and advancement of diabetes[9]. However, polyphenols have the ability to alter the diversity and makeup of the gut microbiome, which may have repercussions for the metabolism of fats and carbohydrates[10]. Consuming polyphenols has been shown to enhance lipid profiles, which are crucial for the cardiovascular health of people with diabetes because they lower triglycerides, total cholesterol, and low-density lipoprotein (LDL) cholesterol[9]. Additionally, they provide protection against the onset of complications associated with diabetes, like diabetic nephropathy and neuropathy[9]. Polyphenols can improve insulin sensitivity by reducing hepatic glucose production and boosting glucose absorption in peripheral tissues such as muscle and adipose tissue[10].

1.3 Management of Diabetes using Zinc Oxide

Zinc is a trace mineral that is necessary for several physiological functions, such as the synthesis of insulin, the metabolism of glucose, and antioxidant defense mechanisms[11]. Due to these special qualities, zinc oxide (ZnO) is a promising option for the treatment of diabetes. Numerous studies have shown that ZnO has a beneficial effect on glucose homeostasis, insulin secretion and oxidative stress markers[14]. The appropriate synthesis, storage, and secretion of insulin by pancreatic beta cells depend on zinc, which is an essential component of insulin[11]. Supplementing with ZnO improves glycemic control and insulin sensitivity in both human and animal models. The possible therapeutic effects of ZnO may also be attributed in part to its antioxidant activity. Diabetes is often associated with increased oxidative stress, which exacerbates disease progression. Due to its potent antioxidant properties, ZnO can mitigate the negative effects of reactive oxygen species and free radicals[12]. Another typical consequence of diabetes is diabetic neuropathy, which is characterized by reduced nerve function and nerve damage. ZnO confers neuroprotective effects, thereby enhancing nerve function and lowering the risk of diabetic neuropathy[11,12]. Diabetics often experience impaired wound healing leading to formation of persistent, non-healing ulcers. [13] ZnO has demonstrated wound-healing qualities which can find use in the treatment of diabetic foot ulcers and other diabetic skin problems.

1.4 Biosynthesised nanoparticles from polyphenols

Nanomaterials are classified according to their particle size and dimensions that are within the nanoscale range (1-100nm). Using this context we can categorize three different types of nanomaterials. First, nanoparticles (NPs) are nanostructures with all 3 dimensions below 100nm, second; nano fibers are structures where 2 dimensions are below 100nm and finally; nanofilms depict structures with only one dimension in the nanoscale range[15]. At the nanoscale, the behavior of materials no longer conforms to the principles of classical physics and may consequently be very different from their macromolecular structures. Nanometric structures may exhibit characteristics unrelated to their macro molecular analogue materials due to the unique properties obtained at this scale, including particle size, shape and other physico-chemical characteristics[16]. This change modulates characteristics including optical, toxicity, catalytic and mechanical properties. This super low particle size may amplify material bioactivity and allow them to be incorporated in formulations at much lower amounts with magnified activity. The possibility above has been widely explored and has found utility in many applications including dosage form design.

NPs can be synthesized through various chemical and physical pathways. The dominant chemical methods, however, generate toxic materials mostly from reagents, unreacted starting materials and by products from side reactions[17]. The toxicity issues as well as high costs of purification have limited their application in mainstream pharmaceutical product development. These drawbacks have recently been overcome by the use of polyphenols as reducing and capping agents in place of synthetic inorganic reagents. Polyphenols are characterized by the presence of more than one phenol group per molecule. It is this ready availability of the reactive –OH groups, that make polyphenols suitable reducing, capping and stabilization agents in the synthesis of NPs[15]. Techniques utilizing natural products such as plant phenolics are referred to as biosynthesis or green synthesis of nanomaterials. In addition to being ecofriendly and providing safer products, green synthesis also lowers the fabrication costs of nanomaterials. Many technical reports confirm that this approach has been successfully used to fabricate NPs from many plants, rich in polyphenolic secondary metabolites[16,17,18]. The added novelty is in the ability to pre- determine the desirable physical characteristics and activity of these NPs by optimizing the reaction conditions. The primary objective of this work therefore was to qualitatively

UNDER PEER REVIEW

determine the major phytoconstituents of *A. stenophylla* root extracts that can mediate in the biosynthesis of ZnONPs, and to characterize the physico-chemical properties of the biogenic ZnONPs. The acute toxicity profile of the *A. stenophylla* hydro-ethanolic root extract was also determined.

2 Materials and methods

2.1 Materials, equipment and facilities

All chemicals, associated reagents, equipment and facilities for the *in-vivo* acute oral toxicity assays, the biosynthesis and the activity determinations of ZnONPs were obtained from the University of Zimbabwe, Faculty of Medicine and Health Science laboratories, Harare, Zimbabwe. For the characterisation of NPs, all chemicals and equipment were availed by the University of California, Los Angeles, Department of Chemistry and Biochemistry.

2.1.1 Animal use approval

Prior to the investigations, animal use and research ethics approvals were obtained from the Joint Parirenyatwa Research Ethics Committee (JREC) which is the local research Institutional Review board for the University of Zimbabwe.

2.1.2 Collection and preparation of plant material

A. stenophylla was harvested from Chiweshe, Mashonaland central in Zimbabwe (16° 53' 0" S · 31° 7' 20" E), observing the guidelines for Zimbabwe's Sustainable Harvesting of Traditional Medicinal Plants. The plant was taxonomically authenticated by the National Herbarium and Botanical Garden in Harare, Zimbabwe.The plant roots were separated from the plant and washed with distilled water to remove dirt and other contaminants. The roots were then shade-dried for three weeks until constant weight was obtained, and ground to a fine powder.

The extraction was done by adding 200g plant powder into 1000ml of 70% (v/v) ethanol in a 2-litre sterile amber bottle and macerated at room temperature for 5 days with 1 minute physical shaking twice a day. The extracts were filtered (Whatman filter paper number 1) and evaporated under low pressure (Rotavapor® R-300, Buchi, Switzerland), followed by lyophilization (Lyovapor I-200, Buchi, Switzerland) under 140Pa pressure and -50 °C.

2.2 Phytochemical Screening of *A. stenophylla*

In a 200ml round bottomed flask, 10g of the lyophilized hydro-ethanolic extracts of *A. stenophylla* were dissolved in 100g of distilled water and subjected to various phyto-screening techniques to confirm the presence or absence of relevant phytoconstituents of pharmacological interest to this study. The following qualitative tests were conducted on the extract liquor.

2.2.1 Tests for alkaloids

The Mayer's test was used to determine the presence of alkaloids. In this assay, to 2 ml of the lyophilized extract, two drops of Mayer's reagent were slowly added along the sides of the test tube. The presence of alkaloids was then identified by the appearance of a white creamy precipitate[19].

2.2.2 Tests for tannins

The ferric chloride test was used to determine the presence of tannins and phenolic compounds in the lyophilized sample. In a test tube, 1 ml of the hydro-ethanolic extract was added to 2 ml of distilled water. Followed by 2-3 drops of ferric chloride. The test sample was checked for the development of a green-blue colour which indicates the presence of Catechic tannins and blue-black indicated the presence of Gallic tannins[20].

2.2.3 Test for flavonoids

Flavonoids were detected by means of the alkaline reagent test. To 2ml of the lyophilized root extract, 2 to 3 drops of sodium hydroxide were added. The initial formation of a deep yellow which gradually fades to colourless after adding a few drops of dilute HCl, indicates the presence of flavonoids[21].

2.2.4 Test for terpenoids

The test for terpenoids was done by dissolving 3 granules of tin metal in 2 ml thionyl chloride solution and then, adding 1 ml of the extract into the test tube. The formation of a pink colour indicates the presence of terpenoids[22].

UNDER PEER REVIEW

2.2.5 Tests for steroids

The test for steroids was confirmed by adding 5 ml of chloroform to 2 ml of the extract followed by the addition of 1 ml of concentrated H_2SO_4. The emergence of a reddish-brown colour confirms the presence of sterols in the test samples[23].

2.2.6 Test for saponins

The simplified foam test was used to determine the presence of saponins. In this assay 2 ml of the extract was added to 20ml distilled water, the mixture was shaken in a graduated cylinder for 15 minutes. The presence of saponins would be confirmed by the formation of form with at least a head height of 1cm[24].

2.2.7 Quantification of Total Phenolic and total tannins content

The total phenolic and total tannins content of the lyophilised *A. stenophylla* were determined according to the Folin-Ciocalteu spectrophotometric as reported in our other previous publcation[25]. To prepare a calibration curve, phenol (Gallic acid) stock solution (5 mg/mL) was added into 100 mL volumetric flasks, and then diluted to volume with water. From each calibration solution, 0.25 mL was mixed with 1.25 mL of 10-fold diluted Folin-Ciocalteu's phenol (1 mL Folin reagent and 9 mL deionized water) reagent and allowed to react for 5 min. Then, 1 mL of 7.5% Na_2CO_3 solution was added, and the final volume was made up to 5 mL with deionized water. After 1 h of reaction at room temperature, the absorbance at 760 nm was determined by spectrophotometer (Lambda 35 UV/Vis-Spectrometer, Perkin Elmer Instruments). The test was done in triplicate. Calibration curves were plotted to determine the level of phenolics and tannins in the samples. The results were expressed as Gallic acid equivalents (GAE, mg/g) of either of the *A. stenophylla extracts* (comparisons were made between ethanolic and methanolic extracts in this assay)[25].

2.2.8 Quantification of total flavonoids

The total flavonoid content of the lyophilised *A. stenophylla* was estimated spectrophotometrically at 510 nm. In the test, 1mg of extract was dissolved in 2mL of distilled water as per methods reported in our previous publication[26]. To this solution, 0.5mL of 1M sodium nitrite was added together with 2ml of a 1M NaOH solution, distilled water was then added to make the volume to 10ml. The solution was shaken and allowed to stand at room temperature for 15 min and the absorbance was subsequently measured. The total flavonoid content was estimated as mg of quercetin equivalent (mg QE/g extract) on a dry weight basis using the standard curve[26].

2.3 Biosynthesis of ZnO Nanoparticles (ZnONPs)

The ZnONPs were biosynthesized using the lyophilized extracts from A. stenophylla as bio reducing, caping and stabilization agents. The lyophilized extract (2g) was dissolved in 50ml distilled water and stirred for 15 minutes and heated at 50oC. To this liquor 1g of zinc acetate-2-hydrate (Zn (CH3COO)2·2H2O) salt was added. The brown solution was mixed with constant stirring at this temperature for approximately 60 minutes. The mixture was left overnight, and a thick precipitation was observed in the solution. The solution with precipitation was centrifuged at 4000rpm for 30 minutes. After that the upper phase was decanted, and the residual precipitate was washed three times with 70%v/v ethanol. The resulting ZnONPs were dried in an oven at 100oC for 24 hours and the dried ZnONPs were calcined in a furnace at 500oC for three hours.

2.3.1 Characterisation of ZnONPs.

The biosynthesized ZnONPs were confirmed by UV-Vis spectrophotometer (Hitachi, UH5300) and further analysed by Transmission electron microscope (TEM)). The TEM was used to obtain images of the synthesized nanoparticles and determine their size and shape. The NPs obtained were diluted 1:10 and 1:100 before being dropped onto the copper grids. Drops of the ZnONPs suspension were placed on a carbon-coated copper grid covered with a formvar film, which were then allowed to dry by evaporation in air. After drying the samples were then subsequently loaded onto the specimen holder. The TEM measurements were conducted at a voltage of 100 kV (A LEO912 AB OMEGA transmission electron microscope). The particle size distribution and average particle sizes were determined by Dynamic light scattering (DLS) (Zetasizer ultra).

UNDER PEER REVIEW

2.4 Acute oral toxicity evaluation of *A. stenophylla* and the biosynthesised nanoparticles

The acute oral toxicity evaluation of A. stenophylla lyophilized extract was done using a modified OECD technical guideline 425 (The up and down test) methodology27. The test consisted of single ordered dose progressions in which animals were dosed, in sequence, at 48-hour intervals. The first animal received a dose below a randomly selected estimated LD50. When animals survived the dose, the next animal received an increased dose subject to our observations on the determined condition of the previous animal over 48 hours. In the present toxicity assay for the A. stenophylla root extract a high start dosage of 250mg/kg body weight was used, followed by doubled subsequent doses, up to the limit of 2000mg/kg body weight. A total of 12 female rats were used for each test. Only female nulliparous rats were used because literature indicates that in conventional toxicity profile evaluations there is usually very small notable differences in observed sensitivity between animal sexes and in the instances where significant differences were noted, it was observed, that female rats were slightly more sensitive to toxicity than males. So, it was therefore decided to use a worst-case scenario. The selected animals were marked so as to facilitate individual identification. The nulliparous rats were kept in the experimental rodent facilities for 10 days prior to dosing. The experimental animals were fasted for 18 hours with water. The A. stenophylla was orally gavaged in a water solution in 4 incremental doses of: 200, 400, 800, and 2000 mg/kg body weight. The female rats were observed by a veterinary specialist for mortality. In the absence of mortality, the animals were observed for any changes and clinical signs and symptoms of toxicity every 1 hour up to 12 hours on day 1, and thereafter, once daily for up to 14 days27.

3 Results and discussion

3.1.1 Qualitative Phytoscreening

Table 1: Secondary metabolites present in A. stenophylla, hydro-ethanolic and distilled extracts

Metabolites	Hydro-ethanolic extract	Hydro extract
Saponins	++	+
Phytosterols	++	+
Phenolics	+++	+
Tannins	++	++
Flavonoids	+++	+
Diterpenes	-	-
Alkaloids	+++	+

(+): Indicates the presence of chemical constituents
(++): Indicates moderate quantities of chemical constituents
(+++): Indicates large quantities of chemical constituents
(-): Indicates absence of chemical constituents

3.1.2 Quantitative selective secondary metabolites analysis of A. stenophylla

Plant phytoconstituents are loosely grouped into either primary or secondary metabolites. Both these categories are pivotal to plant growth and defence processes and therefore cardinal to their survival. Primary metabolites are defined as those that play a role in essential cellular processes contributing to the plant's growth and development like photosynthesis and respiration. Examples include amino acids (vital and non-vital), carbohydrates, lipids and proteins[7,28]. Variations of primary metabolites within the same genus or species are therefore minimal since the generation of primary metabolites is mostly determined by genetics. On the other hand, secondary metabolites are multifunctional entities primarily responsible for defence from environmental threats. Plants utilise intrinsic sensors and receptors when exposed to environmental stresses such as diseases, hostile conditions and foreign agents. They then produce secondary metabolites to ensure their survival [28]. Due to variations in environmental and

UNDER PEER REVIEW

geographical factors, the secondary metabolites present and their relative concentrations may therefore vary within the same genus or even the same species depending on the specific threat signals detected in the environment. Since the same environmental threats including bacteria, fungi, as well as environmental stresses that disrupt metabolic processes like poor nutrition also affect other organisms including humans, plant secondary metabolites are also widely used by humans to boost immunity and defence mechanisms. This is the concept behind the use of plants rich in secondary metabolites as traditional medicines and herbal remedies. Typically, secondary plant metabolites are classified based on functional groups and chemical structures. The most prevalent groups include polyphenolic compounds, flavonoids, tannins, sterols, saponins, terpenes, alkaloids and others[29]. Numerous scientific studies have validated that these secondary metabolites are the arsenal that contribute significantly to defence against stressful situations for all organisms including humans through functional groups that ensure stress adaptation, restoration of metabolic pathways and organ function[30]. In the present study we confirmed that *A. stenophylla* is rich in secondary metabolites including flavonoids, tannins and alkaloids that are potentially beneficial to human health (Table 1). We also established that the root extract possesses high polyphenolics levels, with functional groups capable of bio reducing, capping and stabilising metallic salts in green synthesis processes. The abundance of secondary metabolites especially polyphenolic compounds is in line with previous studies, and therefore validates the traditional medicinal use of *A. stenophylla* (Table 2)[31]. Although the specific identities of the phytochemicals was not confirmed in our study, Figure 4 shows the structures of some of the bioactive phytocompounds which have been isolated by previous scholars from extracts of *A. stenophylla*.

In our comparison between the ethanolic and the methanolic extract, there was no significant difference between the extractable constituents (table 2, and figure 2). These are both polar solvents, though methanol is slightly more polar (0.762) than ethanol (0.654). Due to the toxicity issues associated with methanol use, the hydro-ethanolic extracts were selected for the study.

Table 2 Total phenolics, tannins and flavonoids content of the lyophilized A. stenophylla

Extract	Total phenol	Total Tannins	Total Flavonoids
	mgGAE/g extract	mgGAE/g extract	mgQE/g extract
A. stenophylla- Hydroethanolic	79	16	0.088
A. Stenophylla - Aqueous	48	12	0.062
A. stenophylla- Hydro methanolic	81	10	0.089

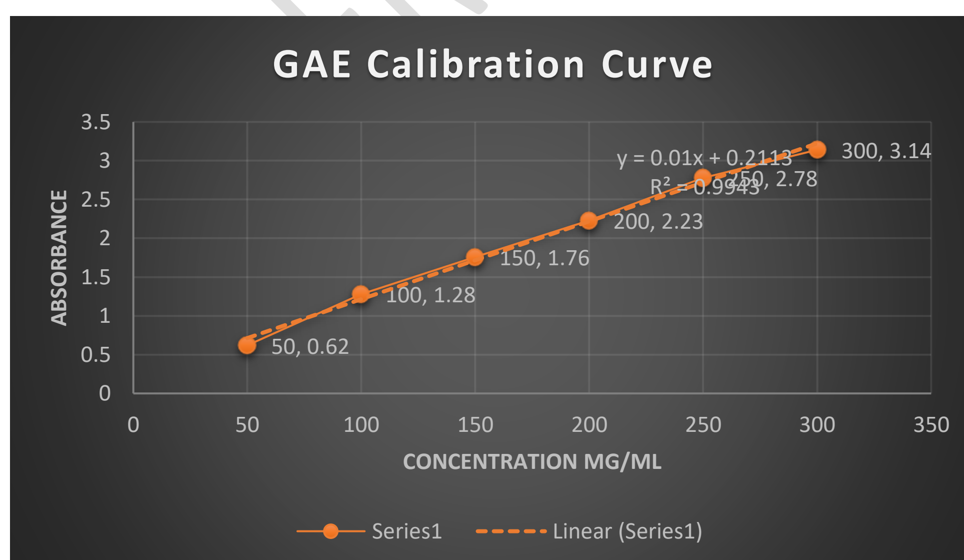

Figure 2: A. stenophylla, Gallic acid standard curve for Folin-Ciocalteu assay

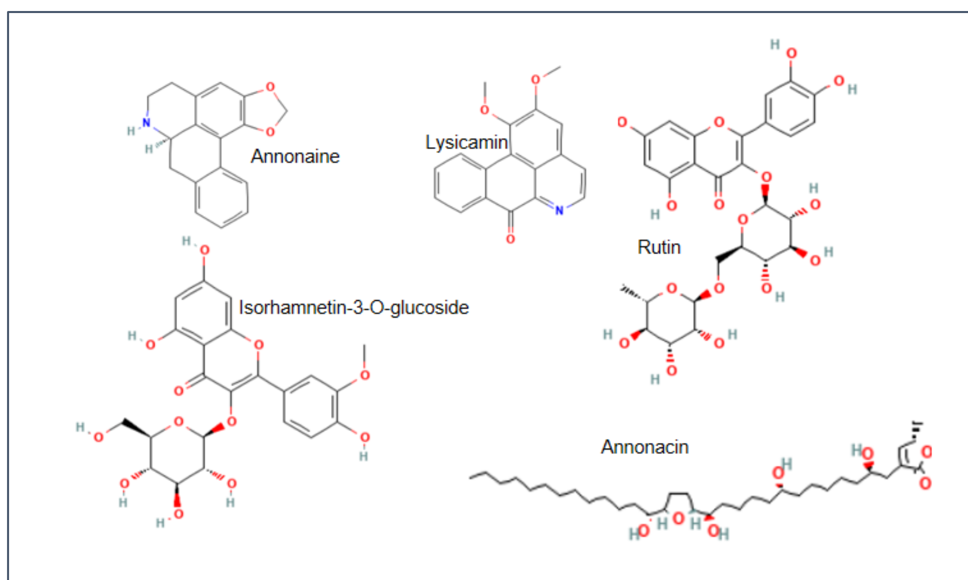

Figure 3: Some prevalent phytoconstituents found in A. stenophylla

3.2 Biosynthesis and characterisation of *A. stenophylla* mediated ZnONPs

In the present study the green synthesis of ZnONPs from *A. stenophylla* root extract offered an eco-friendly one pot technique to successfully fabricate ZnONPs. As predicted, the phytochemicals of *A. stenophylla* including the abundant polyphenols have many functional groups with the capacity to reduce metallic ions. The bio reduction of the Zinc ions into the nanoparticles can technically be linked to the possible tautomeric transformation in the polyphenols from enol- form to keto- form. During such a transition a reactive hydrogen species is released, which has the capacity to reduce the zinc ions resulting in the formation of zinc nanoparticles[32]. The Zn $(CH_3COO)_2{\cdot}2H_2O$ was therefore reduced to zinc hydroxide (ZnOH) by the polyphenols in the lyophilised extract.

Visual observation was the first confirmation that the biosynthesis of nanometric ZnO was successful. After adding the precursor *zinc acetate-2-hydrate* to the *A. stenophylla* lyophilised extract, the colour of the solution changed from dark brown to cream. Similar colour changes of synthesized ZnONPs employing *Pelargonium odoratissimum* leaf extract were observed by Abdelbaky (2022)[33], and in similar studies by Shaba et al (year) [34]. The UV-VIS spectra confirmed the synthesis of ZnONPs, with strong absorption bands between 370 nm to 390nm most likely due to the plasmon resonance phenomenon (SPR). These absorption peaks correspond well to the expected synthesized ZnONPs now present, which is consequent of the electron transition of the semi-conductor to the conduction band from the valence band. There was also another absorption band around 270nm which can be attributed to the polyphenolics in the *A. stenophylla* extract[35]. This is confirmation to the presence of polyphenols which then acted as capping agents that stabilised the fabricated ZnONPs. The poly dispersed range of the observed peaks is also testament to the varied polyphenols in the *A. stenophylla* extract (Figure 6).
The transmission electron microscopy (TEM), images revealed that the ZnO nanostructures fabricated were nearly spherical in shape with slight variation in thickness and an average diameter of 40 -60nm (Figure 5). DLS confirmed that average size of the nanoparticles was 50.5nm. On closer observation the TEM images also show numerous nanoparticle aggregates and scattered nanometric structures with varying morphologies, which most probably indicate the participation of competing functional groups from the extract for Zn ions during the reaction phase. This is shown by an increase in the reduction rate as a function of time.

UNDER PEER REVIEW

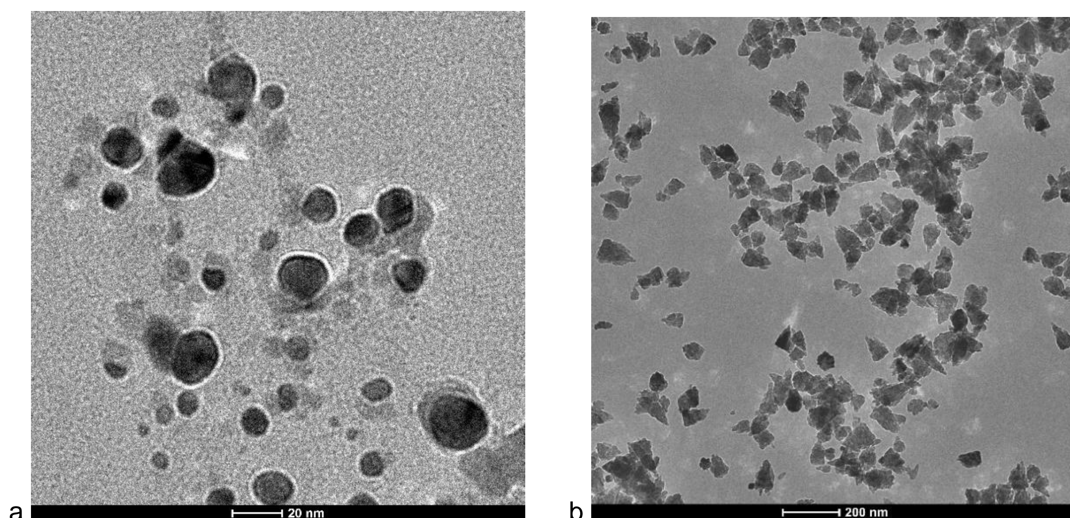

Figure 4: TEM images of A. stenophylla mediated ZnONPs showing the spherical nanostructures obtained

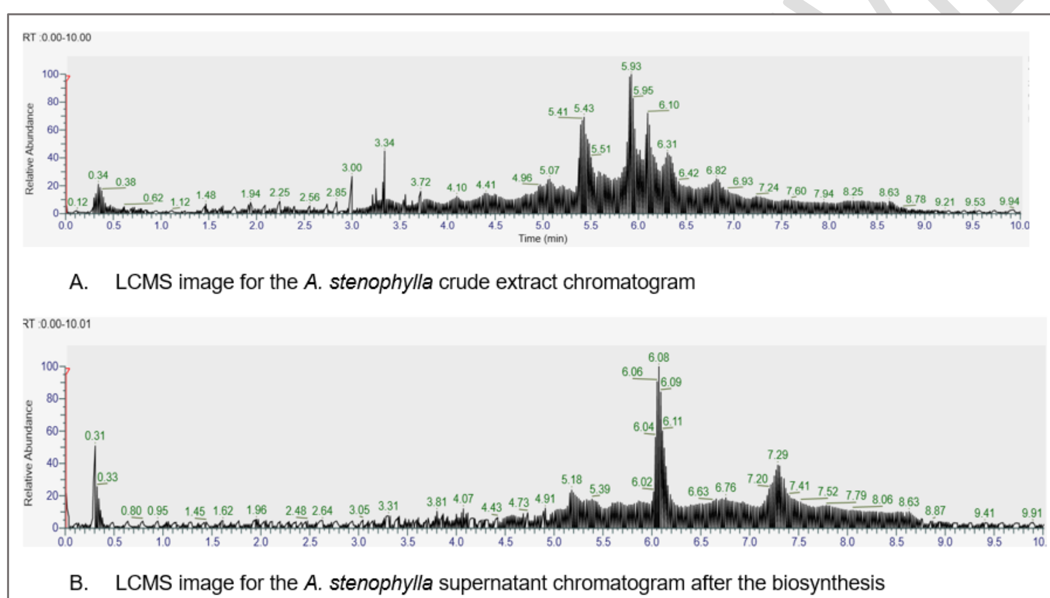

Figure 5: LCMS images of A. stenophylla chromatograms for the crude extract (A) above and the supernatant (B) below after nanoparticle fabrication: disappeared peaks represent the phytoconstituents consumed in the biosynthesis.

The LCMS chromatograms (Figure 6) of the crude extract and the supernatant after the synthesis reveal the disappearance of many peaks at certain elution times. These peaks indicate the major phytoconstituents consumed in the synthesis process. No new major peaks emerged in the LCMS supernatant which confirms the complete removal of the developed ZnONPs. Further work is imperative to identify the exact participating polyphenols involved in the bio reduction and capping noted by these chromatograms.

3.3 Acute oral toxicity evaluation

Table 3: Observations for behaviour and appearance of rats during studies

Dose of *A Stenophylla* gavaged in mg/kg body weight					
A. stenophylla	**250mg**	**500mg**	**1000mg**	**2000mg**	**Control**
Food intake	Normal	Normal	Normal	Normal	Normal
Water intake	Normal	Normal	Normal	Normal	Normal
Death	Alive	Alive	Alive	Alive	Alive
Breathing	Normal	Normal	Normal	Normal	Normal
Diarrhoea	Not observed	Not observed	Not observed	Not observed	Not observed
Urination	Normal	Normal	Normal	Normal	Normal
Skin colour	Normal	Normal	Normal	Normal	Normal
Drowsiness	Not observed	Not observed	Not observed	Not observed	Not observed
Erection of Fur	Not observed	Not observed	Not observed	Not observed	Not observed

The test results and observations from the toxicity profiling validate that the hydro-ethanolic root extract of *A. stenophylla*, is safe for internal use up to 2000mg/kg body weight (Table 3). These results were expected, as the plant is generally considered to be safe from the accumulated experiences and testimonies of traditional medicinal practitioners over a long history of usage in Southern Africa[4,5,6]. Our literature search could not find any report of any adverse effects arising from consumption of any part of the dwarf plant or its fruit and appendages. This was the justification for us to start off with a moderately high dosage level of 250mg/kg body weight in the experiments. The behavioural factors under assessment which include signs of restlessness among the study animals, painful response to touch, urine characteristics and urination frequency, skin texture, morphology and colour, fur condition and erection, as well as food and water intake were periodically journalised by an experienced veterinary specialist (Table 3). No adverse observations were noted with regards to symptoms and signs of toxicity for all the parameters under review and no deaths were recorded for the entire duration of the testing period. All changes observed in these acute oral toxicity studies were within normal physiological ranges. These results correlate well with similar oral toxicity profiles of *A. stenophylla* reported in other publications. Acute and sub-acute oral toxicity of the hydroethanolic root extract of *A. stenophylla* was also studied in Sprague Dawley rats and there were no adverse reactions noted up to 2000 mg/kg body weight over a 14-day period[6]. The biochemical tests on internal organs and hormonal levels including histopathological tissue sections of the pancreas and liver also revealed no significant subacute damage.

3.3.1 Behavioural pattern and LD50.

The up and down test as outlined by OECD technical guideline 425 with minor modifications was used in this study. As per the guideline, only healthy adult nulliparous and non-pregnant females were chosen for the study[26]. The rats were aged between 8-12 weeks as required by the technical guideline 425. The animals were all fasted before dosing overnight with only water provided for them. Before commencement of dosing, the animals were weighed and checked for any adverse health indications. The acute oral toxicity profile study of the *A. stenophylla*, extract was carried out using Wistar laboratory bred rat models at doses of 250, 500, 1000 and 2000mg/kg body weight. The experimental rats were routinely observed, and their behaviour monitored during the experiments for changes in body weight and other observable indicators of poor health effects.

As reported, above there were no deaths and no withdrawals from the study due to adverse health symptoms of participating animals. There were no notable changes observed in all rats for any of the categories. The study concluded that *A. stenophylla*, was toxicologically safe at 2000mg/kg body weight and therefore LD50 is concluded to be beyond 2000mg/kg body weight. With reference to the Hodge and sterner classification for toxicity, the hydro-ethanolic root extract of *A. stenophylla* is classified as nontoxic[36].

UNDER PEER REVIEW

3.3.2 Bodyweight observations.

As part of the protocol for toxicity studies, during the observation period, the body weights of the Wistar rats participating in the study were monitored routinely and recorded. The weights for all the rats in the observation groups including the control were recorded weekly during the test period starting on the initial day, then on the 7th, 14th and the 21st day thereafter. In all the recorded weights, all 4 treated groups did not exhibit statistically relevant or significant aberrations in body weight in comparison with the control group.

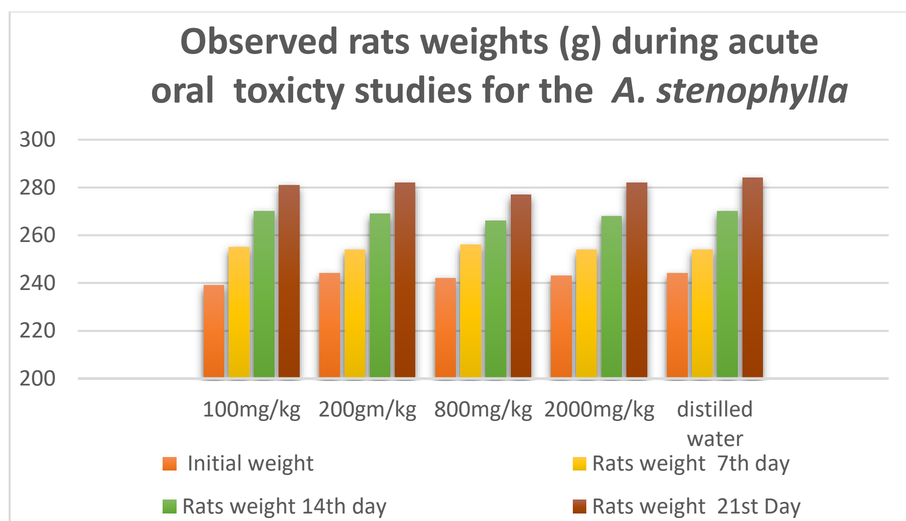

Figure 6: Observations for rat's weights over the experimental period

Weight fluctuations and irregular growth patterns in animals are usually indicative of underlying health conditions and physical stress. Disturbances and changes in metabolism characteristic of internal failures of the homeostatic processes and unexplained gains or losses in weights can be symptoms of toxicity[37]. Sick animals struggle to eat, and many scientific reports suggest that the reduction of food intake in sick animals is controlled by inflammatory cytokines, which relay a signalling message from the immune system to the endocrine system and central nervous system[38]. It therefore is apparent that appetite loss and failure to feed is directly related to adverse health conditions latent or otherwise. The initial weights for all the rats selected for the study were within specifications for their age. The observations from this study confirmed the expected gradual weight increase over a progressive three-week period of normal feeding expected from normal healthy animals. The body weight changes among the groups and the control were not statistically significant. The normal progressive body weight increases observed, and the continued normal feeding appetite is a confirmation of the absence of any toxicity effects on the rats from *A. stenophylla* and correlates well with the absence of any deaths among the animals under the tests. This gives confidence that *A. stenophylla* root extracts do not interfere with the normal metabolism and health of the animals.

4 Conclusions

In this study we presented the biosynthesis of ZnONPs using *A. stenophylla* extracts via an ecofriendly green synthesis route. The abundant polyphenols acted as the bio reducing, capping and stabilising agents. The ZnONPs obtained were spherical, between 40 and 60nm in diameter and showed a characteristic UV-VIS absorption spectra peak at 370 nm. Related peaks at 270nm indicate the participation of polyphenolic compounds in the extract. Furthermore, the phytoscreening confirmed the presence of pharmacologically active secondary metabolites which correlate with the traditional use of A. stenophylla in the management of diabetes. The absence of any symptoms and signs of toxicity on the laboratory animals confirm the safety of the hydro ethanolic extracts of *A. stenophylla.* Our findings therefore suggest the possibility of combining the proven anti-diabetic effects of ZnO with traditionally acknowledged anti-diabetic lyophilised plant extracts through biosynthesis of novel, safe and effective *A. stenophylla* mediated ZnONPs. Future work should investigate the hypoglycaemic effects mechanisms of the *A. stenophylla* biosynthesized ZnONPs.

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