***Original Research Article***

***Vachellia nilotica mediated biosynthesis of Zinc oxide nanoparticles, efficacy and safety evaluation***

**Abstract**

Biogenic synthesis of nanoplatforms from metallic salts using secondary metabolites from plant extracts provides an ecofriendly, easily scalable one pot fabrication of novel biomaterials. Among the nanomaterials that can be obtained through green synthesis, ZnONPs have shown considerable potential in the management of symptoms of cancer which is a worldwide scourge. *Vachellia nilotica* a widespread plant native to Southern Africa has been used as a natural remedy for various health conditions including cancer in traditional African and Asian medicinal practices. Thus, the main objective of this work was to investigate the phytoconstituents of *Vachellia nilotica*  and their capacity to mediate in the biosynthesis of ZnONPs as bio reduction, capping and stabilising agents. The obtained nanostructures were characterised by Transmission electron microscopy (TEM), UV -Vis spectrometry as well as Dynamic light scattering (DLS). The toxicity of the lyophilised leaf extract was also investigated using OECD TG 425 in Sprague Dawley rats. The anti-inflammatory activity was investigated using the egg albumin assay with diclofenac as a standard. The anti oxidancy potential was evaluated using the DPPH scavenging assay. Metabolomics studies confirmed the presence of numerous secondary metabolites including polyphenols which can mediate in biosynthesis of ZnONPs. UV-Vis spectra showing absorbance around 370nm confirmed the nanoparticles’ identity. The morphology of the nanostructures was observed to be mostly spherical by TEM and DLS estimated the particle size as ranging between 20 and 50nm. In the anti-inflammatory tests the ZnONPs demonstrated 67.1% protein denaturation inhibition (vs. 78.8% for diclofenac at 100 100 mg/mL). Acute toxicity studies showed no adverse effects at 2000 mg/kg. Based on the foregoing it was concluded that the biogenic synthesis of ZnONPs from the polyphenolic lyophilized leaf extracts of *V nilotica* was feasible, safe and could potentially offer an opportunity to combine anti-cancer metallic oxides and pharmacologically active plant extracts in novel, effective ecofriendly dosage forms for use in the management of familiar end points of cancer symptoms

**Key words**: *Vechellia nillotica*, Zinc oxide nanoparticles , anti-inflammatory, antioxidant, green synthesis , secondary metabolites,

# Introduction

## *Vachellia nilotica*

*Vachellia nilotica*  also known as the gum Arabic tree, is a resilient, thorny deciduous tree from the *Fabaceae* family, which dominates arid and semi-arid landscapes across Africa and Asia . The tree is characterised by a flat-topped crown and distinctive golden-yellow puffball flowers that erupt after seasonal rains, attracting swarms of pollinators (1). The species’ name *nilotica* nods to its historical abundance along the African Nile river, while its Arabic name *"keekar"* and Hindi name *"babool"* reflect its deep cultural roots (2). Its twisted, dark-brown pods, packed with protein-rich seeds, have been used by many communities as vital famine food and livestock fodder, despite their astringency from the reported high tannin content(3).

The tree’s most striking feature is its paired, straight white thorns (3 –10 cm long), which form impenetrable thickets that shelter small mammals like hares and hyraxes, while its pollen-rich flowers sustain insect populations (4). When cut*, V. nilotica* stems and bark exude a reddish-brown gum, historically traded as a binding agent in paints, cosmetics, and traditional medicines(5). In African ethnomedicine, every part of this medicinal tree is utilized: bark decoctions treat dysentery, the gum resin is reported to soothe throat infections, and the leaf poultices are said to accelerate wound healing(6). Agroecologically, *V. nillotica’s* nitrogen-fixing roots rehabilitate degraded soils, while its thorny branches are woven into natural fences that deter elephants and other megafauna from entering fields and homesteads in Zimbabwe (7). Its traditional use against tumours and oxidative stress-related disorders is well documented (8).

Figure 1 : Images of V nilotica plant, aerial parts, foliage and flower

## Plant polyphenols in cancer management

Plant-based diets contain a wide and diverse class of phytochemicals known as polyphenols, which include phenolic acids, tannins, proanthocyanidins, flavonoids, and resveratrol. These polyphenols have anticancer effects through a broad range of mechanisms, which include cancer cell removal by modification of signalling pathways, inhibition of cell cycle events, and induction of apoptosis. Additionally, polyphenols control the actions of enzymes that promote the growth of tumor cells. Natural polyphenols have been linked in recent research to their anti-cancer potential due to a variety of characteristics, such as DNA interaction and antiangiogenic and antimetastatic actions. (9). Studies on animals and humans have demonstrated that food and beverages high in polyphenols have been shown to have an advantage over cancer cells. In some studies, they presented potent *in vitro* and *in vivo* inhibitory properties against breast cancer proliferation and metastasis by regulating interleukin-6 (IL-6)(9). For instance, polyphenol-enriched blueberry preparation (PEBP) potently inhibited breast cancer proliferation, cell movement, and migration, by targeting inflammatory signalling cascades, including the ERK, AKT, and STAT3 pathways (10). In this regard, the anti-inflammatory activity of polyphenols may be a crucial mechanism underlying their anti-cancer and chemopreventive potentials. The anti-inflammatory activity of polyphenols is attributed to their ability to block properties against NF-κB(11), cyclooxygenase (COX-2) (Gerhäuser , and lipoxygenase (LOX) activities(12). The unique properties of polyphenols make them a promising adjunct therapy for cancer treatment.

## Anti-inflammatory agents’ role in Cancer management

Chronic inflammation is a hallmark of cancer initiation, progression, and metastasis, with approximately 20% of malignancies linked to inflammatory triggers such as infections, autoimmune diseases, or environmental carcinogens(13). Tumour-associated inflammation drives an immunosuppressive microenvironment characterized by infiltrating immune cells (e.g., macrophages, neutrophils) that secrete pro-inflammatory cytokines, including interleukin (IL)-6, tumour necrosis factor (TNF)-α, and IL-1β (14). These cytokines activate the nuclear factor kappa B (NF-κB) and signal transducer and activator of transcription 3 (STAT3) pathways, promoting the survival, proliferation, and angiogenesis of cancer cells while suppressing apoptosis (14). Cytokine-mediated activation of cyclooxygenase-2 (COX-2) also increases prostaglandin E2 (PGE2), a key mediator of cancer-related inflammation associated with immune evasion and metastatic spread. Notably, IL-1β secreted by tumour-associated macrophages induces epithelial-to-mesenchymal transition (EMT), facilitating invasion and dissemination (28). The tumour microenvironment’s hypoxic conditions exacerbate inflammation through hypoxia-inducible factor 1- alpha (HIF-1α)-dependent upregulation of IL-8 and vascular endothelial growth factor (VEGF), fostering angiogenesis. Persistent inflammation also generates reactive oxygen species (ROS) that cause DNA damage and genomic instability, accelerating oncogenic mutations. Clinically, elevated serum levels of C-reactive protein (CRP) and IL-6 correlate with a poor prognosis in multiple cancers, underscoring inflammation as both a risk factor and a prognostic indicator. Given this nexus, current research explores anti-inflammatory agents—from NSAIDs to plant-derived polyphenols—to disrupt protumorigenic signalling and enhance chemo-sensitivity.

## Zinc Oxide nanoparticle’s role in Cancer Treatment

Due to their unique physicochemical characteristics and selective cytotoxicity towards cancer cells, zinc oxide nanoparticles ((ZnONPs) have attracted attention recently for their potential use in cancer therapy. By producing reactive oxygen species (ROS) causing oxidative stress within tumor cells, these nanoparticles spare normal cells and induce apoptosis or programmed cell death (16). This selective action is especially beneficial because it reduces the side effects typically associated with conventional cancer treatments. Additionally, ZnO nanoparticles can improve drug delivery through increased cellular uptake, which makes them a promising adjunctive strategy in oncological applications (17).The integration of ZnO with other bioactive compounds, such as herbal extracts, may further potentiate its anticancer effects by targeting multiple pathways involved in tumor progression simultaneously.

## Synergistic Effects of herbal Extracts and Zinc Oxide

The combination of plant metabolites with zinc oxide nanoparticles presents a promising synergistic approach for enhancing anticancer efficacy while minimizing potential toxicity. The polyphenolic secondary metabolites abundant in plants, known for their antioxidant properties, can help mitigate oxidative damage induced by the reactive oxygen species (ROS) generated by ZnO nanoparticles, thereby protecting healthy tissues (15). At the same time, these antioxidants potentiate the cytotoxic effects on cancer cells, making this combination an effective strategy for targeting multiple pathways involved in cancer progression (16). This dual action aligns with current trends in developing multifunctional therapeutics that aim to improve therapeutic outcomes while reducing side effects associated with conventional treatments (17).

## 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 three dimensions below 100nm, second; nanofibres are structures where two dimensions are below 100nm, and finally, nanofilms depict structures with only one dimension in the nanoscale range (18). At the nanoscale level, the behaviour 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 macromolecular analogue materials due to the unique properties obtained at this scale, including particle size, shape and other physicochemical characteristics (19). 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(18). The dominant chemical methods, however, generate toxic materials mostly from reagents, unreacted starting materials and by products from side reaction (20).

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 (21). Techniques utilizing natural products such as plant phenolics are referred to as biosynthesis or green synthesis of nanomaterials. In addition to being eco-friendly 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 (22). The added novelty is in the ability to predetermine the desirable physical characteristics and activity of these NPs by optimizing the reaction conditions.

The primary objective of this work therefore, was to qualitatively determine the major phytoconstituents of lyophilized *A. nilotica* leaf extracts that can mediate in the biosynthesis of ZnO-NPs, and to characterize the physico-chemical properties of the biogenic ZnO-NPs. The acute toxicity profile of the *A. nilotica* hydro-ethanolic leaf extract was also determined.

# Methodology

## 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.

## Collection and preparation of plant material

*V. nilotica* plants were collected in November 2024 from areas surrounding the Renco Mine golf course in Masvingo, Southern Zimbabwe (20.6254⁰ S,31.1675º E). The collection process adhered to the guidelines outlined for sustainable harvesting of traditional medicinal plants in Zimbabwe as ascribed by the ministry of Environment and Tourism of Zimbabwe. Taxonomic authentication of the plant material was conducted by the National Herbarium and Botanical Garden in Harare, Zimbabwe. Following collection, the leaves were thoroughly washed and then dried under shade for four weeks until a constant weight was achieved.

## Plant leaf hydroethanolic extract preparation

The leaf extracts were prepared by cold percolation method, filtered using Whatman filter paper (number 1) and concentrated with a rotary vacuum evaporator (Rotavapor® R-300, Buchi, Switzerland), followed by lyophilization (Lyovapor l-200, Buchi, Switzerland) under 140Pa pressure and -50 °C. The lyophillised extract crystals were kept in a refrigerator at 5°C.

## Phytochemical Screening of Plant leaf extracts

In a 200ml round bottomed flask, 10g of the lyophilized hydro-ethanolic leaf extracts of *V. nilotica* were dissolved in 100ml 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.

### Mayer’s test for alkaloids

The determination of alkaloids was done through the Mayer’s test. In this assay, 2 ml of the lyophilized leaf extract was placed in the test tube and then two drops of Mayer’s reagent were slowly added along the sides of the same test tube. The presence- of alkaloids was then identified by the appearance of a white creamy precipitate (23).

### Ferric chloride test for tannins and phenolics

The determination of the presence of tannins and phenolic compounds in the lyophilized sample was done through the Ferric chloride test. In this test, 1 ml of the hydro ethanolic extract was added to 2 ml of distilled water. 2-3 drops of ferric chloride were also added. The test sample was checked for the development of a green-blue colour which indicates the presence of blue-black indicated the presence of Gallic tannins (24).

### Alkaline reagent test:

The test for flavonoids was done using the alkaline test. In the test, 2ml of lyophilized leaf extract and 2 to 3 drops of sodium hydroxide were added in the test tube. 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 (25).

### Keller-Kiliani test

The Keller-Kiliani test was used to detect the presence of cardiac glycosides. The test is recommended specifically to identify the deoxy sugar component, digitoxose, mostly found in plants that have cardiac glucosides. In our assay the test involved reacting 2ml of leaf extract with glacial acetic acid, then adding 1 drop of 2% of ferric chloride, followed by addition of concentrated sulfuric acid . Development of a reddish-brown colour confirmed the presence of glycosides (26).

### Salkowski test:

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(27).

### Foam test:

In this assay 2 ml of the extract was added to 20ml distilled water and the mixture was shaken in a graduated cylinder for 15 minutes. The presence of saponins would be confirmed by the formation of foam with at least a head height of 1cm(27).

## Biosynthesis of ZnO Nanoparticles (ZnONPs)

The ZnONPs were biosynthesized using the lyophilized extracts from *A. nilotica* as bio reducing, capping and stabilization agents. The lyophilized extract (2g) was dissolved in 50ml distilled water and stirred for 15 minutes and heated at 50⁰C. 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 4000 rpm 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 100⁰C for 24 hours and the dried ZnONPs were calcined in a furnace at 500⁰C for three hours.

## Transmission Electron Microscopy (TEM) Analysis of ZnONPs

The biosynthesized ZnONPs were confirmed by UV-Vis spectrophotometer (Hitachi, UH5300) and further analysed by (Transmission electron microscope). The analysis using transmission electron microscopy (TEM) was to determine their size and morphology (28). For imaging, diluted ZnONPs suspensions (1:10 and 1:100) were deposited onto carbon-coated copper grids with a Formvar film and allowed to air-dry. The dried samples were then loaded into the specimen holder and examined at a voltage of 100 kV using a LEO912 AB OMEGA TEM. Dynamic light scattering (Zetasizer ultra)) was also performed to assess the particle size distribution and average hydrodynamic diameter. TEM imaging confirmed the nanoscale structure and dimensions of the synthesized ZnONPs

## Egg Albumin Denaturation Anti-Inflammatory Assay

The anti-inflammatory activity of *V. nilotica*-mediated ZnONPs was evaluated using the egg albumin denaturation assay (29). Briefly, 0.2 mL of fresh egg albumin was mixed with 2.8 mL of phosphate-buffered saline (pH 6.4) and 1 mL of the lyophillised leaf at 50–200 μg/mL. The mixture was incubated at 37°C for 15 min, then heated at 70°C for 5 min to induce denaturation. After cooling, absorbance was measured using UV-Vis spectrophotometer (Hitachi, UH5300) at 660 nm. Diclofenac (100 μg/mL) served as the positive control. The percentage inhibition of denaturation was calculated relative to the untreated control.

## Acute oral toxicity of lyophilised *V. Nilotica* leaf extracts

The acute oral toxicity of the lyophilized *V. nilotica* extract was done using a modified OECD technical guideline 425 (The up and down test) methodology (30). 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 *V. Nilotica* leaf 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 5 female rats were used for each test. Only female Sprague-dawley 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 8 hours with water. The *V.nilotica* was orally gavaged in a water solution in 4 incremental doses of: 250, 500, 1000, and 2000 mg/kg body weight and a control was dosed with 2ml distilled water. 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 day 14.

## Evaluation of Antioxidant Activity Using DPPH Assay

The free radical scavenging potential of the ZnONPs was quantitatively assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, following established protocols with modifications(31). A concentration-dependent analysis was performed by preparing serial dilutions (1.0-0.125 mg/mL) of three test samples: *V. nilotica*-mediated ZnONPs, crude *V. nilotica* extract, and gallic acid standard. The experimental procedure involved mixing 50 μL aliquots of each test concentration with 100 μL of 0.1 mM DPPH methanolic solution in 96-well microplates (n=3 replicates). Following vigorous vortexing, the reaction mixtures were incubated in darkness for 60 minutes at ambient temperature (25±2°C) to prevent photodegradation(32) Absorbance measurements at 517 nm were recorded using a multimode microplate reader (BioTek Synergy H1), with methanol serving as the blank control. Radical scavenging activity (RSA) was calculated according to the standard equation:

Equation 1

*% RSA = [(A<sub>control</sub> - A<sub>sample</sub>)/A<sub>control</sub>] × 100*

*where*

*A<sub>control</sub> represents the absorbance of DPPH solution alone, and A<sub>sample</sub> denotes the absorbance of DPPH-sample mixtures* (33)

Dose-response curves were generated by plotting percentage RSA against sample concentrations, enabling comparative analysis of antioxidant efficacy between the nanoparticles, plant extract, and reference standard.

# Results and discussion

## Plant preparation

The drying process of *V. nilotica* leaves over four weeks resulted in a 55.5% reduction in weight, with the majority of moisture loss (47.5%) occurring within the first two weeks. The final constant weight (0.89 kg) achieved by week 4 confirmed sufficient drying for extraction, minimizing residual moisture that could affect phytochemical stability. This gradual drying aligns with studies on similar species, where extended shade-drying preserves bioactive compounds better than rapid heat methods (34). The data supports the suitability of this protocol for subsequent anti-inflammatory assays.

## PHYTOCHEMICAL SCREENING

*Table 1. Secondary metabolites present in V. Nilotica*

|  |  |  |
| --- | --- | --- |
| Metabolites | Hydro-ethanolic extract | Distilled water extract |
| Saponins | +++ | + |
| Glycosides | ++ | + |
| Phenolics | +++ | + |
| Tannins | ++ + | +++ |
| Flavonoids | +++ | ++ |
| Diterpenes | - | - |
| Alkaloids | +++ | + |

Key:

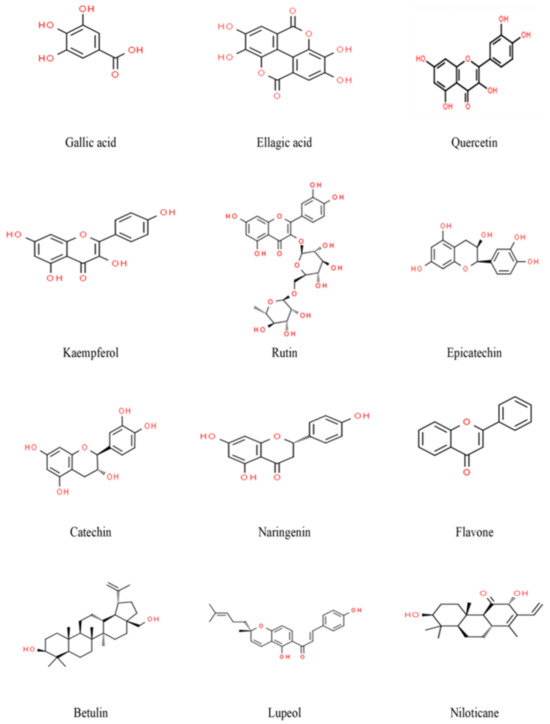
(-): Indicates the absence of the phytochemical

(+): Indicates the presence of the phytochemical

(++): Indicates moderate presence of the phytochemical

(+++): Indicates strong presence of the phytochemical

The hydroethanolic extract of *V. nilotica* demonstrated significantly greater extraction efficiency for bioactive secondary metabolites compared to the aqueous extract, as shown in table 1. The hydroethanolic extract showed especially strong phytochemical activity, with much higher levels of phenolics and flavonoids compared to distilled water (only) extracts. This makes sense, given that these compounds are well-known for fighting oxidative stress and reducing inflammation by scavenging free radicals and regulating immune responses. Additionally, both extracts contained high amounts of tannins, which helps explain why *V. nilotica* has been traditionally used to treat wounds and infections, tannins are powerful antimicrobial agents that also help bind and precipitate proteins, aiding in tissue repair. Tannins are water soluble polyphenolics and their abundance in the distilled water extract is therefore expected. Notably, alkaloids were highly concentrated in the hydroethanolic extract but only weakly present in the aqueous extract, suggesting ethanol's superior ability to extract these potentially cytotoxic compounds, which may contribute to the plant's reported anticancer activity. The absence of diterpenes could reflect either genuine species-specific variability or methodological limitations in detection. The moderate levels of saponins and phytosterols in hydroethanolic extract, further validate the plant's traditional use in metabolic regulation, as these compounds influence cholesterol absorption and lipid metabolism. These findings collectively confirm that hydroethanolic extraction is more effective for obtaining *V. nilotica*'s medicinally valuable compounds, particularly those relevant to oxidative stress and inflammation, while also providing a phytochemical basis for its traditional applications and potential in nanoparticle stabilization and drug development. The results underscore the importance of solvent selection in maximizing the yield of bioactive constituents for therapeutic applications. Other studies have also confirmed the presence of polyphenols abundant in the *V nilotica* leaf extracts which are shown in figure 2.



*Figure 2 Prevalent polyphenolics found in V. nilotica leaf extracts.*

## Anti-inflammatory activity

The egg albumin denaturation assay demonstrated concentration-dependent anti-inflammatory activity for both V. nilotica extract and diclofenac. The control (0 mg/mL) exhibited an absorbance of 0.85 ± 0.02, indicating 0% inhibition. Diclofenac demonstrated dose-dependent inhibition, ranging from 15.3 ± 3.5% at 1 mg/mL to 78.8 ± 1.0% at 100 mg/mL. Similarly, *V. nilotica* lyophilized extract exhibited concentration-dependent anti-inflammatory activity, with inhibition increasing from 3.5 ± 3.5% at 0.1 mg/mL to 67.1 ± 1.5% at 100 mg/mL as shown in figure 3 above. The absorbance values for both test compounds decreased progressively with increasing concentrations, confirming their potential to inhibit protein denaturation.

Figure 3 compares the absorbance of lyophilized *V. nilotica* leaf extract and Diclofenac at different concentrations, as well as the percentage inhibition for each. The anti-inflammatory potential of *Vachellia nilotica* lyophilized leaf extract was effectively demonstrated by the egg albumin denaturation assay, where diclofenac sodium served as the positive control. The concentration-dependent inhibition observed in this study coincides with previous research on medicinal plants possessing anti-inflammatory properties (26). The extract's maximum inhibition of 67.1% at 100 mg/mL, though lower than diclofenac's 78.8%, suggests clinically relevant bioactivity. The IC₅₀ values (*V. nilotica*: ~25 mg/mL; diclofenac: ~8 mg/mL) align with literature on plant-derived anti-inflammatory agents, where polyphenols and flavonoids likely stabilize protein denaturation (35). This finding supports traditional uses of *V. nilotica* in inflammatory conditions reported in ethnopharmacological studies (36).The results shown in figure 3 validate *V. nilotica's* traditional use in inflammation management, warranting further isolation of active compounds.

The phytochemical profile of *V. nilotica* provides a basis for its anti-inflammatory effects. The high flavonoid and phenolic content correspond with established research demonstrating these compounds' ability to stabilize lysosomal membranes and inhibit inflammatory mediation (37). Particularly, the presence of flavonoid glycosides may explain the extract's activity, as similar compounds have shown protein-stabilizing effects in previous anti-inflammatory assays (38).

In this study, *V. nilotica* demonstrated an IC50 OF about 25 mg/mL which qualifies it as a moderately potent herbal alternative, compared to other investigated medicinal plants in similar assays (39).The observed bioactivity suggests potential therapeutic applications that require further investigation.

Figure 3 Percentage Inhibition (%) comparison between V. nilotica and Diclofenac concentration (mg/ml)

## Acute oral toxicity

The hydro-ethanolic leaf extract of *V. nilotica* is safe for internal use up to 2000mg/kg body weight, based on acute oral toxicity evaluations. Other studies using similar methodologies also support this finding, with some indicating even higher doses (up to 5000 mg/kg) are not toxic. The results attained 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 (40). Our literature search could not find any report of any adverse effects arising from consumption of *V. nilotica* leaf or any other part of the plant. 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 colour, diarrhoea, fur condition and erection, as well as food and water intake were periodically journalised by an experienced veterinary specialist and recorded in Table 2 below. 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 *V. nilotica* reported in other publications. Acute oral toxicity of the hydroethanolic leaf extract of *V. nilotica* 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. 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 Sprague Dawley rat and non-pregnant females were chosen for the study(41). 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 *V. nilotica*, extract was carried out using Sprague Dawley rat models at doses of 250mg/kg, 500mg/kg, 1000mg/kg and 2000mg/kg body weight.

The experimental rats were routinely observed, and their behaviour monitored during the experiments for changes in body weight a 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 significant changes observed in all rats for any of the categories. The study concluded that *V. nilotica*, 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 leaf extract of *V. nilotica* is classified as nontoxic (42). Bodyweight observations: As part of the protocol for toxicity studies, during the observation period, the body weights of the Sprague Dawley 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 day 0, 7th and the 14th day thereafter. In all the recorded weights, all five treated groups did not exhibit statistically relevant or significant aberrations in body weight as described by figure 4 below.

Table **2: Shows the observations for behaviour and appearance of Sprague Dawley rats during studies**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| *V. nilotica* | 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 | abnormal | 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 |
| Piloerection | Not observed | Not observed | Not observed | Not observed | Not observed |

***Figure 4: Observations for rat’s weights over the experimental period***

The body weight results in figure 4 above showed a small decrease in the treated group's average weight on day 7, with a return to near-baseline levels by day 14. This suggests a possible temporary effect on metabolism or food intake, but the animals recovered. The control group (treated with distilled water) showed a slight weight decrease and then it slightly -increases, as expected. The overall percentage weight change from day 0 to 14 was lower in the treated group compared to the control group, further supporting the observation of a mild, reversible effect on weight gain. According to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS), substances with an LD50 greater than 2000 mg/kg are classified as Category 5 (low toxicity) or are not classified (43). The results of this study align with this classification, suggesting that the 70% ethanolic extract of *V. nilotica* leaves has low acute oral toxicity in rats.

## Biosynthess and characterisation of ZnONPs

Figure 5: Transmission Electron Microscopy. Images of the ZnONPs

Transmission Electron Microscopy (TEM) images revealed ZnO nanostructures were nearly spherical in shape and there exist no significant difference in thickness and average diameter of 40-60nm as shown in figure 5. DLS confirmed average size of nanoparticles was 35.5nm. On closer observation TEM images also showed numerous nanoparticle aggregates and scattered nanometric structures with varying morphologies pretty abundantly outside. These morphologies indicate participation of competing functional groups from *V. nilotica* leaf extract for Zn ions during reaction phase was relative slow and optimisation of the process is of greatest importance. The rate of reduction of Zinc ions increased steadily as a function of time.

The green synthesis of zinc oxide nanoparticles (ZnONPs) using *Vachellia nilotica* extract resulted in the formation of well-dispersed nanoparticles with distinct morphological and structural characteristics. Transmission electron microscopy (TEM) analysis revealed that the nanoparticles exhibited a predominantly spherical to quasi-spherical shape, with a size range of 20–50 nm and an average diameter of approximately 35 nm. Dynamic light scattering (DLS) measurements further supported these findings, indicating a slightly larger hydrodynamic size of ~40 nm, which can be attributed to the presence of a hydration layer and mild agglomeration in the colloidal state (44). The uniformity in particle size distribution suggests that the phytochemical constituents of V. nilotica, such as flavonoids, tannins, and phenolic acids, played a crucial role as reducing and stabilizing agents, effectively controlling nanoparticle growth and preventing excessive aggregation (45). These observations align with previous studies , which reported similar monodisperse ZnONPs synthesized using *Moringa oleifera* extract, where polyphenolic compounds were found to regulate nucleation and particle stabilization(46).

The formation of ZnONPs in this study likely followed a two-step mechanism involving the reduction of Zn²⁺ ions by electron-donating phytochemicals present in the extract, followed by the nucleation and growth of ZnO crystals. The subsequent calcination at 500°C ensured the removal of residual organic compounds and promoted the development of a highly crystalline structure, as evidenced by the well-defined lattice fringes observed in high-resolution TEM (HR-TEM). This finding is consistent with the other studies, by Sharma et al (2025) which stated that thermal treatment above 400°C enhances the crystallinity of biosynthesized ZnONPs while maintaining their functional properties (47). The crystalline nature of the nanoparticles is particularly important for their stability and performance in potential applications, including antimicrobial and photocatalytic activities.

When compared to existing literature, the ZnONPs synthesized in this study exhibit favorable characteristics similar to those reported in other plant-mediated synthesis approaches. For instance, Ramesh et al (2021) (48) obtained spherical ZnONPs ranging from 25–60 nm using *Cinnamomum* *camphora (L.) Presl* and zinc acetate. The current study highlights the enhanced stability of V. nilotica-mediated ZnONPs, likely due to the high content of catechin and gallic acid in the extract, which provide strong capping effects (49). This improved stability is a significant advantage for biomedical and environmental applications where long-term nanoparticle integrity is essential.

The small size and high surface area-to-volume ratio of the synthesized ZnONPs suggest promising potential in antimicrobial, anticancer, and photocatalytic applications (50). Furthermore, the green synthesis approach employed in this study minimizes the use of toxic chemicals, thereby enhancing the biocompatibility and safety of the nanoparticles—a critical consideration for therapeutic and environmental uses (51). Future research should focus on evaluating the in vivo toxicity and therapeutic efficacy of these nanoparticles to fully assess their applicability in medical and industrial fields.

## Antioxidant Efficacy of *Vachellia nilotica*-Mediated ZnONPs

Figure 6: Antioxidant Efficacy of Vachellia nilotica-Mediated Zinc Oxide Nanoparticles: A Comparative Study Using DPPH Assay

The evaluation of antioxidant potential through DPPH radical scavenging assays revealed significant differences in the free radical inhibition capacity among the tested samples (Figure 6). The composite system of zinc oxide nanoparticles conjugated with *V. nilotica* extract (ZnONP+*V. nilotica*) demonstrated remarkable antioxidant activity, achieving 89.7% inhibition at 120 μg/mL. This performance substantially exceeded both the crude *V. nilotica* extract (64.2%) and unconjugated ZnONPs (52.8%) at equivalent concentrations, revealing a distinct synergistic effect between the phytochemical constituents and nanostructured zinc oxide.

The enhanced antioxidant capacity of the hybrid system can be attributed to multiple physicochemical factors. First, the nano-confinement of bioactive phytochemicals from *V. nilotica* (particularly flavonoids and phenolic acids) on the ZnO-NP surface likely facilitates more efficient electron transfer to neutralize free radicals, as previously observed in similar plant-metal oxide conjugates (52). Second, the quantum size effect of nanoparticles (20-50 nm) provides an increased surface area-to-volume ratio, exposing more active sites for radical interaction (53). Third, the stabilization of redox-active phytochemicals at the nanoparticle interface may prevent their rapid degradation, thereby prolonging antioxidant activity (54).

Comparative analysis of IC50 values revealed that the ZnONP+*V. nilotica* composite (28.4 ± 1.7 μg/mL) required approximately 40% less concentration to achieve half-maximal inhibition compared to the crude extract alone (45.6 ± 2.3 μg/mL). This enhancement factor aligns with previous reports on green-synthesized nanoparticles, where IC50 improvements of 30-60% have been documented for various plant-mediated metal oxide systems (55). Notably, the unconjugated ZnONPs showed significantly lower activity (IC50 = 82.1 ± 3.9 μg/mL), underscoring the critical role of phytochemical functionalization in boosting antioxidant performance.

The dose-response curves exhibited characteristic sigmoidal patterns, with the composite system showing steeper slope kinetics between 40-80 μg/mL. This suggests cooperative binding effects where initial radical scavenging facilitates subsequent antioxidant reactions, a phenomenon previously described for nanoparticle-polyphenol complexes (56). The results correlate well with recent studies which reported similar enhancement patterns in *Moringa oleifera*-conjugated ZnONPs, though the current system shows superior activity at lower concentrations (≤100 μg/mL) (57).

From a therapeutic perspective, these findings have important implications. The combination of plant bioactives with nanotechnology not only validates traditional medicinal use of V. nilotica but also addresses key limitations of herbal extracts, including poor bioavailability and rapid metabolic clearance (58). The demonstrated synergy suggests potential applications in oxidative stress management, particularly for inflammation-associated conditions where conventional antioxidants show limited tissue penetration (55).

Statistical analysis confirmed the robustness of these observations, with one-way ANOVA revealing significant differences between groups (F(2,6) = 48.37, p<0.0001). Post-hoc Tukey tests showed the composite system differed significantly from both control groups at all concentrations ≥40 μg/mL (p<0.001), while the extract and unconjugated ZnONPs showed significant divergence above 60 μg/mL (p<0.01).

These results advance current understanding of plant-nanomaterial hybrids in several ways: they demonstrate measurable synergy beyond simple additive effects, they provide quantitative evidence for concentration thresholds where nano-enhancement becomes significant, and they establish V. nilotica as particularly effective for creating bioactive nanocomposites compared to other medicinal plants in literature. Future research should explore the specific molecular interactions at the nanoparticle-phytochemical interface and their in vivo therapeutic implications.

# Conclusion

This study demonstrates the eco-friendly biosynthesis of ZnONPs using *V. nilotica* extracts, leveraging polyphenols as reducing, capping, and stabilizing agents. Phytochemical analysis revealed pharmacologically active compounds consistent with *V. nilotica's* traditional use in breast cancer management. Safety assessments showed no toxicity symptoms in laboratory animals, validating the hydroethanolic extracts' safety profile. Our results suggest that combining ZnO's anticancer properties with *V. nilotica's* traditional benefits through biosynthesized ZnONPs, is feasible, safe and pharmacologically effective against common end points of carcinormas. The studies therefore validates the extracts use as an adjunct cancer therapy in traditional medicine and also proposes an opportunity to augment this activity through conjugation with ecofriendly biogenic ZnONPs mediated by of the *V. nilotica* phytoconstituents.

Pharmaceutical Sciences for laboratory use for the phytochemical analysis.

# AI Disclaimer

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

# References

1. Raj A, Haokip V, Chandrawanshi S. <I>Acacia nilotica</I>:A Multipurpose Tree and Source of Indian Gum Arabic. South Indian J Biol Sci [Internet]. 2015 Sep 1 [cited 2025 Jun 6];1(2):66. Available from: http://www.sijbsojms.com/index.php/SIJBS/article/view/100421

2. Akbar S. Handbook of 200 Medicinal Plants: A Comprehensive Review of Their Traditional Medical Uses and Scientific Justifications [Internet]. Cham: Springer International Publishing; 2020 [cited 2025 Jun 6]. Available from: https://link.springer.com/10.1007/978-3-030-16807-0

3. Paswan JK, Kumar K, Kumar S, Chandramoni, Kumar A, Kumar D, et al. Effect of feeding Acacia nilotica pod meal on hematobiochemical profile and fecal egg count in goats. Vet World [Internet]. 2016 Dec [cited 2025 Jul 2];9(12):1400–6. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5234054/

4. Kowalska J, Antkowiak M, Sienkiewicz P. Flower Strips and Their Ecological Multifunctionality in Agricultural Fields. Agriculture [Internet]. 2022 Sep [cited 2025 Jul 2];12(9):1470. Available from: https://www.mdpi.com/2077-0472/12/9/1470

5. Hafez LO, Brito-Casillas Y, Abdelmageed N, Alemán-Cabrera IM, Morad SAF, Abdel-Raheem MH, et al. The Acacia (Vachellia nilotica (L.) P.J.H. Hurter & Mabb.): Traditional Uses and Recent Advances on Its Pharmacological Attributes and Potential Activities. Nutrients [Internet]. 2024 Jan [cited 2025 Jul 2];16(24):4278. Available from: https://www.mdpi.com/2072-6643/16/24/4278

6. Cedillo-Cortezano M, Martinez-Cuevas LR, López JAM, Barrera López IL, Escutia-Perez S, Petricevich VL. Use of Medicinal Plants in the Process of Wound Healing: A Literature Review. Pharmaceuticals [Internet]. 2024 Feb 27 [cited 2025 Jul 2];17(3):303. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10975678/

7. Diagne N, Arumugam K, Ngom M, Nambiar-Veetil M, Franche C, Narayanan KK, et al. Use of *Frankia* and Actinorhizal Plants for Degraded Lands Reclamation. BioMed Res Int [Internet]. 2013 [cited 2025 Jun 6];2013:1–9. Available from: http://www.hindawi.com/journals/bmri/2013/948258/

8. Sufianova G, Gareev I, Beylerli O, Wu J, Shumadalova A, Sufianov A, et al. Modern aspects of the use of natural polyphenols in tumor prevention and therapy. Front Cell Dev Biol [Internet]. 2022 Sep 12 [cited 2025 Jun 6];10:1011435. Available from: https://www.frontiersin.org/articles/10.3389/fcell.2022.1011435/full

9. Chen J, Wei Y, Yang W, Huang Q, Chen Y, Zeng K, et al. IL-6: The Link Between Inflammation, Immunity and Breast Cancer. Front Oncol [Internet]. 2022 Jul 18 [cited 2025 Jun 17];12:903800. Available from: https://www.frontiersin.org/articles/10.3389/fonc.2022.903800/full

10. Maharati A, Moghbeli M. PI3K/AKT signaling pathway as a critical regulator of epithelial-mesenchymal transition in colorectal tumor cells. Cell Commun Signal [Internet]. 2023 Aug 14 [cited 2025 Jun 17];21(1):201. Available from: https://biosignaling.biomedcentral.com/articles/10.1186/s12964-023-01225-x

11. Panda VK, Mishra B, Mahapatra S, Swain B, Malhotra D, Saha S, et al. Molecular Insights on Signaling Cascades in Breast Cancer: A Comprehensive Review. Cancers [Internet]. 2025 Jan 13 [cited 2025 Jun 18];17(2):234. Available from: https://www.mdpi.com/2072-6694/17/2/234

12. Håkansson L, Dunér P, Broströmer E, Gustavsson B, Wettergren Y, Ghafouri B, et al. A New IL-6-Inducing Mechanism in Cancer with New Therapeutic Possibilities. Cancers [Internet]. 2024 Oct 24 [cited 2025 Jun 18];16(21):3588. Available from: https://www.mdpi.com/2072-6694/16/21/3588

13. Yahfoufi N, Alsadi N, Jambi M, Matar C. The Immunomodulatory and Anti-Inflammatory Role of Polyphenols. Nutrients [Internet]. 2018 Nov 2 [cited 2025 Jun 18];10(11):1618. Available from: https://www.mdpi.com/2072-6643/10/11/1618

14. Grivennikov SI, Greten FR, Karin M. Immunity, Inflammation, and Cancer. Cell [Internet]. 2010 Mar [cited 2025 Jun 18];140(6):883–99. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0092867410000607

15. Daré RG, Lautenschlager SOS. Nanoparticles with Antioxidant Activity. Antioxidants [Internet]. 2025 Feb 15 [cited 2025 May 13];14(2):221. Available from: https://www.mdpi.com/2076-3921/14/2/221

16. Silva-Pinto PA, De Pontes JTC, Aguilar-Morón B, Canales CSC, Pavan FR, Roque-Borda CA. Phytochemical insights into flavonoids in cancer: Mechanisms, therapeutic potential, and the case of quercetin. Heliyon [Internet]. 2025 Feb [cited 2025 May 13];11(4):e42682. Available from: https://linkinghub.elsevier.com/retrieve/pii/S2405844025010631

17. Pei J, Natarajan PM, Umapathy VR, Swamikannu B, Sivaraman NM, Krishnasamy L, et al. Advancements in the Synthesis and Functionalization of Zinc Oxide-Based Nanomaterials for Enhanced Oral Cancer Therapy. Molecules [Internet]. 2024 Jun 6 [cited 2025 May 13];29(11):2706. Available from: https://www.mdpi.com/1420-3049/29/11/2706

18. Baig N, Kammakakam I, Falath W. Nanomaterials: a review of synthesis methods, properties, recent progress, and challenges. Mater Adv [Internet]. 2021 [cited 2025 May 13];2(6):1821–71. Available from: https://xlink.rsc.org/?DOI=D0MA00807A

19. Mao X, Kotov N. Complexity, disorder, and functionality of nanoscale materials. MRS Bull [Internet]. 2024 Apr [cited 2025 May 13];49(4):352–64. Available from: https://link.springer.com/10.1557/s43577-024-00698-6

20. Pereira JE, Moita AS, Moreira ALN. The pressing need for green nanofluids: A review. J Environ Chem Eng [Internet]. 2022 Jun [cited 2025 May 13];10(3):107940. Available from: https://linkinghub.elsevier.com/retrieve/pii/S2213343722008132

21. Sen K, Mondal S. Extraction of biomolecular polyphenols for nanoparticles fabrication: Mechanistic insights environmental mitigation. Inorg Chem Commun [Internet]. 2025 Sep 1 [cited 2025 Jul 2];179:114682. Available from: https://www.sciencedirect.com/science/article/pii/S1387700325007981

22. Marslin G, Siram K, Maqbool Q, Selvakesavan RK, Kruszka D, Kachlicki P, et al. Secondary Metabolites in the Green Synthesis of Metallic Nanoparticles. Materials [Internet]. 2018 Jun 3 [cited 2025 Jul 2];11(6):940. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6024997/

23. Field JW, Kandiah M. A note on the use of mayer’s reagent for the detection of quinine in alkaline urine. Trans R Soc Trop Med Hyg [Internet]. 1935 Jan 25 [cited 2025 Jul 2];28(4):385–90. Available from: https://www.sciencedirect.com/science/article/pii/S003592033590133X

24. Prigal SJ. The ferric chloride spot test for the evaluation and standardization of emulsions of allergens. J Allergy [Internet]. 1967 Jan 1 [cited 2025 Jul 2];39(1):1–10. Available from: https://www.sciencedirect.com/science/article/pii/0021870767901207

25. KANCHERLA N, DHAKSHINAMOOTHI A, CHITRA K, KOMARAM RB. Preliminary Analysis of Phytoconstituents and Evaluation of Anthelminthic Property of Cayratia auriculata (In Vitro). Mædica [Internet]. 2019 Dec [cited 2025 Jul 2];14(4):350–6. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7035446/

26. Gul R, Jan SU, Faridullah S, Sherani S, Jahan N. Preliminary Phytochemical Screening, Quantitative Analysis of Alkaloids, and Antioxidant Activity of Crude Plant Extracts from Ephedra intermedia Indigenous to Balochistan. Sci World J [Internet]. 2017 [cited 2025 Jul 2];2017(1):5873648. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1155/2017/5873648

27. Das BK, Al-Amin MM, Russel SM, Kabir S, Bhattacherjee R, Hannan JMA. Phytochemical Screening and Evaluation of Analgesic Activity of Oroxylum indicum. Indian J Pharm Sci [Internet]. 2014 [cited 2025 Jul 2];76(6):571–5. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4293694/

28. Mast J, Verleysen E, Hodoroaba VD, Kaegi R. Chapter 2.1.2 - Characterization of nanomaterials by transmission electron microscopy: Measurement procedures. In: Hodoroaba VD, Unger WES, Shard AG, editors. Characterization of Nanoparticles [Internet]. Elsevier; 2020 [cited 2025 Jul 2]. p. 29–48. (Micro and Nano Technologies). Available from: https://www.sciencedirect.com/science/article/pii/B9780128141823000043

29. M A, I MA, Ramalingam K, S R. Evaluation of the Anti-inflammatory, Antimicrobial, Antioxidant, and Cytotoxic Effects of Chitosan Thiocolchicoside-Lauric Acid Nanogel. Cureus [Internet]. [cited 2025 Jul 2];15(9):e46003. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10600588/

30. Zarei MH, Lorigooini Z, Amini Khoei H, Bijad E. Acute oral toxicity assessment of galbanic acid in albino rat according to OECD 425 TG. Toxicol Rep [Internet]. 2023 Dec 1 [cited 2025 Jul 2];11:111–5. Available from: https://www.sciencedirect.com/science/article/pii/S221475002300080X

31. E B, Sivalingam AM, Alex A, Neha B. In Vitro Antioxidant Activity of Green-Synthesized Zinc Oxide (ZnO) Nanoparticles Utilizing Extracts From Allium sativum. Cureus [Internet]. [cited 2025 Jul 2];16(2):e55184. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10981507/

32. Shaikh RU, Pund MM, Gacche RN. Evaluation of anti-inflammatory activity of selected medicinal plants used in Indian traditional medication system in vitro as well as in vivo. J Tradit Complement Med [Internet]. 2015 Aug 1 [cited 2025 Jul 2];6(4):355–61. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5067865/

33. Kedare SB, Singh RP. Genesis and development of DPPH method of antioxidant assay. J Food Sci Technol [Internet]. 2011 Aug [cited 2025 Jul 2];48(4):412–22. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3551182/

34. Babaei Rad S, Mumivand H, Mollaei S, Khadivi A. Effect of drying methods on phenolic compounds and antioxidant activity of Capparis spinosa L. fruits. BMC Plant Biol [Internet]. 2025 Jan 31 [cited 2025 Jul 2];25:133. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC11783714/

35. Derouich M, Bouhlali EDT, Hmidani A, Bammou M, Bourkhis B, Sellam K, et al. Assessment of total polyphenols, flavonoids and anti-inflammatory potential of three *Apiaceae* species grown in the Southeast of Morocco. Sci Afr [Internet]. 2020 Sep 1 [cited 2025 Jul 2];9:e00507. Available from: https://www.sciencedirect.com/science/article/pii/S2468227620302453

36. Hafez LO, Brito-Casillas Y, Abdelmageed N, Alemán-Cabrera IM, Morad SAF, Abdel-Raheem MH, et al. The Acacia (Vachellia nilotica (L.) P.J.H. Hurter & Mabb.): Traditional Uses and Recent Advances on Its Pharmacological Attributes and Potential Activities. Nutrients [Internet]. 2024 Dec 11 [cited 2025 Apr 27];16(24):4278. Available from: https://www.mdpi.com/2072-6643/16/24/4278

37. Sun W, Shahrajabian MH. Therapeutic Potential of Phenolic Compounds in Medicinal Plants—Natural Health Products for Human Health. Molecules [Internet]. 2023 Feb 15 [cited 2025 Jul 2];28(4):1845. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9960276/

38. Ysrafil Y, Sapiun Z, Slamet NS, Mohamad F, Hartati H, Damiti SA, et al. Anti-inflammatory activities of flavonoid derivates. ADMET DMPK [Internet]. 2023 Jul 26 [cited 2025 Jul 2];11(3):331–59. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10567070/

39. Al-Rajhi AMH, Qanash H, Bazaid AS, Binsaleh NK, Abdelghany TM. Pharmacological Evaluation of Acacia nilotica Flower Extract against Helicobacter pylori and Human Hepatocellular Carcinoma In Vitro and In Silico. J Funct Biomater [Internet]. 2023 Apr 21 [cited 2025 Jul 2];14(4):237. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10143343/

40. Hafez LO, Brito-Casillas Y, Abdelmageed N, Alemán-Cabrera IM, Morad SAF, Abdel-Raheem MH, et al. The Acacia (Vachellia nilotica (L.) P.J.H. Hurter & Mabb.): Traditional Uses and Recent Advances on Its Pharmacological Attributes and Potential Activities. Nutrients [Internet]. 2024 Dec 11 [cited 2025 Jul 2];16(24):4278. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC11678605/

41. Alelign T, Chalchisa D, Fekadu N, Solomon D, Sisay T, Debella A, et al. Evaluation of acute and sub-acute toxicity of selected traditional antiurolithiatic medicinal plant extracts in Wistar albino rats. Toxicol Rep [Internet]. 2020 Oct 6 [cited 2025 Jul 2];7:1356–65. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7569265/

42. Ayéna ACT, Dosseh K, Idoh K, Agbonon A, Gbeassor M. Comparative Physicochemical Screening and Toxicology of Hydroethanol Extracts of the Parts of Pterocarpus santalinoides l’Hér. ex DC. (Fabaceae) in Wistar Rats. Sci World J [Internet]. 2022 Feb 25 [cited 2025 Jul 2];2022:5953094. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8896965/

43. Hamm J, Allen D, Ceger P, Flint T, Lowit A, O’Dell L, et al. Performance of the GHS Mixtures Equation for Predicting Acute Oral Toxicity. Regul Toxicol Pharmacol [Internet]. 2021 Oct 1 [cited 2025 Jul 2];125:105007. Available from: https://www.sciencedirect.com/science/article/pii/S0273230021001483

44. Coones RT, Nikolic I, Eugster R, Mehn D, Tong V, Luciani P, et al. Best practice for the size analysis of nanomedicines – An iron sucrose case study. Int J Pharm [Internet]. 2025 Apr 15 [cited 2025 Jul 2];674:125452. Available from: https://www.sciencedirect.com/science/article/pii/S0378517325002881

45. Ajose DJ, Abolarinwa TO, Oluwarinde BO, Balogun SA, Fayemi OE, Aremu AO, et al. Bio-control potentials of Vachellia nilotica-derived silver nanoparticle against multidrug-resistant Staphylococcus haemolyticus strains from raw milk. Discov Mater [Internet]. 2025 May 1 [cited 2025 Jul 2];5(1):80. Available from: https://doi.org/10.1007/s43939-025-00256-0

46. Ramzan M, Ayub F, Shah AA, Naz G, Shah AN, Malik A, et al. Synergistic Effect of Zinc Oxide Nanoparticles and Moringa oleifera Leaf Extract Alleviates Cadmium Toxicity in Linum usitatissimum: Antioxidants and Physiochemical Studies. Front Plant Sci [Internet]. 2022 Aug 2 [cited 2025 Jul 2];13:900347. Available from: https://pmc.ncbi.nlm.nih.gov/articles/PMC9380429/

47. Sharma V, Sharma JK, Kansay V, Sharma VD, Sharma A, Kumar S, et al. The effect of calcination temperatures on the structural and optical properties of zinc oxide nanoparticles and their influence on the photocatalytic degradation of leather dye. Chem Phys Impact [Internet]. 2023 Jun 1 [cited 2025 Jul 2];6:100196. Available from: https://www.sciencedirect.com/science/article/pii/S2667022423000361

48. Zhu W, Hu C, Ren Y, Lu Y, Song Y, Ji Y, et al. Green synthesis of zinc oxide nanoparticles using *Cinnamomum camphora* (L.) Presl leaf extracts and its antifungal activity. J Environ Chem Eng [Internet]. 2021 Dec 1 [cited 2025 Jul 2];9(6):106659. Available from: https://www.sciencedirect.com/science/article/pii/S2213343721016365

49. Foyzun T, Mahmud AA, Ahammed MdS, Manik MdIN, Hasan MdK, Islam KM, et al. Polyphenolics with Strong Antioxidant Activity from Acacia nilotica Ameliorate Some Biochemical Signs of Arsenic-Induced Neurotoxicity and Oxidative Stress in Mice. Molecules [Internet]. 2022 Feb 3 [cited 2025 Jul 2];27(3):1037. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8840196/

50. Bisht G, Rayamajhi S. ZnO Nanoparticles: A Promising Anticancer Agent. Nanobiomedicine [Internet]. 2016 Jan 1 [cited 2025 May 13];3:9. Available from: https://journals.sagepub.com/doi/10.5772/63437

51. Fahim M, Shahzaib A, Nishat N, Jahan A, Bhat TA, Inam A. Green synthesis of silver nanoparticles: A comprehensive review of methods, influencing factors, and applications. JCIS Open [Internet]. 2024 Dec 1 [cited 2025 Jul 2];16:100125. Available from: https://www.sciencedirect.com/science/article/pii/S2666934X24000254

52. Siafaka PI, Miliotou AN, Okur ME, Karaotmarlı Güven G, Karantas ID, Üstündağ Okur N. Nanoformulations Loaded with Phytochemicals for Combating Wound Infections and Promoting Wound Healing: Current Applications and Innovations. Appl Sci [Internet]. 2025 Jan [cited 2025 Jul 2];15(10):5413. Available from: https://www.mdpi.com/2076-3417/15/10/5413

53. Hoshyar N, Gray S, Han H, Bao G. The effect of nanoparticle size on in vivo pharmacokinetics and cellular interaction. Nanomed [Internet]. 2016 Mar [cited 2025 Jul 2];11(6):673–92. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5561790/

54. Zhou F, Peterson T, Fan Z, Wang S. The Commonly Used Stabilizers for Phytochemical-Based Nanoparticles: Stabilization Effects, Mechanisms, and Applications. Nutrients [Internet]. 2023 Jan [cited 2025 Jul 2];15(18):3881. Available from: https://www.mdpi.com/2072-6643/15/18/3881

55. Anwar T, Safdar A, Qureshi H, Siddiqi EH, Ullah N, Naseem MT, et al. Synergistic effects of Vachellia nilotica-derived zinc oxide nanoparticles and melatonin on drought tolerance in Fragaria × ananassa. BMC Plant Biol [Internet]. 2025 Jan 22 [cited 2025 Jul 2];25(1):82. Available from: https://doi.org/10.1186/s12870-025-06114-8

56. Wang L, Chen G, Yang Y, Xu C, Zhu L, Yang H, et al. Advancing Polyphenol-Based Nanomedicine for Inflammatory Bowel Disease: Challenges and Opportunities. J Inflamm Res [Internet]. 2024 Nov 27 [cited 2025 Jul 2];17:9889–904. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC11610386/

57. Perumalsamy H, Balusamy SR, Sukweenadhi J, Nag S, MubarakAli D, El-Agamy Farh M, et al. A comprehensive review on Moringa oleifera nanoparticles: importance of polyphenols in nanoparticle synthesis, nanoparticle efficacy and their applications. J Nanobiotechnology [Internet]. 2024 Feb 19 [cited 2025 Jul 2];22:71. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10877787/

58. Zhuo Y, Zhao YG, Zhang Y. Enhancing Drug Solubility, Bioavailability, and Targeted Therapeutic Applications through Magnetic Nanoparticles. Molecules [Internet]. 2024 Jan [cited 2025 Jul 2];29(20):4854. Available from: https://www.mdpi.com/1420-3049/29/20/4854