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BIOACTIVITY AND SAFETY OF HYDRO-ETHANOLIC *ZANTHOXYLUM HUMILE*

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ABSTRACT: Introduction: *Zanthoxylum humile*, a medicinal plant belonging to the Rutaceae family, has been used for many years in traditional medicine to treat various illnesses. *Zanthoxylum* plants have been used to treat infections caused by parasites (malaria, leishmaniasis, trypanosomiasis, and other parasitic diseases), as well as sickle cell disease, tumors, and bacterial, fungal, and viral infections. However, there is very little scientific research on the ethnomedical claims around *Z. humile*. The present study aims to explore the phytoconstituents of the hydroethanolic bark extract, screen for antioxidant activity, and determine the plant's safety. **Methods:** *Zanthoxylum humile* bark was subjected to maceration using hydroethanolic solvent to obtain an extract. Phytochemical screening was performed using gas chromatography–mass spectrometry (GC–MS) to identify bioactive compounds. The antioxidant activity was evaluated using the DPPH assay. Acute oral toxicity was evaluated in accordance with the OECD 425 guideline to determine the safety profile of the extract. **Results:** GC–MS analysis revealed multiple bioactive phytochemicals, including 2-Methoxy-4-vinylphenol, resorcinol, and copaene, supporting the therapeutic relevance of *Zanthoxylum humile*. The extract exhibited strong antioxidant activity, with an IC₅₀ value of 8.59 µg/ml in the DPPH antioxidant assay. The OECD 425 acute toxicity study demonstrated an LD₅₀ greater than 2500 mg/kgBW with no mortalities or observable clinical signs of toxicity, indicating a favourable safety profile. **Conclusion:** *Zanthoxylum humile* bark extract demonstrated strong antioxidant activity and a good safety profile, indicating its potential as a source of therapeutic agents.

INTRODUCTION: *Zanthoxylum humile* (E.A. Bruce) P.G. Waterman belongs to the Rutaceae family. It is commonly known as Hairy knob-wood (E) and *Monokwane* in Sotho. The roots and bark are traditionally used to treat bacterial infections¹.

Z. humile roots are used in Zimbabwean traditional medicine to treat chest pains and flu². The plant is covered in short grey hairs and thorns along the branches. The tree is deciduous and grows to a height of 3 meters.

Z. humile grows in different places, from KwaZulu-Natal, Limpopo, and Mpumalanga provinces in South Africa to Mozambique and the southern parts of Zimbabwe. The plant can be male or female, with males producing only flowers while females produce both flowers and fruits³.

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Zanthoxylum Genus in Traditional Medicine:

The genus *Zanthoxylum* has gained attention for demonstrating strong antibacterial properties across multiple studies⁴. *Zanthoxylum* species decoctions have long been used to treat a variety of infections, particularly parasitic infections (malaria, leishmaniasis, trypanosomiasis, and other parasitic diseases), as well as sickle cell disease, tumours, and bacterial, fungal, and viral infections. A lot of medicinal properties have been reported for members of the *Zanthoxylum* genus⁵. Papo³ mentioned that, traditionally, decoctions of the root bark are used to treat flu, colds, sore throat, diarrhoea, diabetes, hypertension, wound healing, and toothaches in areas of Limpopo, Zimbabwe, and Mozambique.

The *Zanthoxylum* genus has many plants that are used in food and traditional medicine. The *Z.* species are found in many places across Asia, America, and Africa. Phytochemicals isolated from *Z.* species have been reported to have many pharmacological properties, such as anti-inflammatory, anti-diabetic, antibacterial, anti-tumour, anti-viral, anti-malarial, anti-fungal, and anti-parasitic effects³. Decoctions of the leaves, roots, seeds, and bark are used in traditional medicine. The plant may also be chewed raw or ground into a powder and applied directly to wounds. Seeds and fruit are also used in Asia as a spice for cooking³. In a study by Wekesa *et al.*⁶ on the drug-likeness and Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADMET) properties of 406 secondary metabolites isolated from the genus *Zanthoxylum*, they reported that 81% of the phytochemicals met Lipinski's rule of five and exhibited very high oral bioavailability. It was also predicted that 9.1% of the secondary metabolites were lead-like, making them promising candidates for drug development.

Plant Secondary Metabolites: Secondary metabolite synthesis occurs during periods of stress, and since ancient times, they have been used to treat various ailments⁷. Phytochemicals are secondary metabolites that possess antibacterial, antiviral, anti-inflammatory, antioxidant, antifungal, anthelmintic and antitumour properties^{7, 8}. The classes into which various phytochemicals fall include polyphenols, saponins, and

phytosterols. Their natural origin may confer less toxicity as compared to synthetic drugs⁸. The pharmacological value of phytochemicals arises from their diverse chemical structures, which allow them to interact with several biochemical pathways⁹. A major contributor to the pharmacological utility of phytochemicals is their potent antioxidant activity. Phenolics and terpenoids are effective radical scavengers that protect cellular machinery from oxidative damage by neutralizing reactive oxygen species¹⁰. They can also activate endogenous defence mechanisms, such as the Nrf2-HO-1 signalling pathway, which upregulates antioxidant enzymes to maintain cellular redox homeostasis. These properties are crucial for the prevention and management of chronic conditions that are caused by oxidative stress¹¹.

Many studies have noted the potential that phytochemicals have as antimicrobial agents. Phytochemicals, alone or in combination with antibiotics, can enhance antibacterial activity against several bacteria¹². Phytochemicals possess antibacterial properties and employ various mechanisms such as inhibition of drug efflux pumps and inhibition enzymes responsible for drug degradation; restraining bacteria DNA and RNA synthesis¹³. They also kill bacterial cells by increasing membrane permeability and binding to DNA phosphate groups e.g. P-coumaric phenolic compound¹⁴; interference with microbial DNA synthesis and respiration (Naphthaquinolone), disruption of microbial membrane and interfering with biofilm formation (emodin and rhein), interfering with microbial cell signalling metabolism and mycelian structure¹⁵. Reserpine alkaloid has the effect to reverse multidrug resistance by altering efflux pumps. Michellamine B another alkaloid, functions by inhibiting vital enzymes resulting in poor cellular metabolism and eventually death¹⁶. Terpenes also exhibit activity against a range of bacterial and mycobacteria. α -terpineol, terpinen-4-ol, δ -terpineol, and 1,8-cineole function by destabilizing the cytoplasmic membrane¹⁷.

Secondary Metabolites in *Zanthoxylum* Plants:

Zanthoxylum plants possess a wide variety of phytochemicals, including alkaloids, terpenes,

flavonoids, lignans, amides, and coumarins, with alkaloids constituting the largest group.

Among the alkaloids that have been found in the *Z.* species are Sanguinarine, Fagarine I (*Z. gillettii*), Skimmianine (*Z. zanthoxyloides*), Zanthoxyline (*Z. fagara*), N-Methylcanadine, Zanthosimuline, Zanthobugeanine (*Z. bungeanum*), Oxychelerythrine (*Z. nitidum*), and Bocconoline (*Z. davyi*). The *Z.* species is rich in flavonoids, namely Quercitrin (*Z. zanthoxyloides*), Hesperidin, Eriocitrin, Datiscin, and Quercetin-3-O-Glucopyranoside (*Z. zanthoxyloides*)⁶. Coumarins that have been found in the *Z.* species include 8-Formylalloxanthoxyletin, Avicennol, Xanthoxyletin, Scopoletin (*Z. avicennae*), Hydrangetin, and Amottianamide (*Z. schinifolium*). Phenols and phenolic compounds that have been found in the *Z.* species include 2-Methoxy-4-hydroxyphenyl - 1 - O - α - L - rhamnopyranosyl- (100 $\rightarrow$ 6 0)- β -D-glucopyranoside (*Z. armatum*), Cuspidiol, Caffeic acid, Chlorogenic acid, Hydrocuspidiol (*F. zanthoxyloides*). The *Z.* species are rich in secondary metabolites⁵.

This study focuses on screening phytochemicals, evaluating the antioxidant activity, and assessing the safety of the *Z. humile* bark extracts.

MATERIALS AND METHODS:

Materials, Equipment and Facilities: Ethanol (99.9%, Sigma Aldrich); Orbital shaker (Stuart Scientific Orbital shaker SO1); Whatman filter paper (Number 1); Rotar evaporator (Rotavapor® R-300, Buchi, Switzerland); Distiller (A20, BIOBASE, China); Sulphuric acid (98%, Sigma Aldrich); Glacial acetic acid (Sigma Aldrich); Ferric chloride (Sigma Aldrich); Sodium chloride; Gelatin solution; Wagner's reagent; Ammonia (Sigma Aldrich); Methanol (Sigma Aldrich); Gas Chromatography Mass Spectrometer (Agilent 5977B MSD); ThermoScientific Microplate photometer (Multiskan FC, China). The chemicals and equipment used to carry out extraction, *in-vitro* bioactivity assays, and *in-vivo* toxicity tests were obtained from the University of Zimbabwe Faculty of Medicine and Health Sciences Laboratory and the University Animal House.

***Zanthoxylum humile* Plant Material Collection and Preparation:** The plant material was collected

from Zvishavane (20°19'36" S, 30°03'59" E). Collection was done from 5 different plants in observance of the rules of Zimbabwe's sustainable Harvest of Traditional Medicinal plants. Plant authentication was conducted at the National Herbarium on 15 October 2025 by KB Mutasa at Botanical Garden in Harare, Zimbabwe. The Specimen was deposited at SRGH as voucher specimen FT0001. The *Z. humile* bark was thoroughly washed to remove debris and shade-dried at room temperature (approximately 25-30°C) until a constant weight for three weeks.

Extraction of Phytochemical Constituents: The dried bark was then pulverized in a mortar and pestle and sifted to a coarse powder. Phytochemical extraction was carried out by adding 450g of powdered bark to 2300ml hydro-ethanolic solvent (70% ethanol and 30 % distilled water v/v) in a clean flat-bottomed flask at a plant-to-solvent ratio of 1:5 and macerated for 72 hours, with constant shaking on an orbital shaker (Stuart Scientific Orbital shaker SO1). Extraction was carried out with a single maceration cycle, and the marc was not subjected to further extraction.

The mixture was initially filtered through muslin cloth to remove coarse particles; further clarification was achieved by filtration through Whatman No. 1 filter paper. The filtrate was then concentrated under reduced pressure at 50°C (Rotavapor® R-300, Buchi, Switzerland), followed by air-drying in a fume hood. The extract was then stored in a sample bottle, wrapped in parafilm, and kept at 4 $\pm$ 0.2 °C in a refrigerator until required and used within 2 months for all subsequent tests. The percentage yield of phytochemicals was calculated using Equation 1,

$$\text{Percentage yield \%} = (\text{Mass of extract / g}) / (\text{Mass of coarse plant powder / g}) \times 100 \dots\dots\dots 1$$

Qualitative Phytochemical Screening of *Zanthoxylum humile*: The presence of pharmacologically active phytochemical constituents was confirmed by conducting qualitative phytoscreening tests. A stock solution was prepared by dissolving 10g of the extract in 100ml of distilled water. The following qualitative tests were carried out:

Determination of Tannins using Braymer's

Test: The Braymer's test was carried out by adding 3 ml of 10% ferric chloride solution to 1 ml of the extract. The presence of Tannins was confirmed by the appearance of a blue green colour¹⁸.

Determination of Glycosides using Keller-

Killani Test: The Keller-Killani test was carried out by adding to 1 ml of the extract, 1.5 ml glacial acetic acid and a few drops of 5% ferric chloride were added as well as conc. H₂SO₄. Presence was confirmed by a reddish-brown ring at the interface¹⁸.

Determination of Saponins by the Simplified

Foam Test: The foam test was performed by adding 1 ml of the extract to 10 ml of distilled water in a stopped test tube and shaking vigorously for 30 seconds. The presence of saponins would be confirmed by the formation of foam with a height of at least 1 cm¹⁹.

Determination of Phenolic Compounds by

Gelatin Test: The Gelatin test was done by adding 5 ml of 1% gelatin solution and 5 drops of 10% NaCl to 2ml of the extract. The presence of the phenolic compounds was identified by the appearance of a precipitate²⁰.

Determination of Alkaloids by Wagner's Test:

Wagner's test was done by adding a few drops of Wagner's reagent to 3 ml of the extract. The presence of alkaloids was confirmed by the appearance of a brown precipitate¹⁸.

Determination of Flavonoids by Alkaline Test:

The alkaline test was carried out by adding a few drops of 20% NaOH to 2 ml of plant extract. The presence of flavonoids was confirmed by the emergence of a yellow colour which disappeared upon addition of HCl¹⁸.

Phytochemical Screening with Gas Chromatography Mass Spectrometry (GC-MS):

GC-MS was carried out using an Agilent Technologies 7890B gas chromatograph coupled to a 5977A mass selective detector (MSD) equipped with a 7693 autosampler. Separation was carried out using a capillary column (HP-5MS, 30 m × 0.250 mm internal diameter, 0.25 µm film thickness). The injector temperature was

maintained at 250 and 1 µl of sample was injected in split mode (split ratio 5:1). Helium was used as the carrier gas at a flow rate of 1.0 mL/min. The oven temperature was programmed from 40 °C (1-minute hold) to 280 °C (5-minute hold) at 4 °C/min, then to 300 °C (5-minute hold) at 10 °C/min. The mass spectra were acquired at 70 eV, and data were acquired over an m/z range of 35 to 550. The National Institute of Standards and Technology (NIST) library was used as a reference for compound identification.

The GC-MS report provided data on retention time (RT, min), area, CAS number, match score (%), and chemical formula. The area was used to calculate the % area as shown in Equation 2,

$$\text{Area (\%)} = \text{Area} / (\text{Total area}) \times 100 \dots\dots\dots 2$$

Where, Total area is the sum of all the areas for chemical species obtained from the plant by GC-MS. The retention index (I) was obtained from the NIST Chemistry WebBook, SRD 69, using the CAS number, instrument name, and analysis parameters. Values were selected if they match or are close to the capillary column characteristics, polarity (non-polar column), and testing conditions.

Acute Oral Toxicity Evaluation of *Zanthoxylum*

humile: The OECD test guidelines 425²¹ with modifications were used to evaluate the oral toxicity of hydroethanolic *Zanthoxylum humile* bark extract. Evaluation was performed using female nulliparous Sprague-Dawley rats, 5 in total, at 8 weeks of age. The rats had been left to acclimatize for 6 days prior. The rats were divided into groups: the control group, which received only distilled water, and the test groups. The plant extracts were dissolved in water and administered by oral gavage to rats that had been fasted overnight.

The first animal was given a starting dose of 250 mg/kg body weight, which was doubled when the animal survived after 48 hours following the study by Chifamba *et al.*²². The rats were then observed for any visible changes and signs and symptoms of toxicity every hour for the first 24 hours. Monitoring was then continued for the next 14 days. The weight of the rats was weight using an analytical balance. The initial weight of the rats (at

day 0) was 215.4 ± 21.6 , and the weight of all animals was within the $\pm 20\%$ difference. The rats were kept on recommended conditions, temperature $25 \pm 5^\circ\text{C}$ and lighting (12 hrs dark and 12 hours light) as recommended by OECD 425 guidelines²¹. The rats were only separated shortly after drug administration and were placed back in the group when they did not show elevated signs of distress after being administered plant extract doses.

Main test was carried out since there were no established limit LD50 values regarding that *Z. humile* extract. Nevertheless, the maximum dose was set at 2500 mg/kg BW (slightly above the standard 2000 mg/kg BW), then reduced to 1000, 500, and lastly 250 mg/kg BW as the minimum in accordance to Chifamba *et al.*²². The OECD 425²¹ likelihood function was used to determine the LD50 by observing the number of mortalities at each concentration using functions in Julia programming language (v1.12.6). The overall mortality was estimated by multiplying the probability of individual animals' outcome,

$$L = L_1 \times L_2 \times \dots \times L_5 \dots \dots \dots 3$$

The individual likelihood (L_i) of each rat (i) depend on whether it lived or died. Thus, Equation 4 (if the rat died) and Equation 4 (if it lived):

$$L_i = F(Z_i) \dots \dots \dots 3$$

$$L_i = 1 - F(Z_i) \dots \dots \dots 4$$

Where, F is the standard normal cumulative distribution function, and $Z_i = (\log d_i - \mu) / \sigma$ with d_i as the dose concentration and μ as the LD50 value. The Equation 4 is usually converted to logarithmic expression for easy computation, Equation 5,

$$\ln L = \sum \ln (L_i) = \sum \ln [F(Z_i)] (1 - F(Z_i)) \dots \dots \dots 5$$

The weight (W) of the rats was also monitored at day 1, 7, and 14. The Overall gain, Average weight gain (AWG) and Relative gain (%), were calculated as shown in Equations 6–8, respectively.

$$\text{Overall gain (g)} = (W_{14} - W_1) \dots \dots \dots 6$$

$$\text{Average weekly weight gain (AWG, g)} = (\text{week 1 gain} + \text{week 2 gain}) / 2 \dots \dots \dots 7$$

and,

$$\text{Relative gain (\%)} = (W_{14} - W_1) / W_1 \times 100\% \dots \dots 8$$

Antioxidants Activity: The antioxidant activity of *Z. humile* was evaluated using the DPPH radical-scavenging assay, a simple and widely used method for assessing the antioxidant activity of natural products. The assay is based on the changes in colour of DPPH following reduction through donation of a hydrogen from an antioxidant compound, from an intense violet colour to a pale yellow. The change in colour corresponds to a decrease in absorbance, signalling the antioxidant nature of the compound²³. The antioxidant activity of *Z. humile* used in this study was obtained from Yazdani *et al.*²⁴ with slight modifications. *Z. humile* extract concentrations ranging from 100 $\mu\text{g/ml}$ to 0.78 $\mu\text{g/ml}$ were tested using a freshly prepared 1,1-diphenyl-2-picryl-hydrazil (DPPH) solution in methanol in a 96-well plate. The assay was carried out by adding 200 μl of DPPH to 20 μl of *Z. humile* extract. Ascorbic acid was used as a positive control. Control wells for DPPH (200 μl DPPH and 20 μl methanol) and plant control wells (20 μl of plant extract and 200 μl of DPPH) were also prepared. The plate was left to incubate in the dark for 30 mins, and absorbance was measured using an ELISA plate reader (Thermo Scientific Multiskan FC, China) at 492nm. The analysis was done at 492nm in the present while it is normally carried out at 517nm mainly since an older model of Elisa reader was used which only had the filter 492nm however, according to literature when 517 or 520nm filters analysis can still be reliably done at 492nm to determine antioxidant activity as shown by studies by (Ellen *et al.*²⁵ and Ravipati *et al.*²⁶). Each test was replicated 3 times for *Z. humile* plant extract and twice ascorbic acid. Percentage radical-scavenging activity (RSA) was calculated (Equation 9),

$$\% \text{RSA} = (A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}} \times 100 \dots \dots 9$$

Where A_{control} is absorbance of DPPH and methanol and A_{sample} is absorbance of DPPH and extract or ascorbic acid.

The concentration required to scavenge 50% of the radicals (IC50) was estimated by determine the model that fits well with our experimental data based on the value of coefficient of determination, R^2 . Fraser-Spears *et al.*²⁷ and Olugbam *et al.*²⁸ indicated that most concentrations response models either follows linear models for low range

concentrations and sigmoid (saturation or S - shaped) curve for wider concentration range. This study considered both the linear model and sigmoid curve as the starting point.

The linear regression model assumes monotonic relationship between independent variable concentration or log concentration (for a semi-log model) and % RSA (dependent variable) as illustrated in Equation 1028,

$$\%RSA = G \times \text{Concentration} + I \dots\dots\dots 10$$

Where G is the gradient and I is the intercept. The IC50 was then calculated from Equation 11 as follows,

$$IC50 = (\%RSA50 - I) / G \dots\dots\dots 11$$

Where, %RSA50 is 50% radical scavenging percentage.

Fraser-Spears *et al.*²⁷ explains the sigmoid curve as a 4 -parameter logistic curve (4PL), Equation 12,

$$\%RSA = p1 + (p2 - p1) / (p3 / \text{concentration}) \times p4 \dots\dots 12$$

Where, p1 –is the bottom plateau (minimum %RSA), p2 – is the top plateau (maximum %RSA), p3 – is the inflection point (IC50) and p4 – is the hill slope (steepness of the curve). Thus, p3 = IC50 value.

Statistical Analysis: Calculations of variables such as % Radical scavenging activity (%RSA) was done in Excel (version 2605, Microsoft 365). The comparison between 3 or more groups was done using generalised linear models (GLM) for all data with replicates, Equation 13³¹,

$$Y_{ij} = \mu + K_{pi} + B_{fj} + (F_p \times B_f)_{ij} + r_{ij} \dots\dots\dots 13$$

Where Y_{ij}-is the response variable (%RSA, %Inhibition, % Deviation, weights) for treatment i

and j, μ -is the overall mean, K_p -is the effect of the ith treatment (dose level, antioxidant type, formulation), B_f-is the blocking factor (concentration). The term (F_p×B_f)_{ij} is the interaction term between treatment and blocking factor.

In cases where there was no blocking factor (B_{fj}), GLM procedure was conducted using the treatment and interaction term was not expected. The GLM procedure gives results for the least-squares mean (LS Mean), standard error, ANOVA, and post hoc analysis using Tukey HSD. The comparison of at least 2 groups was performed using Welch's t-test, which assessed significant differences between means of %RSA values of *Z. humile* plant extract and ascorbic acid. Statistical significances were considered at 95% confidence interval. Data analysis, manipulation and graph plotting was done in Julia programming language (version 1.12.6). The RC all. jl interface was utilized to execute functions from the R (version 4.6.0) ecosystem.

RESULTS AND DISCUSSION:

Extraction of Phytochemicals: A weight of 60.1 g of *Zanthoxylum humile* extract was obtained per 450.0 g, corresponding to a yield of 13.4% **Table 1.** The percentage yield of the extract obtained in this study was within the range compared with values for *Zanthoxylum* species. For instance, Ayangla *et al.*³⁰ mentioned that the crude phytochemical extract from *Zanthoxylum* species ranges from 10.0 – 25.0 % of plant dry weight. In a study on *Zanthoxylum zanthoxyloides* (Lam.) Zepernick and Timler in Burkina Faso, Ouédraogo *et al.*³¹ obtained a slightly lower % yield of 10.78±1.1% from stem and root bark using methanol extraction. In another study on five *Zanthoxylum* species, Ayangla *et al.*³⁰ obtained percentage yields with the range of 7.78 – 14.21% in leaves and seeds using ethanol solvent.

TABLE 1: SUMMARY OF PHYTOCHEMICAL EXTRACTION OF HYDROETHANOLIC *Z. HUMILE* EXTRACT

Solvent system	Plant part	Initial weight (g)	Extract weight (g)	% yield	Colour	Consistency
Hydroethanolic	Bark	450	60.1	13.4%	Reddish- brown	Solid

The slight % yield difference comparison with other *Zanthoxylum* species can be attributed to species, the area in which the plants were obtained, and the extraction methods used^{30, 31}. For instance, Tourabi *et al.*³² and Cendrowski *et al.*³³ showed

that extraction with a hydroethanolic solvent often yields more phenolic compounds and other phytochemicals as compared to absolute solvents.

Phytochemical Screening:

Qualitative Profiling: Phytochemical screening of *Zanthoxylum humile* showed the presence of various classes of phytochemicals, namely flavonoids, alkaloids, phenols, glycosides, tannins, and saponins **Table 2**. The results showed the presence of tannins, alkaloids, saponins, and flavonoids, phenols, and glycosides **Table 2**. These compounds are pharmacologically active and act as antioxidants, antibacterial, antifungal, antiviral, antitumour, antidiabetic ^{5, 22}.

TABLE 2: QUALITATIVE PHYTOCHEMICAL PROFILE OF ZANTHOXYLUM HUMILE

Phytochemical class	Presence in hydroethanolic extract
Flavonoids	+
Tannins	+
Phenols	+
Alkaloids	+
Glycosides	+
Saponins	+

(-) Indicating absence, (+) indicating presence,

Ayangla *et al.* ³⁰ studies on *Zanthoxylum* species (*Zanthoxylum armatum*, *Zanthoxylum oxyphyllum*,

Zanthoxylum oxyphyllum, *Zanthoxylum rhetsa* and *Zanthoxylum rhetsa*) also showed the presence of flavonoids, phenols, tannins and glycosides using ethanol extract. However, Ayangla *et al.* ³⁰ indicated the presence of coumarins in the *Zanthoxylum* species above, which were not detected in this study; rather, we found saponins. Other studies ⁵ showed the presence of saponins (10– 500 µg/ml) in bark, fruits, and leaves of *Zanthoxylum* species, e.g., *Zanthoxylum armatum*, after ethanol extraction.

GC-MS Profiling: The GC-MS results revealed the various compounds in *Zanthoxylum humile*, which are responsible for the pharmacological activity of *Z. humile*. The compounds detected included various polyphenols, terpenes, alkaloids, and phytosterols. **Table 3** shows a summary of some of the compounds identified using the NIST library as a reference with match scores above 80.0%.

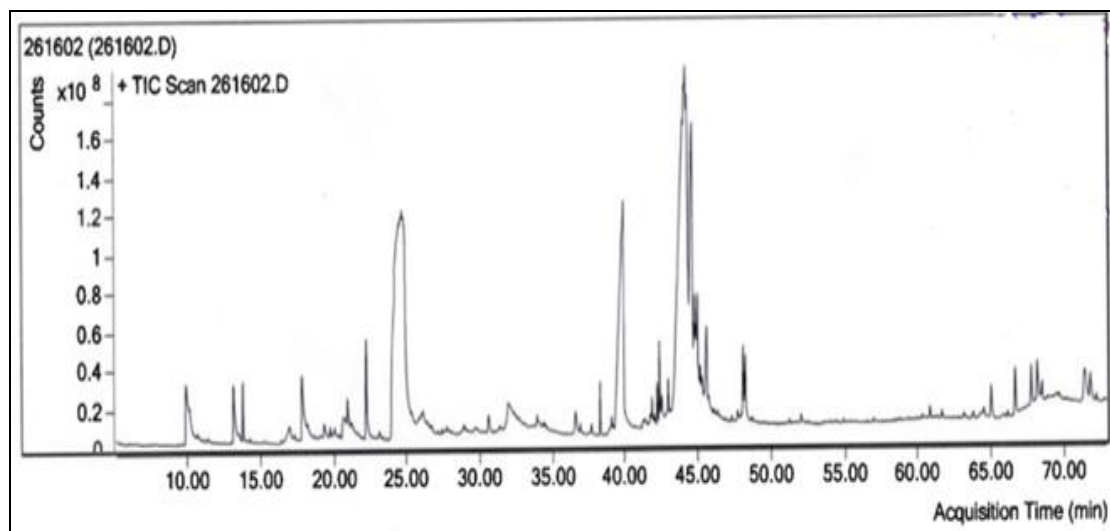


FIG. 1: GC-MS CHROMATOGRAM OF *Z. HUMILE* BARK EXTRACT

According to the results obtained, the extract had a variety of terpenes as shown by the presence of compounds such as delta.-Elemene (sesquiterpene), Guaiazolene (sesquiterpene), Lup-20(29)-en-3-one (pentacyclic triterpene), Copaene (sesquiterpene), Phenol, and 4-ethyl-2-methoxy- (sesquiterpene). The present findings were also consistent with previous studies on phytochemical screening of plants of the genus *Zanthoxylum*. For instance, compounds such as Lup-20(29)-en-3-one has been identified in *Z. gilleti* ⁴² and *Z. rhetsa*

⁴³. Calacorene has also be reported in *Z. schinifolium* ⁴⁴. The presence of terpenes supports the antioxidant activity of *Z. humile* extract due to their ability to directly and indirectly scavenge for free radicals. Terpenes with conjugated double bonds act by electron delocalisation ⁴⁵ or hydroxyl group substitution ⁴⁴. *Z. humile* extract also showed the presence of alkaloids 1H-Indole, 1-methyl- and 3-methyl- indole. The findings were consistent with literature, where 1H-Indole, 1-methyl- was identified in *Z. nitidum* ⁴⁷.

The nitrogen atoms in alkaloids act as hydrogen bond acceptors and electron donors. Alkaloids mainly function via direct radical scavenging,

inhibiting prooxidant enzymes, and transition metal chelation^{48, 49}.

TABLE 3: SUMMARY OF SOME OF THE COMPOUNDS DETECTED FROM GC-MS

Retention time	Compound name	Class	Match score %	%Area	Cas number	Chemical Formula	RI _{NIST}
44.052	Geranyl Acetate	Monoterpenoid ester	95.2	2.163	2000188-26-9	C ₁₂ H ₂₀ O ₂	NA
20.960	2-Methoxy-4-vinylphenol	Phenol	96.2	0.42	7786-61-0	C ₉ H ₁₀ O ₂	1315 ³²
34.479	Guaiazolene	Sesquiterpene	89.5	0.03	489-84-9	C ₁₅ H ₁₈	1775 ³³
21.191	Resorcinol	Phenol	90	0.78	108-46-3	C ₆ H ₆ O ₂	1180 ³⁴
20.406	1H-Indole, 1-methyl-	Alkaloid	95.9	0.05	120-72-9	C ₉ H ₉ N	1292 ³⁵
21.577	.delta.-Elemene	Sesquiterpene	82.3	0.008	20307-84-0	C ₁₅ H ₂₄	1336 ³⁶
63.763	Stigmasterol	Phytosterol	88.2	0.14	83-48-7	C ₂₉ H ₄₈ O	3286 ³⁷
66.124	Lup-20(29)-en-3-one	Terpene	86.0	0.13	1617-70-5	C ₃₀ H ₄₈ O	1048 ³⁸
23.370	3-methyl- indole	Alkaloid	80.4	0.02	2000041-11-6	C ₉ H ₉ N	NA
25.994	Copaene	Sesquiterpene	87.6	0.01	3856-25-5	C ₁₅ H ₂₄	1376 ³⁸
27.725	4 - vinyl - syringol	Phenol	85.7	0.09	2000148-84-2	C ₁₀ H ₁₂ O ₃	NA
27.997	Calacorene	Sesquiterpene	96.7	0.02	2000125-38-3	C ₁₃ H ₁₆	NA
60.306	.beta.-Tocopherol	Diterpene	87.8	0.02	148-03-8	C ₂₈ H ₄₈ O ₂	2974 ³⁸
19.764	Phenol, 4-ethyl-2-methoxy-	Phenol	96.1	0.17	2785-89-9	C ₉ H ₁₂ O ₂	1275 ³⁹
31.953	2,5-dimethoxybenzene-1,4-diol	Phenol	87.2	0.85	2000117-49-3	C ₈ H ₁₀ O ₄	NA

*NA – the retention index was not available in NIST registry.

The extract was particularly rich in phenolic compounds, as exhibited by the presence of compounds such as 2-Methoxy-4-vinylphenol, resorcinol, 4 - vinyl – syringol, Phenol, 4-ethyl-2-methoxy-, and 2,5-dimethoxybenzene-1,4-diol with 2-Methoxy-4-vinylphenol having been previously identified in *Z. bangeanum* and *Z. schinifolium*⁵⁰. The GC-MS analysis of *Z. humile* extract showed a diverse profile of phytochemicals; however, most of the pharmacologically active phytochemicals, as shown in **Table 3**, had peak areas of less than 1%. These findings are consistent with literature, where nearly half of the pharmacologically active phytochemicals in medicinal plants are frequently found to be minor components with chromatographic abundance of less than 1%⁵¹. The presence of these minor compounds suggests that the biological activity of the plant was a result of the multitarget interactions and synergism of the compounds instead of the effect of a single compound^{52,53}. Hence, the minor constituents identified in *Z. humile* should be considered as significant contributors to *Z. humile*'s therapeutic potential.

Oral Acute Toxicity: Despite the widespread use of *Z. humile* in traditional medicine, there is limited information regarding the safety of the plant. Female rats were used as they are more sensitive

due to their limited capacity to detoxify compounds as compared to males^{21, 54}. The doses used in this study (0 – 2500 mg/kg) did not show any detrimental effect on the behaviour or health of animals used for a 14-day period **Table 4**.

All animals showed normal food and water intakes, normal respiration, faeces consistency, urination, skin colour, and respiration. There were no mortalities observed for rats treated at all doses. Since no mortality was observed, the likelihood that the rat will die, F(Zi) term in Equation 4, tends to 0, as the likelihood for survival (1-F(Zi)) at that dose tends to 1.

This consequently means that the maximum dose at which the rats experience mortality was beyond the range of concentrations used in this study. Thus, LD₅₀ > 2500 mg/kg. It was impossible to obtain the confidence interval (CI) since the variance boundary used in calculating CI used the observed death rate. That is, Variance boundary $\propto \ln\left(\frac{0}{10}\right)$, where $\ln 0 \rightarrow -\infty$. These results are consistent with studies that have been done on its sister plant *Zanthoxylum chalybeum* that has been studied extensively. In a study by Obakiro *et al.*⁵⁵ on the acute oral toxicity of *Z. chalybeum* root bark extract on Wistar albino rats, they reported an LD₅₀ > 2000 mg/kg bodyweight.

TABLE 4: BEHAVIOURAL PATTERNS OBSERVED IN RATS FOLLOWING ACUTE ORAL TESTING WITH HYDROETHANOLIC ZANTHOXYLUM HUMILE BARK EXTRACT

Parameter observed	Doses of <i>Z. humile</i> in mg/kg body weight				
	250	500	1000	2500	Control
Food intake	Nl	Nl	Nl	Nl	Nl
Water intake	Nl	Nl	Nl	Nl	Nl
Mortality	Nf	Nf	Nf	Nf	Nf
Respiration	Nl	Nl	Nl	Nl	Nl
Faeces Consistency	Nl	Nl	Nl	Nl	Nl
Urination	Nl	Nl	Nl	Nl	Nl
Skin colour	Nl	Nl	Nl	Nl	Nl
Drowsiness	Nb	Nb	Nb	Nb	Nb
Erection of fur	Nb	Nb	Nb	Nb	Nb

Nl – normal, Nb – not observed, Nf – not found

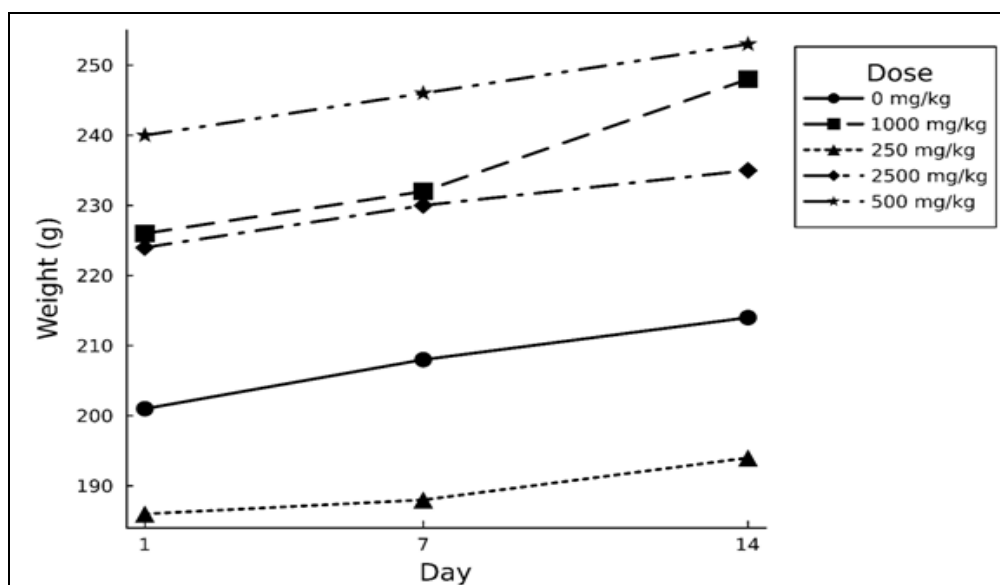
The results showed that, in general, body weight linearly increased as the number of days increases from day 1 to 14 **Fig. 2**. This indicates that the animals were gaining weight as the number of days increase. Morita *et al.*⁵⁶ and Ha *et al.*⁵⁷ showed that increase in weight for animals under toxicity evaluation is a rough confirmation that food intake and nutrition utilization was not affected by the plant extract. Morita *et al.*⁵⁶ indicates weight gains may be a result of fluid retention or organ swelling and hypertrophy (fat accumulation).

However, in these cases the animals should have shown respiratory difficulties, for example, fluid retention usually results in dyspnoea, rattling, wheezing, porphyrin staining and faeces become soft stools or diarrhoea or mucoid stools⁵⁸. **Table 5** indicated that there was a positive change in weight of rats treated with all doses. Nevertheless, 1000 mg/kg showed relative higher weekly gains (+11.0 g), total gain (+22.0 g) and relative gain (+9.73%) as compared to other treatments.

TABLE 5: WEIGHT DISTRIBUTION OF RATS AS NUMBER OF DAYS INCREASED

Dose (mg/kg)	Rat ID	Weight per day			AWG (g)	Total gain (g)	Relative gain (%)
		1	7	14			
0	1	201	208	214	+6.50	+13.00	+6.47
250	2	186	188	194	+4.00	+8.00	+4.30
500	3	240	246	253	+6.50	+13.00	+5.42
1000	4	226	232	248	+11.00	+22.00	+9.73
2500	5	224	230	235	+5.50	+11.00	+4.91

*AWG -Average weight gain

**FIG. 2: CHANGES OF FEMALE RATS' BODY WEIGHT WITH TIME**

Antioxidant Assay: The antioxidant activity of *Zanthoxylum humile* bark extract in comparison to ascorbic acid was investigated by firstly determining if the antioxidant type, concentration and their interaction have effect on the radical scavenging activity percentage (%RSA). The results in **Table 6** indicates that all factors antioxidant type ($p < 0.05$), concentration ($p < 0.001$) and their interaction ($p < 0.01$, Antioxidant * Concentration) have significant effect on % RSA.

TABLE 6: SIGNIFICANCE OF ANTIOXIDANT TYPE AND CONCENTRATION ON THE % RSA

Factor	% RSA
Antioxidant Type	*
Concentration	***
Antioxidant * Concentration	**

RSA -radical scavenging activity. Significant levels: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). ns -not significant ($p > 0.05$).

Table 6 suggest that the radical scavenging activity for ascorbic acid (control) and *Z. humile* behaves

differently, either performance (radical scavenging power) or unique chemical difference as noted by Christodoulou *et al.*⁵⁹. **Fig. 3** shows that the distribution of *Z. humile* curve showed lower %RSA values from concentrations between 6.25 – 25.0 µg/ml.

This may suggest lower radical scavenging power as compared to ascorbic acid. The %RSA for both antioxidants increases significantly as the concentration increases **Table 7**.

The results for pair wise differences (Weich's T - test) between ascorbic acid and *Z. humile* showed that most %RSA values per each concentration were significantly the same at 95.0% confidence interval **Table 7**.

Statistical differences ($p < 0.05$) between ascorbic acid and *Z. humile* %RSA values were observed at a concentration of 12.5 µg/ml.

TABLE 7: MEAN VALUES FOR %RSA FOR ASCORBIC ACID AND Z. HUMILE AND VARIOUS CONCENTRATIONS

Concentration	Radical scavenging activity (% RSA)	
	Ascorbic Acid	<i>Z. Humile</i>
	Mean±SE	Mean±SE
100.0	67.97±1.1 ^a	68.98±0.6 ^a
50.0	66.97±1.1 ^a	66.89±1.6 ^a
25.0	66.94±0.6 ^a	64.23±3.1 ^a
12.5	65.85±0.9 ^a	52.72±3.1 ^b
6.25	65.53±0.08 ^a	48.39±6.6 ^a
3.12	46.73±3.2 ^a	40.16±1.1 ^a
1.56	32.02±1.5 ^a	36.19±0.8 ^a
0.78	25.93±1.3 ^a	32.03±0.5 ^a
Group means	54.7±4.2 ^a	51.20±2.6 ^a
Number of observations	16	32

^{a, b} values in the same row differ significantly ($p < 0.05$) using Welch's t-test.

The mathematical modelling results for estimating IC₅₀ showed lower R^2 values for the linear models, i.e., 0.31 for ascorbic acid and 0.57 for *Z. humile* **Table 8**. Changing the concentration to a logarithmic scale (log Concentration, base 10) increases the prediction power. **Table 8** showed

that the semi-log model has slightly higher R^2 values of 0.781 and 0.875, respectively. However, the R^2 for ascorbic acid was slightly lower than the 0.80 recommendation. Moreover, Fraser-Spears *et al.*²⁷ indicated that the R^2 for the control should be above 90% for better IC₅₀ estimation.

TABLE 8: MODEL PARAMETERS FOR LINEAR REGRESSION BETWEEN %RSA AND CONCENTRATION

Model parameters						
Model	Antioxidant	Intercept (I)	Slope (G)	IC ₅₀ (µg/mL)	R^2	CI (LB, UB)
Linear	Ascorbic acid	47.70	0.28	8.09	0.311	
	<i>Z. humile</i>	42.80	0.34	21.34	0.572	
	Ascorbic acid	34.92	20.95	5.25	0.781	
	<i>Z. humile</i>	32.92	19.32	7.58	0.875	
		p1	p2	p3 (IC ₅₀)	p4	
Logistic	Ascorbic acid	26.51	67.33	3.05	3.30	0.987 (2.69, 3.41)
	<i>Z. humile</i>	29.64	72.37	8.59	1.08	0.871 (3.08, 14.10)

*Units -intercept (%), slope (%/(µg/mL), IC₅₀ (µg/mL). *Abbreviations – CI -confidence interval at 95.0%, LB -lower boundary, UP -upper boundary.

The results, **Table 8**, indicated that the 4-parameter logistic model (4PL) was the best fit model with higher R^2 values for both ascorbic (pseudo $R^2 = 0.987$) and *Z. humile* (pseudo $R^2 = 0.871$). Thus, further evaluation of IC_{50} were done using results obtain from the logistic model. **Fig. 3** shows that

for both antioxidants, %RSA increased rapidly at lower concentration ($<6.5 \mu\text{g/mL}$) and flattens out as concentration increases to $100 \mu\text{g/mL}$. The %RSA values showed less change after reaching about $65.53 \pm 0.08\%$ (ascorbic acid) and $48.39 \pm 6.6\%$ (*Z. humile*), **Table 8**.

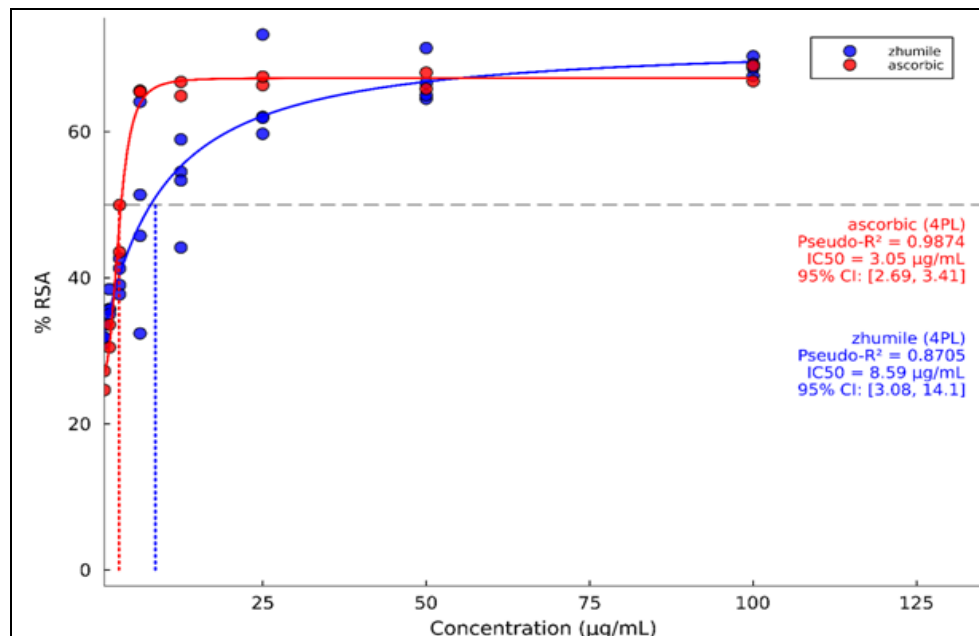


FIG. 3: CONCENTRATION RESPONSE CURVE FOR ASCORBIC ACID AND *Z. HUMILE* RADICAL SCAVENGING ACTIVITY

The estimated inhibitory concentration at 50.0% RSA (IC_{50}) for ascorbic acid and *Z. humile* were 3.05 and $8.59 \mu\text{g/mL}$, respectively **Table 8, Fig. 3**. According to a commonly used classification, compounds with IC_{50} values of $50 \mu\text{g/mL}$ or lower are considered highly potent. Activity in the range of $50\text{--}100 \mu\text{g/mL}$ is classified as strong, $101\text{--}250 \mu\text{g/mL}$ is moderate, $250\text{--}500 \mu\text{g/mL}$ is weak, and greater than $500 \mu\text{g/mL}$ is inactive⁶⁰. The results suggests that *Z. humile* demonstrated considerable high potent antioxidant activity. This indicates that relatively low amounts of the extract were required to scavenge 50% of the radicals. As expected, the positive control, ascorbic acid, produced very low IC_{50} values of $3.05 \mu\text{g/mL}$, significantly lower than that of *Z. humile* extracts. The difference in the IC_{50} values can also be attribute to purity difference instead of just molecular differences. While the results show that the positive control was more efficient at scavenging free radicals, the plant extract's antioxidant activity can still be very high. The present findings were also consistent with previous studies on related plants. In a study by Kaigongi *et al.*⁶¹ they reported IC_{50} values of 10

$\mu\text{g/mL}$ or lower for *Z. chaylbeum*, *Z. gilleti*, *Z. holtzianum*, *Z. paracanthum*, and *Z. usambarensis* stem bark extracts. The strong antioxidant activity of *Z. humile* is attributed to the presence of phytochemicals that can donate electrons or hydrogen, thereby stabilizing free radicals. Antioxidant activity is particularly desirable as it counters the effects of oxidative stress, which has been linked to cellular damage, aging, neurodegenerative diseases, inflammation, and cardiovascular disease²³. Hence, supporting the potential medicinal value of *Z. humile*. Moreover, GS-MS indicated the presence of resorcinol, calacorene, stigmasterol, Lup-20(29)-en-3-one, copene, betatocopherol and 2-methoxy-4-vinylphenol molecules, which are active radical scavenging molecules.

CONCLUSION: The present study provided a comprehensive preliminary evaluation of *Zanthoxylum humile*'s phytochemical profile, antioxidant activity, and acute oral toxicity, creating a strong foundation for further pharmacological exploration. GC-MS revealed

some interesting compounds already known for their antioxidant and therapeutic activity. Antioxidant activity screening further confirmed the bioactivity of the extract's phytochemicals, as evidenced by a favourable IC₅₀, reinforcing the plant's potential as a source of pharmacologically active compounds. The acute oral toxicity showed that there was no treatment-related toxicity in the rats during the study, indicative of a favourable preliminary acute oral toxicity profile. However, our study did not include other studies, such as hematological evaluation, histopathological examination of major organs, etc., necessary to fully characterise *Z. humile*'s toxicological profile. Further studies should include isolating bioactive compounds and exploring mechanisms of action. Studies must be extended to antimicrobial and anti-inflammatory evaluation to explore the full extent of the extract's therapeutic activity. A more comprehensive toxicity evaluation expanding to histopathology, hematology, subacute, subchronic, and chronic toxicity studies should also be explored in further studies.

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Animal use Approval: Before commencing the study, animal use and research ethics approvals were obtained from the Joint Parirenyatwa Research Ethics Committee (JREC), the local research Institutional Review board for the University of Zimbabwe.

CONFLICT OF INTEREST: No conflict to declare.

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