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Original Article

Hepatoprotective Properties of *Acacia nilotica* Bark Extract against H_2O_2 Induced Oxidative Damage in Normal Liver (LO2) and Cancer (HepG2) Cells

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ABSTRACT

Background: Liver cancer's severity has prompted interest in herbal remedies for hepatotoxicity and the disease. *Acacia nilotica (Vachellia nilotica)*, valued for its traditional medicinal properties, particularly in its aerial components, is being investigated for its hepatoprotective and anti-cancer effects. However, research on *A. nilotica* bark extracts and their mechanisms of action is limited. **Materials and Methods:** The study aimed to determine the Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and antioxidant properties of *A. nilotica* bark extract using biochemical assays. Additionally, it examined the protective effects of *A. nilotica* bark extract on normal Liver Cells (LO2) and its toxicity against liver cancer cells (HepG2) using cell culture techniques. **Results:** The extract showed significant antioxidant properties (EC_{50}-16.34), with a total phenolic content of 159.98±9.91 mg GAE/DW and a flavonoid content of 16.93 mg QE/g dry weight. It exhibited moderate toxicity towards HepG2 cells at concentrations >100 µg/mL but was non-toxic to LO2 cells. Moreover, it prevented oxidative damage in LO2 cells induced by H_2O_2. **Conclusion:** These findings highlight the therapeutic potential of *A. nilotica* bark extract in liver cancer and hepatotoxicity treatment, warranting further clinical investigation.

Keywords: *A. nilotica*, cytotoxic, DPPH, H_2O_2, HepG2, LO2.

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INTRODUCTION

The liver, an essential organ integral to myriad physiological processes, plays a pivotal role in detoxification and the expulsion of xenobiotics.[1] The incessant exposure to diverse xenobiotics, pharmaceuticals, and noxious agents *via* the portal blood circulation renders the liver exceptionally vulnerable to impairments. Preserving optimal hepatic health is of paramount importance for holistic human well-being, particularly in light of the widespread occurrence of liver diseases worldwide. Various compounds, encompassing hydrogen peroxide, carbon tetrachloride, lipopolysaccharide, alcohol, free radicals and paracetamol present potential hazards to hepatic cells, culminating in progressive dysfunction.[2] Oxidative stress, distinguished by a perturbed redox equilibrium between the generation of pro-oxidants, notably Reactive Oxygen Species (ROS), and the defense mechanisms of antioxidants, intricately participates in the processes underpinning hepatotoxicity and pathogenesis.[3] The excessive production of ROS precipitates oxidative stress, assuming the role of a mediator in the infliction of cellular damage to various cellular components, including structures, lipids, membranes, proteins, and DNA.[4]

Hydrogen Peroxide (H_2O_2), recognized as a catalyst of oxidative stress and cytotoxicity in human hepatic cells, has been linked to diverse modifications, including alterations in anti- and pro-apoptotic proteins and caspases.[5] Despite notable advancements in therapeutic strategies and contemporary medical practices, hepatic diseases endure as a formidable global health concern. The pursuit of innovative therapeutic agents for the management of liver disorders remains an urgent imperative, particularly given the carcinogenic propensities and potential hepatotoxicity attributed to numerous synthetic pharmaceuticals.[6]

Contemporary research initiatives have pivoted towards the preservation of hepatic well-being and the attenuation of undesirable side effects through the exploration of naturally occurring compounds derived from botanical sources.[7] Numerous plant extracts, along with their constituent elements, have consistently exhibited pronounced hepatoprotective effects, showcasing substantial antioxidant activity against chemically induced liver damage.[8] This study seeks to make a substantive

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contribution to this burgeoning domain by examining the hepatoprotective attributes of *Acacia nilotica* bark extracts in the context of H_2O_2-induced oxidative damage in both normal liver (LO2) and malignant liver (HepG2) cells.

Acacia nilotica (L.), now recognized as *Vachellia nilotica* (L.), is a pioneer tree with a preference for light and possesses nitrogen-fixing qualities, exhibiting rapid growth in arid environments. This tree holds particular significance as a riverine species in India, Sudan, and Senegal, where it is deliberately cultivated for its valuable timber.[9] *Acacia nilotica* has gained popularity as a versatile farm tree, serving multiple purposes such as providing timber, fuelwood, fodder, tannins, and gum, while also being easily propagated. In addition to its utilitarian uses, *Acacia nilotica* holds a revered position in traditional medicine, where every part of the plant is ascribed with auspicious medicinal properties. A comprehensive literature survey has unveiled a diverse array of phytoconstituents in Acacia species, with tannins, alkaloids, fatty acids, polysaccharides, polyphenols emerging as major classes. The chemical composition includes polyphenolic compounds like tannin, phlobatannin, phenolics such as gallic acid, ellagic acid, catechin, and flavonoids, providing the plant with antioxidative capabilities.[10] Furthermore, *Acacia nilotica* boasts a distinctive profile of psychoactive alkaloids, such as dimethyltryptamine and N-methyltryptamine, in conjunction with compounds like tryptamine, β-carbolines, mescaline, bufoteinine, and nicotine.[11] This rich chemical repertoire aligns with the traditional uses of the plant in addressing a spectrum of ailments, from impotence and tumours to tuberculosis and toothaches. Scientific investigations into its pharmacological potential have uncovered activities such as antihypertensive and antispasmodic effects, as well as noteworthy antibacterial, antifungal, and antiplasmodial properties.[12] The multifaceted nature of *Acacia nilotica* positions it as a valuable resource with ecological significance and diverse pharmacological potentials. Although studies have revealed the diverse potential of *A. nilotica*, research on its hepatoprotective properties and liver toxicity is limited, particularly with emphasis on the bark. This paper elaborates the research and findings of hepatoprotective properties of *A. nilotica* bark extract against oxidative damage induced by hydrogen peroxide in normal liver (LO2) cells and cancer (HepG2) cells.

MATERIALS AND METHODS

Sample Collection and Extraction

The powdered stem bark of *A. nilotica* was sourced from India, meticulously authenticated, and its voucher specimen duly recorded at Qingdao Municipal Hospital, Qing Dao, China. To outline the extraction process, air-dried barks (317.01g) of *A. nilotica* were finely powdered and subjected to extraction by dissolving in 2L methanol within a 2000 mL conical flask. The mixture underwent sonication at 20 W for 30 min, followed by an overnight settling period and subsequent filtration. The resulting methanol extract (28.8 g) was then efficiently concentrated using a rotary evaporator at 40°C under reduced pressure of 250 mbar. All the chemicals and reagents used are of analytical grade.

Total Phenolic Content (TPC)

The Folin–Ciocalteu method (Santhanam *et al.*, 2022)[13] was employed to assess the total phenolic content of *A. nilotica* bark extract. In this method, 50 µL of the methanolic crude extract sample, 50 µL of distilled water, 50 µL of 10% Folin-Ciocalteu reagent, and 50 µL of 1 M sodium carbonate solution were combined and added to a 96-well microplate. The resulting mixture was incubated at room temperature for 60 min in the dark and the reading was observed using a microplate reader at 750 nm. The determination of total phenolic content involved employing a standard curve created with gallic acid at various concentrations, and the experiment was conducted in triplicates.

Total Flavonoid Content (TFC)

The total flavonoid content of *A. nilotica* bark was assessed using a spectrophotometric method as described by Santhanam *et al.* (2022).[13] In this method, 100 µL of methanolic crude extract was mixed with 100 µL of 2% $AlCl_3$ solution in 96-well microplates, followed by incubation at room temperature for 60 min. Absorbance was then measured at a wavelength of 415 nm using a microplate reader. The total flavonoid content was determined through a standard curve created with various concentrations of quercetin (0-200 µg/mL) as the standard,[14] and the experiment was conducted in triplicates.

Free Radical Scavenging Assay

The free radical scavenging properties of the methanolic crude extract from *A. nilotica* bark were assessed through the DPPH free radical scavenging assay, as described by Santhanam *et al.* (2022).[13] In this assay, a combination of 0.1 mM DPPH and various concentrations of the methanolic crude extract sample (ranging from 0 to 200 µg/mL)/positive control, Ascorbic Acid (AA)[15] was mixed at a 1:1 ratio (100 µL each). The resulting mixture was thoroughly blended using a vortex mixer and then incubated in a dark room at room temperature for 30 min. Subsequently, the absorbance of the mixture was measured at 517 nm using a spectrophotometer. The experiment was conducted in triplicates.

Cell Culture

LO2 and HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) high-glucose media, following literature protocols with minor adjustments. The media were enriched with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin, and the cells were maintained in a humidified 5% CO_2 incubator at 37°C. Upon reaching 70-80% confluence, the cells were used for seeding and subsequent treatment.

Cell Viability assay

The MTT assay was employed to assess cell viability and cytotoxicity. LO2 and HepG2 cells were seeded at a density of 1×10^4 cells/well in 96-well plates. Upon reaching 80% confluence, 100 µL of varying concentrations (0-200 µg/mL) of the methanol extract of *A. nilotica* was added to the cells. After a 24 hr incubation period, 20 µL of MTT was introduced, and the cells were further incubated at 37°C for 4 hr. Subsequently, the medium was replaced with 100 µL DMSO, and the absorbance for each well was measured at 570 nm using a microplate reader. The cytotoxicity assay aimed to establish the concentration range and all experiments were conducted in triplicates. In the case of HepG2 cells, Curcumin served as the positive control.

Protective Effect of Samples against H_2O_2-Induced Oxidative Damage in LO2 cells

The protective effects of *A. nilotica* against H_2O_2-induced oxidative damage were investigated following previous literature.[16] LO2 cells were seeded at a density of 1×10^5 cells per well in 96-well plates, with a 24 hr incubation period at 37°C. Once reaching confluence, the culture medium was replaced with 100 µL of *A. nilotica* samples and the positive control (ascorbic acid) at varying concentrations (ranging from 0 to 200 µg mL-1) and incubated for an additional 18 hr. Subsequently, the cells were exposed to H_2O_2 (150 µM) for 6 hr, with the concentration based on prior literature. Following H_2O_2 exposure, MTT solution was added, and cells were further incubated for 4 hr. Finally, formazan crystals were dissolved in DMSO (100 µL per well), and absorbance was measured at 540 nm using a microplate reader (Tecan Infinite F200 Pro plate, USA). Ascorbic acid served as the positive control, and all experiments were conducted in triplicates.

Statistical Analysis

Data were expressed as mean ± SD and analyzed using GraphPad Prism version 5. Results with a significance level of less than 0.05 were considered statistically significant. For the cytotoxicity assay involving HepG2 cells, comparisons between the two groups (curcumin and *A. nilotica* extract) were performed using an unpaired t-test. For the protective properties against H_2O_2-induced damage in LO2 cells, comparisons among the four groups (H_2O_2 as negative control, ascorbic acid as positive

Table 1: Total Phenolic and Flavonoid Content in the bark extract of *A. nilotica*.

Sample	Total Phenolics mg GAE/g DW	Total flavonoids mg QE/g DW
A. nilotica (methanol crude extract)	159.98 ± 9.91	16.93 ± 0.81

Data expressed as mean ± SD (*n*=3).

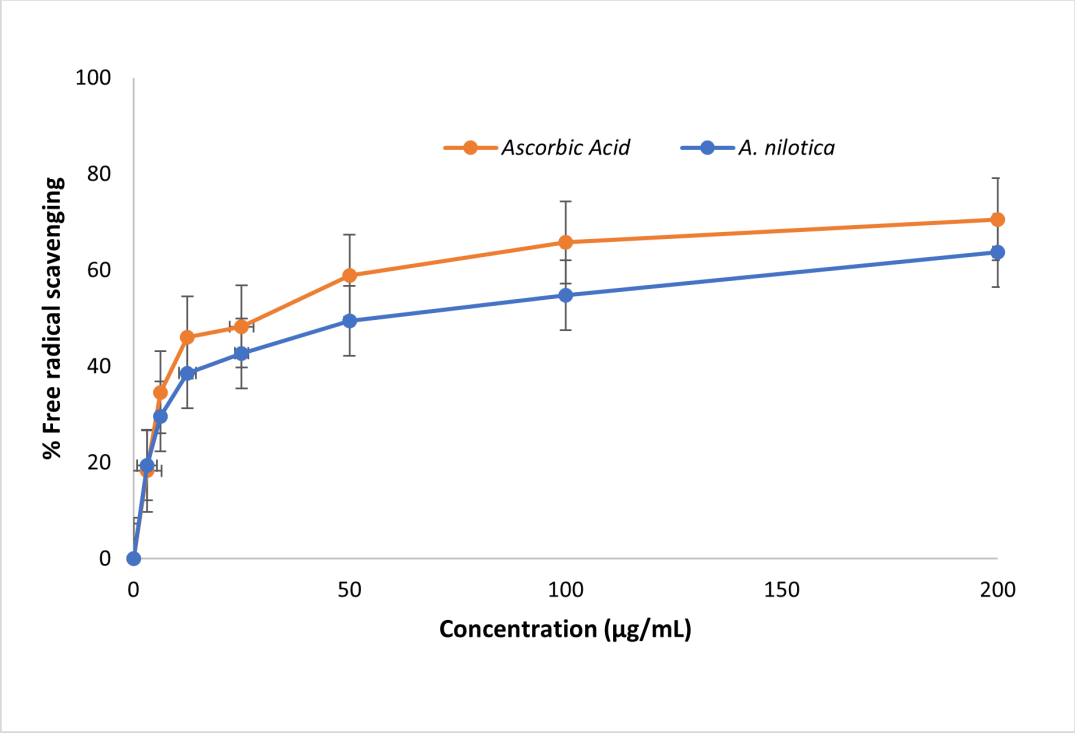

Figure 1: DPPH Free radical scavenging of *A. nilotica* bark methanol extract compared with positive control AA. Data expressed as Mean ± SD (*n*=3).

control, *A. nilotica* extract, and untreated) were performed using one-way ANOVA followed by Dunnett's *post-hoc* test to compare each treatment group to the negative control.

RESULTS

Total Phenolic and Flavonoid content of *A. nilotica* bark extract

The total phenolic and flavonoid content in the crude methanol extract of *A. nilotica* is shown in Table 1. From the table, it is evident that the the crude methanol extract of *A. nilotica* bark exhibited a high phenolic content, approximately 160 mg of gallic acid equivalent per gram (159.98±9.91 mg GAE/g DW), and 16.93 mg of quercetin equivalent flavonoids per gram of dry weight. This indicates a significant presence of phenolic compounds, which are known for their various biological properties, including antioxidant effects. The flavonoid content in the bark extract was comparatively lower than in other parts of the plant, suggesting differential distribution of secondary metabolites within *A. nilotica*.

DPPH Free radical scavenging properties of *A. nilotica* bark extract

Free radicals are molecules characterized by high reactivity and instability, naturally produced by the body as a byproduct of regular metabolic processes, which is one of the major concerns for illness.[17] In this study, the DPPH free radical scavenging properties of the *A. nilotica* bark extract were determined within the concentration range of 0-200 µg/mL and compared with the positive control, Ascorbic Acid (AA), as depicted in Figure 1. The results revealed that the crude methanolic bark extract of *A. nilotica* exhibited strong antioxidant properties, with an EC_{50} value of 16.34±3.60 µg/mL, comparable to that of the positive control AA (8.67±1.62 µg/mL). At a concentration of 100 µg/mL, the *A. nilotica* bark extract exhibited a DPPH free radical scavenging activity of 54.80 ± 0.62%. Comparative analysis with literature findings by Abdel-Farid *et al.* (2014) revealed that the leaves, flowers, and pods demonstrated DPPH scavenging activities of 65.86%, 61.85%, and 63.86%, respectively.[18] These results suggest that *A. nilotica* possesses a diverse array of bioactive compounds with potent antioxidant capabilities, which may be attributed to the presence of phenolic and flavonoid compounds, as supported by previous literatures.[19]

Cytotoxicity of *A. nilotica* bark extract against LO2 and HepG2 cells

Various concentrations (0-200 µg/mL) of crude methanol extract of *A. nilotica* were tested for their cytotoxic properties against LO2 and HepG2 cells, as shown in Figure 2 and Figure 3. From the graphs, it is clear that concentrations up to 200 µg/mL of *A. nilotica* were non-toxic towards LO2 cells. However, at concentrations >12.5 µg/mL, the extract exhibited dose-dependent cytotoxicity towards HepG2 cells. This result suggests that the bark extract of *A. nilotica* possesses selective cytotoxicity towards HepG2 cells at higher concentrations. At a concentration of 200 µg/mL, cell viability was reduced to 65%. The IC_{50} value of the *A. nilotica* bark extract against HepG2 cells is 186.7 µg/mL, whereas for the positive control curcumin, the IC_{50} value is 44.2 µg/mL. Previous studies on the *A. nilotica* flower methanol extract also showed a similar pattern of results against HepG2 cells, with an IC_{50} value of 176.15 µg/mL.[20]

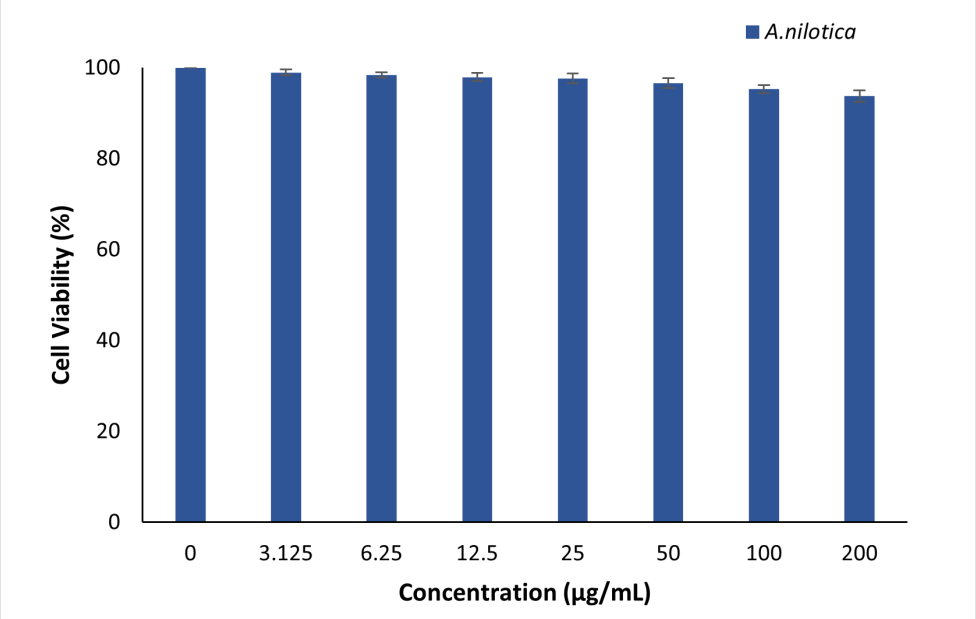

Figure 2: Cytotoxic properties of *A. nilotica* bark extract against LO2 cells. Data expressed as Mean ± SD (*n*=3).

Protective effects of *A. nilotica* bark extract against H_2O_2 induced damage in LO2 cells

Pre-treatment with *A. nilotica* bark extract demonstrated significant protective effects against H_2O_2-induced oxidative damage in LO2 cells. As illustrated in the graph Figure 4, the extract effectively prevented oxidative damage induced by 150 µM H_2O_2, comparable to the positive control Ascorbic Acid (AA). At a concentration of 200 µg/mL, both *A. nilotica* bark extract and AA exhibited considerable protection against H_2O_2-induced cell death, with cell viabilities reaching 66% and 72%, respectively. In contrast, untreated cells exposed to H_2O_2 alone showed significantly reduced cell viability of only 13.71%. These results suggest that the chemical constituents present in the *A. nilotica*

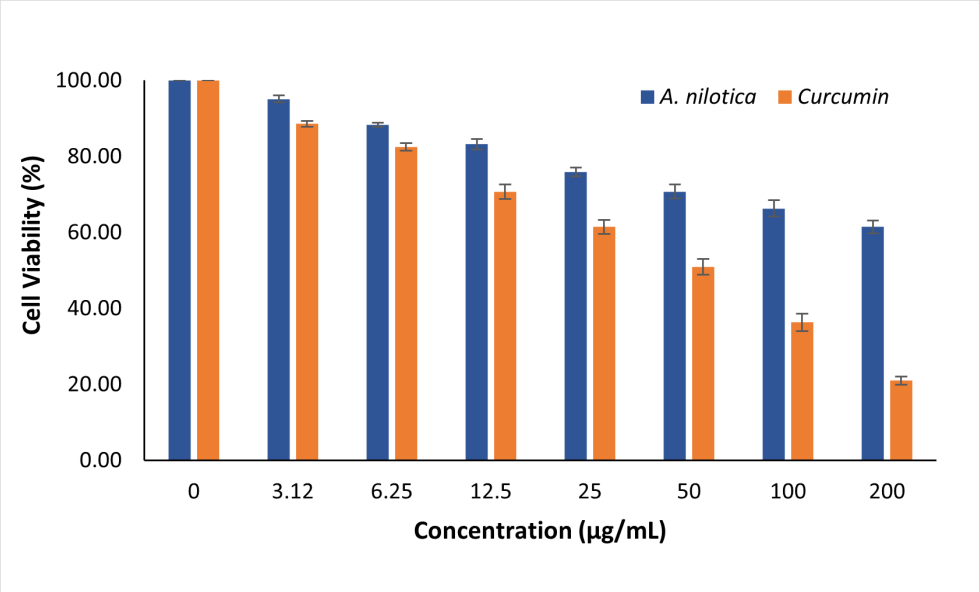

Figure 3: Cytotoxic properties of *A. nilotica* bark extract against HepG2 cells. Data expressed as Mean ± SD (*n*=3). Comparisons between the two groups (curcumin and *A. nilotica* extract) were performed using an unpaired t-test.

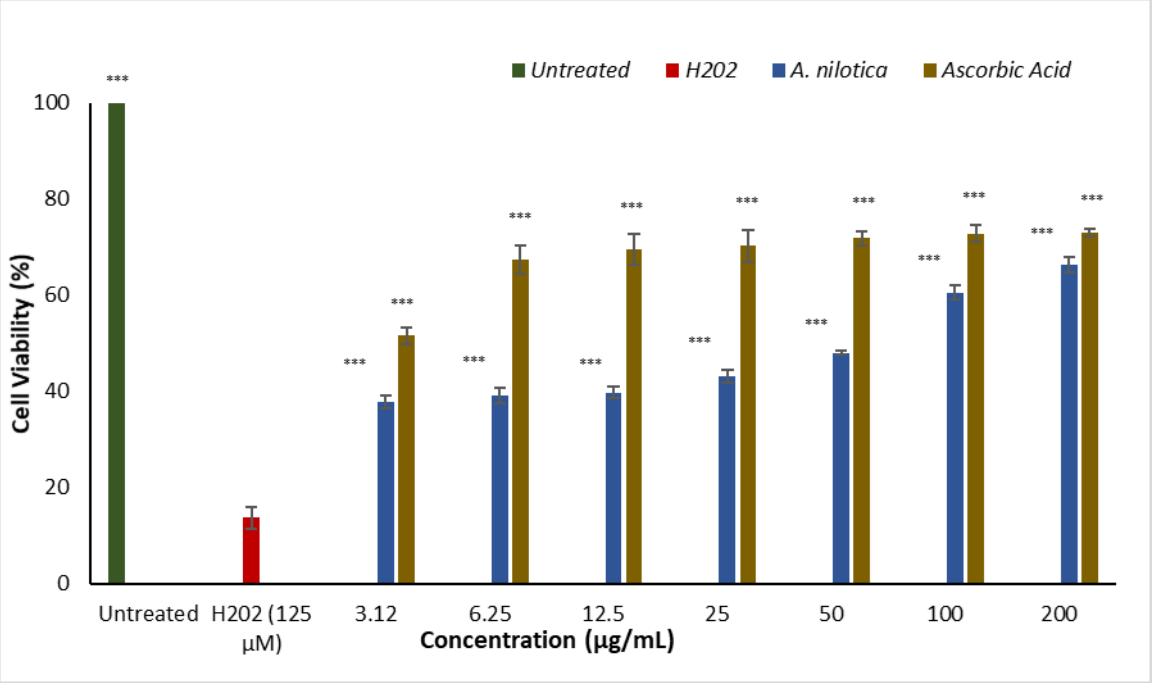

Figure 4: Protective properties of *A. nilotica* bark extract against H_2O_2 induce damage in LO2 cells. Data expressed as Mean ± SD (*n*=3). Comparisons among the four groups (H_2O_2 as negative control, ascorbic acid as positive control, *A. nilotica* extract, and untreated) were performed using one-way ANOVA followed by Dunnett's post-hoc test to compare each treatment group to the negative control.

bark extract possess potent antioxidant properties, capable of scavenging free radicals and mitigating oxidative stress-induced cell death.

DISCUSSION

Phenolic acids and flavonoids constitute a diverse group of plant-derived compounds that are abundantly present across various plant species. These phytochemicals are synthesized through biochemical pathways involving phenylalanine and tyrosine, typically known as the shikimic acid pathway.[21] Their crucial role in various biological properties makes measuring the phenolic acid and flavonoid content in plant species mandatory. Polyphenolic compounds such as gallic acid, catechin, chlorogenic acid, quercetin, and kaempferol found in *A. nilotica* exhibit potent antioxidant activity, crucial for neutralizing harmful free radicals and reducing oxidative stress.[15,25] These antioxidants play a pivotal role in maintaining cellular health and protection against various oxidative stress-related conditions. Our findings underscore the significant free radical scavenging potential of *A. nilotica* bark extract through DPPH assay, suggesting its therapeutic value in combating oxidative stress-induced damage across different cellular systems due to the presence of its phenolic and flavonoid compounds.

In terms of cytotoxicity, the *A. nilotica* bark extract exhibited selective cytotoxicity towards HepG2 cells at higher concentrations, while showing no toxicity towards LO2 cells at concentrations up to 200 µg/mL. This selective cytotoxicity is particularly relevant in the context of liver health, as HepG2 cells are commonly used as a model for studying liver cancer and hepatic cytotoxicity. In addition to its ability to induce cytotoxicity in cancer cells, the ethyl acetate fraction extracted from *A. nilotica* has demonstrated efficacy in alleviating obesity, insulin resistance, and hyperlipidemia in rats.[22] Additionally, there is an emerging evidence suggesting that *A. nilotica* bark extract could serve as a viable substitute for antibiotic growth promoters, aiding in the growth of broilers through the enhancement of immunity and promotion of gut health.[23] In light of the potential benefits of *A. nilotica*, further exploration beyond its bark to include other aerial parts such as leaves, flowers, and pods emerges as a promising avenue for additional research in the field of natural therapeutics, especially concerning liver toxicity.

Hydrogen peroxide (H_2O_2) is a well-known inducer of oxidative stress in cells, leading to DNA damage, lipid peroxidation, and protein oxidation, all of which contribute to cellular dysfunction and various diseases, including liver disorders and cancer.[24] From this research, the protective effects of the *A. nilotica* bark extract against H_2O_2-induced damage in LO2 cells further emphasize its antioxidant properties and cellular protective mechanisms. Our results are consistent with this previous literature, demonstrating that the acetone extract of Acacia species, such as *A. leucophloea, A. dealbata, and A. ferruginea*, effectively inhibits H_2O_2-mediated oxidative stress in HepG2 cells.[25] Oxidative stress induced by H_2O_2 is particularly relevant in liver cells (HepG2 cells) as they are susceptible to ROS due to their high metabolic activity and involvement in detoxification processes. The ability of *A. nilotica* bark extract to mitigate H_2O_2-induced damage in LO2 cells, a model for normal liver cells, suggests its potential in protecting liver tissues from oxidative damage, which is implicated in various liver pathologies, including liver cancer. Further exploration of its mechanisms of action and specific bioactive compounds involved in combating oxidative stress can contribute to the development of targeted therapies for oxidative stress-related conditions, especially those affecting liver health.[26]

Overall, the findings suggest that the *A. nilotica* bark extract possesses significant antioxidant, cytotoxic, and protective properties, making it a promising candidate for further exploration in natural therapeutics and pharmaceutical development, especially in addressing oxidative stress-related conditions and potential applications in cancer therapy.

CONCLUSION

This study highlighted the cytotoxic and antioxidant properties of *A. nilotica* bark extract, indicating its potential usage in hepatotoxicity. The total phenolic and flavonoid content analysis revealed a high concentration of these bioactive compounds in the extract, contributing to its antioxidant capabilities. Additionally, the extract exhibited strong DPPH free radical scavenging activity, comparable to that of the positive control, ascorbic acid. The cytotoxicity assessment demonstrated selective cytotoxicity of the *A. nilotica* bark extract against HepG2 cells. Moreover, the extract showed protective effects against H_2O_2-induced oxidative damage in LO2 cells, further emphasizing its antioxidant properties and potential therapeutic benefits. These findings support the traditional medicinal use of *A. nilotica* and suggest its potential as a natural alternative to synthetic antioxidants and cytotoxic agents. The demonstrated selective cytotoxicity and protective effects against oxidative stress indicate that *A. nilotica* bark extract could play a significant role in the prevention and treatment of liver disorders. Its high phenolic and flavonoid content, along with its potent free radical scavenging activity, underscores its promise as a therapeutic agent. Further research is warranted to identify the chemical constituents, elucidate the underlying mechanisms of action, and explore its therapeutic applications in clinical settings. By doing so, the full potential of *A. nilotica* bark extract in preventing and treating liver disorders can be realized, offering a promising natural alternative to conventional therapies.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

H_2O_2: Hydrogen peroxide; **ROS:** Reactive oxygen species; **LO2:** Normal liver cells; **HepG2:** Malignant liver cells; **DMEM:** Dulbecco's Modified Eagle's Medium; **FBS:** Fetal bovine serum; **DPPH:** 2,2-Diphenyl-1-picrylhydrazyl; **AA:** Ascorbic acid; **MTT:** 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide; **EC_{50}:** Half-maximal effective concentration.

SUMMARY

This research explores the hepatoprotective and antioxidant potential of *Acacia nilotica* bark extract against Hydrogen Peroxide (H_2O_2)-induced oxidative damage in liver cells. The extract, rich in phenolic compounds and flavonoids, exhibited strong antioxidant activity as evidenced by its DPPH free radical scavenging properties. It displayed selective cytotoxicity against HepG2 cancer cells while being non-toxic to normal liver cells (LO2). Moreover, the extract demonstrated significant protective effects against H_2O_2-induced oxidative stress in LO2 cells, highlighting its potential as a natural therapeutic agent for liver disorders and oxidative stress-related conditions.Top of Form

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