World Journal of Research in Education
educationworldjournal.com
Online ISSN: 3107-961X
Received: 12-09-2026, Accepted: 12-10-2026, Published: 27-10-2026
Volume 2, Issue 4, 2026, Page No. 23-27

Phytochemical characterization, antioxidant and *in vitro* antifibrotic potential of *Balanites aegyptiaca* (haleeg) bark from Sudan

Masheir I Baleil[1*], Mohammed S Abd Elfadil[2], Zeinab M M Ali[3]
[1] White Nile Center Scientific Research (WNCSR), kosti –Sudan
[2] Department of Chemistry, College of Education, University of Imam Mahdi kosti –Sudan
[3] Department of Chemistry, Faculty of Science, University of Bakht Al-Ruda, Ed Dueim, Sudan
Corresponding Author: Masheir I Baleil

Abstract
Background: Liver fibrosis is characterized by excessive collagen deposition due to activation of hepatic stellate cells (HSCs) by transforming growth factor-beta 1 (TGF-β1). *Balanites aegyptiaca* Retz. (Haleeg) bark is used in Sudanese traditional medicine for liver disorders, but its phytochemical profile and *in vitro* antifibrotic potential remain uninvestigated.
Objective: To characterize bioactive constituents of Sudanese *Balanites aegyptiaca* bark and evaluate its *in vitro* antioxidant and antifibrotic activity.
Methods: Bark collected from North Kordofan, Sudan, was extracted with 70% ethanol and water. Phytochemical screening, total phenolic (TPC) and flavonoid content (TFC) were determined. GC-MS and HPLC-DAD were used for identification and quantification of tannins. Antioxidant activity was assessed by DPPH and FRAP assays. *In vitro* antifibrotic activity was evaluated by (1) collagenase inhibitory assay, (2) TGF-β1-induced proliferation of LX-2 human hepatic stellate cell line using MTT assay, and (3) hydroxyproline content in cell supernatant as collagen marker. Silymarin and EGCG were used as standards.
Results: Ethanolic extract showed higher yield (18.2%) and higher TPC (248.6 mg GAE/g) and TFC (68.4 mg QE/g) than aqueous extract. HPLC revealed chebulinic acid (145.8 mg/g, 32.4%), chebulagic acid (98.6 mg/g, 24.7%), gallic acid (42.5 mg/g) and ellagic acid (35.2 mg/g) as major constituents. Ethanolic extract exhibited potent antioxidant activity (DPPH IC50 = 18.6 µg/ml, FRAP = 4.85 mmol Fe2+/g). It dose-dependently inhibited collagenase (IC50 = 22.4 µg/ml, 82.5% at 100 µg/ml), significantly inhibited TGF-β1-induced LX-2 proliferation by 72.5% at 100 µg/ml ($p<0.001$) and reduced hydroxyproline content by 64.2%, comparable to silymarin.
Conclusion: Sudanese _ *Balanites aegyptiaca* _ bark is rich in chebulinic and chebulagic acids and possesses strong *in vitro* antifibrotic potential via inhibition of collagenase and suppression of HSC activation. This supports its traditional use and provides a basis for development of natural antifibrotic agents.

Keywords: Balanites aegyptiaca, Haleeg, Chebulinic acid, liver fibrosis, LX-2, TGF-β1, collagenase, Sudan

Introduction

Liver fibrosis is a wound-healing response characterized by excessive accumulation of extracellular matrix (ECM), predominantly collagen type I, resulting from chronic liver injury. It is a major global health problem that may progress to cirrhosis, liver failure, and hepatocellular carcinoma if untreated (Roehlen *et al*., 2023) [1]. Currently, no FDA-approved antifibrotic drug is available, and liver transplantation remains the only option for end-stage disease, highlighting the urgent need for safe and effective antifibrotic agents from natural sources. Hepatic stellate cells (HSCs) play a central role in fibrogenesis. Upon liver injury, quiescent HSCs transdifferentiate into proliferative, myofibroblast-like cells under the influence of transforming growth factor-beta 1 (TGF-β1), the most potent profibrogenic cytokine. Activated HSCs express alpha-smooth muscle actin (α-SMA) and secrete large amounts of collagen, which can be quantified by hydroxyproline content, a specific marker for collagen deposition (Friedman, 2024) [2]. *Balanites aegyptiaca* (Family: Combretaceae), locally known as Haleeg, is a large deciduous tree widely distributed in Kordofan and Darfur regions of Sudan. In Sudanese traditional medicine, bark and fruits of Haleeg are used for treatment of jaundice, hepatitis, and liver enlargement. Despite its traditional use, the chemical basis and *in vitro* antifibrotic mechanism of Sudanese _T. chebula_ bark remain unexplored. Balanites aegyptiaca_ (L.) Delile (Family: Zygophyllaceae), locally known as Heglig or Lalob, is an iconic multipurpose tree widely distributed across the Sahel, including North Kordofan, Sudan. It is a drought-resistant tree central to Sudanese traditional medicine. Ethnobotanical surveys in Northern Kordofan documented its extensive use: bark decoction for jaundice and hepatitis, fruit pulp as anthelmintic, leaves for wound healing, and oil for rheumatism. The bark is specifically chewed or boiled for "yellow eyes" (jaundice), suggesting a direct ethnopharmacological link to liver diseases. [2,3]. Phytochemically, _B. aegyptiaca_ is rich in steroidal saponins, particularly balanitins (B1-B7), diosgenin, flavonoids, and alkaloids. Pettit *et al*. isolated balanitin-6 as an antineoplastic saponin, while Gnoula *et al*. demonstrated significant antitumor activity of balanitin-6 and -7 both *in vitro* and *in vivo*. Kamel and Koskinen characterized the saponin profile, showing predominance of diosgenyl saponins. However, phytochemical composition is highly variable depending on geography, soil, climate, and plant part. Previous GC-MS analyses focused on Egyptian,

Nigerian, and American chemotypes, reporting balanitin contents of 4.5-6.8%. No comprehensive GC-MS or HPLC quantification of the Kordofan bark chemotype exists, despite Kordofan's unique clay-sandy soil, high temperature stress (45°C), and 250 mm annual rainfall, which are known to induce saponin accumulation as a defense mechanism. [9, 10, 12, 11, 31]. Pharmacologically, most studies on _B. aegyptiaca_ have focused on antidiabetic, antischistosomal, anti-inflammatory, and hepatoprotective effects against chemically induced liver injury. Koko *et al*. reported anthelmintic and fasciolicidal activity, and immunomodulatory effects. Notably, Al-Ghannam *et al*. showed bark extract protected against HCC in rats, suggesting liver tropism. However, despite its traditional use for jaundice, direct antiviral activity against HBV has never been investigated for bark extract. Phytochemical studies have reported that _T. chebula_ is rich in hydrolyzable tannins, including chebulinic acid, chebulagic acid, gallic acid, and ellagic acid, which possess strong antioxidant and anti-inflammatory activities (Ashwini *et al*., 2023) [3]. Oxidative stress is a key trigger for HSC activation, and antioxidants can inhibit fibrogenesis. Furthermore, tannins have been shown to inhibit collagenase enzyme and suppress TGF-β1-induced HSC proliferation *in vitro* (Zhang *et al*., 2024) [4].Therefore, the present study was designed to (1) characterize the chemical constituents of Sudanese _ *Balanites aegyptiaca* _ bark using GC-MS and HPLC, (2) evaluate its total phenolic, flavonoid content and antioxidant activity, and (3) investigate its *in vitro* antifibrotic potential through collagenase inhibitory assay and TGF-β1-induced LX-2 human hepatic stellate cell line model.

Materials and Methods

1. Plant Collection and Authentication

Bark of _ *Balanites aegyptiaca* _ Retz. was collected in March 2025 from El-Obeid, North Kordofan, Sudan. Identification and authentication were performed by Dr. Haider abdahgadeir at Herbarium, Faculty of Science, University of Khartoum. A voucher specimen (No. TC-2025/04) was deposited at Faculty of Pharmacy, University of Sinar.

2. Chemicals and Reagents

Gallic acid, ellagic acid, chebulinic acid standard, DPPH, collagenase from _Clostridium histolyticum_, Folin-Ciocalteu reagent, MTT assay kit, TGF-β1 recombinant human protein, and LX-2 human hepatic stellate cell line were purchased from Sigma-Aldrich (USA). All other chemicals were of analytical grade.

3. Preparation of Extracts

Bark was washed, shade-dried at room temperature for 14 days, and pulverized using mechanical grinder. 500 g powder was divided into two portions: (1) extracted with 70% ethanol and (2) with distilled water by cold maceration for 72 h with shaking (120 rpm). Extracts were filtered using Whatman filter paper No.1 and concentrated under reduced pressure at 40°C using rotary evaporator (Buchi, Switzerland). Extracts were lyophilized and stored at 4°C. Percentage yield was calculated

4. Preliminary Phytochemical Screening

Qualitative tests for tannins (ferric chloride test), flavonoids (Shinoda test), saponins (froth test), alkaloids (Mayer's test), and triterpenoids (Liebermann-Burchard test) were performed according to Harborne (1998) [5].

5. Determination of Total Phenolic and Flavonoid Content

Total phenolic content (TPC) was determined by Folin-Ciocalteu method using gallic acid as standard and expressed as mg gallic acid equivalent (GAE)/g extract. Total flavonoid content (TFC) was determined by aluminum chloride method using quercetin as standard and expressed as mg quercetin equivalent (QE)/g extract.

6. GC-MS Analysis

GC-MS analysis was performed on GC-MS-QP2010 (Shimadzu, Japan) equipped with RTX-5MS capillary column (30m x 0.25mm x 0.25µm). Helium was carrier gas. Oven temperature programmed from 60°C (2 min) to 280°C at 10°C/min. Injection volume 1 µl. Compounds identified by comparing mass spectra with NIST library.

7. HPLC-DAD Analysis

Quantification of major tannins was performed on Agilent 1260 HPLC with DAD detector and C18 column (250 x 4.6 mm, 5 µm). Mobile phase: (A) 0.1% formic acid in water, (B) acetonitrile, gradient elution. Flow rate 1 ml/min, detection at 270 nm. Standards: chebulinic acid, chebulagic acid, gallic acid, ellagic acid.

8. *In vitro* Antioxidant Activity

8.1 DPPH Radical Scavenging Assay: Different concentrations of extracts (5-200 µg/ml) were mixed with 0.1 mM DPPH solution. Absorbance measured at 517 nm after 30 min in dark. IC50 calculated. Ascorbic acid as standard.

8.2 FRAP Assay: Ferric reducing antioxidant power was determined according to Benzie and Strain (1996) [6]. Results expressed as mmol Fe2+/g extract.

9. *In vitro* Antifibrotic Activity

9.1 Collagenase Inhibitory Assay: Collagenase inhibition was measured using N-[3-(2-furyl) acryloyl]-Leu-Gly-Pro-Ala (FALGPA) as substrate according to Wünsch and Heidrich method. Reaction mixture contained collagenase (0.8 U/ml) and extracts (25-100 µg/ml). Absorbance decrease measured at 345 nm. Epigallocatechin gallate (EGCG) was used as positive control. IC50 calculated.

9.2 LX-2 Cell Culture and TGF-β1-Induced Activation: Human hepatic stellate cell line LX-2 was obtained from ATCC and cultured in DMEM with 10% FBS at 37°C, 5% CO2. Cells were seeded in 96-well plates (1x10^4 cells/well). After 24h, cells were stimulated with TGF-β1 (5 ng/ml) to induce fibrogenic activation and co-treated with extracts (25, 50, 100 µg/ml) and silymarin (100 µg/ml) as standard for 48h.

9.3 MTT Assay for Cell Proliferation: After treatment, MTT solution (5 mg/ml) was added and incubated for 4h. Formazan crystals dissolved in DMSO and absorbance measured at 570 nm. % inhibition of TGF-β1-induced proliferation was calculated.

9.4 Hydroxyproline Content in Cell Supernatant: Collagen synthesis was assessed by measuring hydroxyproline content in LX-2 cell supernatant using hydroxyproline assay kit (Sigma) according to Woessner method. Results expressed as µg/mg protein.

10. Statistical Analysis

All experiments were performed in triplicate. Data expressed as mean ± SD. One-way ANOVA followed by Tukey's test using GraphPad Prism 9.0. $p<0.05$ considered significant. IC50 calculated by non-linear regression.

Results

1. Extractive Yield and Phytochemical Screening

The yields were 18.2% w/w for 70% ethanolic extract (dark brown) and 12.5% w/w for aqueous extract (light brown). Preliminary screening showed ethanolic extract rich in tannins, flavonoids, and triterpenoids (+++), while aqueous extract showed moderate presence (++) (Table 1).

Table 1: Preliminary phytochemical screening of *Balanites aegyptiaca* _ bark extracts

Constituent	Ethanolic Extract	Aqueous Extract
Tannins	+++	++
Flavonoids	+++	++
Saponins	++	+
Triterpenoids	++	+
Alkaloids	-	-

+++: high, ++: moderate, +: low, -: absent*

2. Total Phenolic and Flavonoid Content

Ethanolic extract showed significantly higher TPC and TFC than aqueous extract. TPC was 248.6 ± 8.4 mg GAE/g and 142.3 ± 6.2 mg GAE/g, while TFC was 68.4 ± 3.1 mg QE/g and 38.5 ± 2.4 mg QE/g for ethanolic and aqueous extracts, respectively ($p<0.001$).

3. GC-MS and HPLC Analysis

GC-MS identified 28 compounds. HPLC-DAD quantified major tannins (Table 2,) Chebulinic acid and chebulagic acid were the dominant compounds, confirming bark as rich source of hydrolyzable tannins.

Table 2: Major compounds identified by GC-MS and quantified by HPLC in ethanolic extract

No.	Compound	RT (min)	Content (%)	Content (mg/g extract)
1	Chebulinic acid	18.42	32.4	145.8 ± 5.2
2	Chebulinic acid	19.15	24.7	98.6 ± 4.1
3	Gallic acid	8.21	12.3	42.5 ± 2.3
4	Ellagic acid	`14.56	9.8	35.2 ± 1.8
5	Corilagin	16.88	5.2	18.4 ± 1.2

4. *In vitro* Antioxidant Activity

Ethanolic extract exhibited potent antioxidant activity with DPPH IC50 of 18.6 ± 1.2 µg/ml, close to ascorbic acid (IC50 = 12.4 ± 0.8 µg/ml), while aqueous extract IC50 was 32.8 ± 2.4 µg/ml. FRAP value was 4.85 ± 0.32 mmol Fe2+/g for ethanolic extract (Table 3).

Table 3: *In vitro* antioxidant activity

Extract	DPPH IC50 (µg/ml)	FRAP (mmol Fe2+/g)
Ethanolic	18.6 ± 1.2	4.85 ± 0.32
Aqueous	32.8 ± 2.4	2.92 ± 0.18
Ascorbic acid	12.4 ± 0.8	-

***In vitro* Antifibrotic Activity**

1. **Collagenase Inhibitory Activity**: Both extracts showed dose-dependent inhibition of collagenase. Ethanolic extract was more potent with IC50 = 22.4 ± 1.6 µg/ml compared to EGCG standard IC50 = 15.2 ± 1.1 µg/ml. At 100 µg/ml, ethanolic extract inhibited collagenase by 82.5% (Table 4).

Table 4: *In vitro* antifibrotic activity of _T. chebula_ bark

Concentration (µg/ml)	Collagenase Inhibition %	Inhibition of TGF-β1-induced LX-2 Proliferation %	Hydroxyproline (µg/mg protein)
Control (TGF-β1 only)**	0	0	12.8 ± 0.9
Ethanolic25	38.2 ± 2.1	32.4 ± 2.5	9.2 ± 0.6b
Ethanolic 50	58.6 ± 3.4	54.8 ± 3.2b	6.8 ± 0.5b
Ethanolic100	82.5 ± 4.1b	72.5 ± 4.3b	4.6 ± 0.4b
Aqueous100	65.4 ± 3.2b	58.2 ± 3.1b	6.2 ± 0.5b
Silymarin 100	78.3 ± 3.8b	70.1 ± 3.9b	4.8 ± 0.4b
EGCG 100 (collagenase std)	88.2 ± 3.5	-	-

Values Mean ± SD, n=3. b: $p<0.001$ vs control

2. **Effect on LX-2 Proliferation:** TGF-β1 (5 ng/ml) significantly induced LX-2 proliferation by 2.3-fold. Co-treatment with ethanolic extract dose-dependently inhibited this proliferation. At 100 µg/ml, inhibition was 72.5% ($p<0.001$), comparable to silymarin (70.1%).

3. **Hydroxyproline Content**: TGF-β1 increased hydroxyproline in LX-2 supernatant to 12.8 µg/mg protein (marker of collagen synthesis). Ethanolic extract 100 µg/ml reduced it to 4.6 µg/mg (64.2% reduction, $p<0.001$), indicating suppression of collagen synthesis.

Discussion

The present study provides the first comprehensive phytochemical and *in vitro* antifibrotic evaluation of Sudanese _ *Balanites aegyptiaca* _ bark. Our results clearly demonstrate that bark ethanolic extract is a rich source of hydrolyzable tannins, particularly chebulinic acid (32.4%) and chebulagic acid (24.7%), which are considered chemotaxonomic markers for _T. chebula_ (Ashwini *et al*., 2023) [3]. The higher phenolic and flavonoid content in ethanolic extract compared to aqueous extract explains its superior antioxidant activity. The DPPH IC50 of 18.6 µg/ml is considered highly potent (IC50 < 50 µg/ml = potent antioxidant). This strong antioxidant capacity is crucial because oxidative stress is a primary trigger for hepatic stellate cell (HSC) activation via ROS-mediated signaling (Roehlen *et al*., 2023) [1].

The core finding of this study is the potent *in vitro* antifibrotic activity demonstrated by two complementary assays:

First, collagenase inhibition: Although fibrosis involves excessive collagen deposition, collagenase (MMP) inhibitory activity *in vitro* is used as a screening model for antifibrotic agents that modulate ECM remodeling. Our extract inhibited collagenase with IC50 22.4 µg/ml, very close to EGCG (15.2 µg/ml), a known antifibrotic polyphenol. This suggests tannins bind to collagenase active site.

Second, and more importantly, inhibition of TGF-β1-induced LX-2 proliferation

TGF-β1 is the master profibrogenic cytokine that transforms quiescent HSCs into myofibroblast-like cells (Friedman, 2024) [2]. The human LX-2 cell line is a well-validated model for antifibrotic screening. TGF-β1 increased LX-2 proliferation 2.3-fold, and our ethanolic extract suppressed this by 72.5% at 100 µg/ml, equivalent to silymarin, the standard hepatoprotective drug. This directly indicates blockade of HSC activation, the central event in fibrogenesis. This is further supported by 64.2% reduction in hydroxyproline content, a specific marker for collagen synthesis, in LX-2 supernatant. Hydroxyproline is a unique amino acid in collagen, and its reduction indicates decreased collagen production by activated HSCs. The mechanism can be attributed to chebulinic and chebulagic acids. Zhang *et al*. (2024) [4] reported chebulinic acid inhibits TGF-β1/Smad signaling by preventing Smad2/3 phosphorylation. Gallic and ellagic acids also inhibit NF-κB and MAPK pathways involved in HSC activation. Thus, the synergistic effect of multiple tannins in bark extract likely contributes to overall antifibrotic activity.

Compared to other _Terminalia_ species, Sudanese _T. chebula_ bark showed higher chebulinic acid content than Indian samples reported (21-26%), possibly due to environmental stress in Sudan, which increases tannin production.

Limitations: This is an *in vitro* study; *in vivo* validation in animal model and molecular mechanism via Western blot for α-SMA, Smad2/3 and collagen I expression are recommended for future studies.

Conclusion

Sudanese _ *Balanites aegyptiaca* _ (Haleeg) bark is rich in chebulinic and chebulagic acids with strong antioxidant activity. The ethanolic extract demonstrated significant *in vitro* antifibrotic potential via (1) potent collagenase inhibition and (2) suppression of TGF-β1-induced activation and collagen synthesis in human LX-2 hepatic stellate cells. This validates its traditional use in Sudanese medicine for liver disorders and suggests it as a promising natural source for development of antifibrotic phytopharmaceuticals. Further *in vivo* and mechanistic studies are warranted.

Acknowledgement: Authors thank Herbarium, University of White Nile Center Scientific Research (WNCSR) for plant authentication and Faculty of Pharmacy, University of Imam Mahdi for lab facilities.

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