Arjunetin as a promising phytotherapeutic molecule: Isolation from Terminalia arjuna bark and therapeutic evaluation for anti-inflammatory, anti-cancer, anti-diabetic, and cytotoxic activities

Gandarvakottai Senthilkumar Arumugam

senthilorganic@gmail.com

Indian Institute of Technology Madras

Sathyanarayana N Gummadi

Indian Institute of Technology Madras

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Arjunetin as a promising phytotherapeutic molecule: Isolation from *Terminalia arjuna* bark and therapeutic evaluation for anti-inflammatory, anti-cancer, anti-diabetic, and cytotoxic activities

Gandarvakottai Senthilkumar Arumugam[1,2]*, Sathyanarayana N. Gummadi[1,2] *

[1]Applied and Industrial Microbiology Lab, Department of Biotechnology, Indian Institute of Technology Madras, Chennai 600 036, India.

[2]International Phytomedicine Innovation Private Limited, 1FA I Floor, Kanagam Road, Taramani, Tamil Nadu, India, IIT Madras Research Park, Chennai-600113, India.

*Corresponding authors: sngummadi@gmail.com, senthilorganic@gmail.com

Abstract

Terminalia arjuna bark ethanolic extract and its bioactive compound, arjunetin, possess diverse therapeutic properties, including anti-inflammatory, anti-diabetic, anti-cancer, anti-malarial, and anti-viral activities. This study developed a novel solvent extraction-based method for isolating arjunetin from *T. arjuna* bark, achieving 95% purity without column chromatography. The isolated arjunetin and direct ethanolic extract (DEE) were evaluated for anti-inflammatory, anti-diabetic, anti-cancer, and cytotoxic activities. Arjunetin demonstrated significant anti-inflammatory activity by reducing nitric oxide production in LPS-stimulated cells, while the ethanolic extract showed 42.4% inhibition at 100 µg/mL. In anti-diabetic assays, arjunetin (0.730–2.289 µg/mL) induced a dose-dependent enhancement of glucose uptake. Similarly, the direct ethanolic extract (DEE) significantly improved glucose utilization in differentiated 3T3-L1 adipocytes, increasing glucose uptake from 56.81% in the insulin-treated control to 72.33% at 100 µg/mL. These findings suggest that both arjunetin and DEE exert potent insulin-sensitizing effects by enhancing insulin-mediated glucose transport, highlighting their promising therapeutic potential for diabetes management. Arjunetin exhibited potent anticancer activity against MCF-7 cells with an IC_{50} of 10.807 µg/mL, whereas the extract showed cytotoxicity against MDA-MB-231 cells (IC_{50} 84.316 µg/mL). Cytotoxicity studies on Vero cells indicated lower toxicity of the extract toward normal cells. These findings validate the traditional medicinal use of *T. arjuna* and highlight arjunetin as a promising therapeutic candidate for inflammation, cancer, and diabetes management.

Keywords: *Terminalia arjuna,* arjunetin, anti-cancer, anti-diabetic, anti-inflammatory.

1. Introduction

World Health Organization reports that medicinal plants remain essential in global healthcare, supporting both traditional and modern medicine. Nearly 80% of the global population relies on traditional medicine, while about 60% of rural India depends on herbal remedies. In India, healthcare is strongly supported by Ayurveda, Siddha, and Unani systems[1].

Growing awareness of medicinal plants has increased research into bioactive compounds. Herbal medicines are valued for their availability, affordability, safety, and effectiveness, often with fewer side effects than synthetic drugs. This has promoted phytochemical drug discovery and integration of traditional knowledge with modern medicine. Although many medicinal plants are described in Indian systems, only a few have been scientifically validated. These plant-based therapeutics show potential in treating cardiovascular diseases, diabetes, ulcers, respiratory disorders, inflammation, infections, skin diseases, and cancer[2].

T. *arjuna* (Roxb.) Wight & Arn., widely recognized as Arjuna, is a prominent medicinal tree with extensive therapeutic significance in traditional medicine. The medicinal value of *T. arjuna* has been recognized for centuries in Ayurveda, with its therapeutic applications documented in classical texts such as the Charaka Samhita. Contemporary research has increasingly validated these traditional claims, establishing its broad-spectrum biological activities, including cardioprotective, anticancer, antidiabetic, anti-inflammatory, antimicrobial, and antioxidant effects [3,4,5].

Arjunolic acid, a major bioactive pentacyclic triterpenoid from *T. arjuna,* has gained significant attention for its broad pharmacological potential, including antioxidant, anti-inflammatory, antidiabetic, cardioprotective, antimicrobial, and anticancer activities. These properties make it a promising lead molecule for developing novel phytopharmaceuticals [4,5].

Growing evidence on *T. arjuna* and its phytochemicals highlights the need for further research to understand their molecular mechanisms and validate therapeutic efficacy. Continued studies on arjunolic acid and related compounds may aid the development of innovative treatments for cardiovascular, metabolic, infectious diseases, and cancer, bridging traditional medicine with modern biomedical science [6,7].

T. arjuna has emerged as a promising medicinal plant with significant anticancer potential due to bioactive constituents such as arjunolic acid, flavonoids, tannins, and

other phytochemicals. Studies show that *T. arjuna* extracts inhibit cancer cell proliferation by inducing apoptosis, cell cycle arrest, modulation of signaling pathways, and reduction of oxidative stress. Its strong antioxidant activity also contributes to anti-carcinogenic effects by suppressing oxidative damage and limiting tumor growth, supporting its potential in anticancer drug development [8,9].

The potent anti-inflammatory activity of *T. arjuna* is mainly linked to triterpenoids, flavonoids, saponins, and phenolic compounds. Bark and leaf extracts suppress acute and chronic inflammation by inhibiting prostaglandin biosynthesis and downregulating pro-inflammatory mediators, supporting its traditional use for inflammatory disorders[10].

T. arjuna also exhibits notable anti-diabetic properties, including significant reduction of blood glucose and protection of pancreatic β-cells. Its phytochemicals act similarly to synthetic antidiabetic agents by inhibiting DPP-IV and improving insulin sensitivity, highlighting its therapeutic potential in diabetes management[11].

The bark of *T. arjuna* is rich in bioactive compounds, with over 45 identified, which demonstrate potential for addressing various health conditions [12]. These compounds can be effectively utilized for their therapeutic benefits in disease management. However, the isolation of individual pure compounds from the complex crude extracts of the bark remains a significant challenge in the field. A critical aspect of botanical research involves isolating active components from plants. Various established methods exist for extracting bioactive substances from *T. arjuna bark. H*owever, isolating pure single compounds from complex plant extracts remains challenging without employing column chromatography In the present study, an innovative technique for separating pure chemicals has been elucidated, eliminating the need for the time-consuming and labor-intensive process of column chromatography. This innovative technique streamlines the traditionally labor-intensive and time-consuming process of isolating arjunetin from *T. arjuna* bark. It facilitates the extraction of a pure single constituent without the need for column chromatography. The method allows for straightforward scaling to industrial production while ensuring high-purity arjunetin yields[13].

Based on a comprehensive literature review and our scientific investigations, no previous studies have documented the antimalarial, antiviral, anticancer, anti-inflammatory, and antidiabetic properties of arjunetin. Thus, this study is the first to report these novel biological activities.

Our principal research objectives stem from laboratory evidence identifying arjunetin as a highly promising therapeutic candidate for the treatment of COVID-19, malaria,

and other viral diseases[14]. Building on this breakthrough, we aim to establish a cost-effective and scalable strategy for isolating pure arjunetin from *T. arjuna* bark without relying on column chromatography. Additionally, we seek to systematically compare the anticancer, anti-inflammatory, and anti-diabetic activities of isolated arjunetin with those of the ethanolic extract, thereby evaluating its therapeutic superiority and translational potential.

2. Materials and Methods

2.1 Materials

The dried stem bark of *T. arjuna* was procured from K. Ramaswamy Chetty Country Drugs Wholesale and Retail Shop, Rasappa Chetty Street, Chennai, Tamil Nadu, India. The plant material was taxonomically authenticated at the Siddha Central Research Institute, Arumbakkam, Chennai, Tamil Nadu, India, and a voucher specimen was deposited (Voucher No. T19081901A). Sephadex LH-20 chromatography matrix was purchased from Merck India and GE Healthcare, India. Hexane, chloroform, and dimethyl sulfoxide-*d*6 (DMSO-*d)*[6] used for extraction and nuclear magnetic resonance (NMR) analysis were of analytical reagent (AR) grade and obtained from commercial suppliers in India. Ethanol (95%, v/v; technical grade) was procured from Hayman, India. Unless otherwise stated, all other chemicals and reagents used in this study were of analytical grade.

2.2 Measurements

UV spectra were recorded on a Jasco V 550 UV-VIS spectrophotometer. IR spectra were obtained using a Perkin Elmer Spectrum One Fourier Transform Infrared spectrometer with KBr pellets. ^{1}H and ^{13}C NMR spectra of compounds were recorded on a Bruker AVANCE III 500 NMR spectrometer in DMSO-d6 with TMS as the internal standard, and chemical shifts (δ) were reported in ppm. HRESIMS were measured on a Agilent Q-TOF micro hybrid quadrupole time of flight mass spectrometer with electrospray ionisation (ESI) in Positive ion mode using methanol as the solvent. Chromatograph HPLC (Shimadzu's RID 10 A), Column, C18 128 Shim-pack XR-ODS II (150 mm (L) x 3 mm (i.d.). Mobile Phase (A: 0.1% Acetic acid in CH_3OH, B: 0.1% Acetic acid in H_2O, Gradient: 2% to 30 % of CH_3OH in 10 minutes,

Flow rate: 1 ml/min, Detection: 254 nm, Injection volume: 20 µl, Column Temperature: 30 ^{0}C.

2.3 Extraction and isolation of arjunetin from *T. arjun b*ark using a solvent extraction method

Air-dried bark of *T. arjuna* (2.5 kg) was pulverized into a fine powder and subjected to sequential solvent extraction using solvents of increasing polarity, namely hexane, chloroform, and ethanol (5 L each), with each extraction carried out for 72 h using a solvent extractor. The extracts were filtered, and the respective solvents were removed under reduced pressure using a rotary evaporator to obtain the corresponding crude extracts. The ethanol extract (250 g) was retained for further purification based on its phytochemical profile and biological activity.

The ethanol extract was subjected to Sephadex LH-20 column chromatography using methanol–water mixtures of increasing methanol concentration as the mobile phase, followed by elution with 100% methanol. Fractions containing the target compound were pooled and concentrated under reduced pressure to yield arjunetin as a white crystalline solid (10 g, 99% purity). The extraction and purification workflow is illustrated schematically in **Fig. 1**.

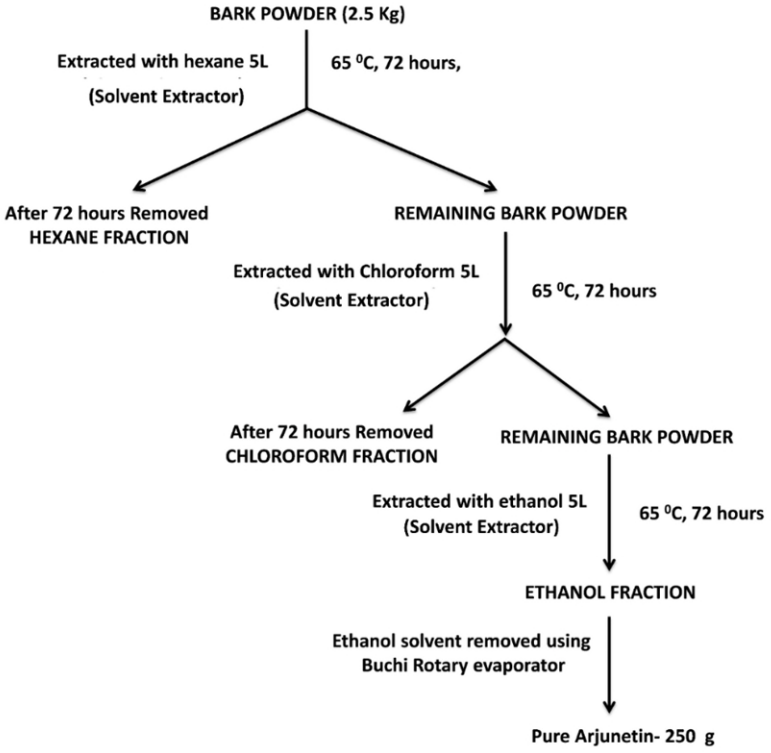


Figure 1. Schematic representation of the sequential extraction of various fractions from *T. arjuna* bark powder.

2.3.1 Preparation of direct ethanol extract of *T. arjuna bark powder (DEE)*

Finely powdered *T.arjuna* bark (100 g) was extracted with 500 mL of absolute ethanol in a 1 L borosilicate reagent bottle under continuous stirring at 80°C for 72 h. Upon completion of the extraction, the mixture was cooled to room temperature and filtered to remove insoluble plant material. The filtrate was concentrated under reduced pressure using a Büchi rotary evaporator to remove the solvent completely. The resulting crude extract, designated as the direct ethanol extract (DEE) of *T. arjuna* bark, yielded 10.0 g of dry extract (10% w/w based on the initial dry bark weight). The dried extract was stored in airtight containers until further use and was employed in all subsequent biological assays.

2.4 Evaluation of anti- inflammatory activity of arjunetin and DEE

The anti-inflammatory activity of arjunetin and the direct ethanol extract (DEE) of *T. arjuna* bark was evaluated by measuring nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophages. RAW 264.7 cells were obtained from the National Centre for Cell Science (NCCS, Pune, India) and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

For the assay, cells were seeded at a density of 1×10^5 cells/mL and allowed to equilibrate for 1 h before treatment with different concentrations of arjunetin or DEE. Following pretreatment, inflammation was induced by the addition of lipopolysaccharide (LPS; 1 µg/mL), and the cells were incubated for an additional 24 h under standard culture conditions. The concentration of nitric oxide released into the culture medium was determined indirectly by measuring nitrite accumulation using the Griess reagent assay[15]. Absorbance was measured at 540 nm using a microplate reader, and nitrite concentrations were quantified from a standard curve generated with sodium nitrite, as described previously [16,17,18].

2.5 Evaluation of anti-diabetic activity of arjunetin and DEE

The glucose uptake activity of arjunetin and the direct ethanol extract (DEE) of *T. arjuna* bark was evaluated in differentiated 3T3-L1 adipocytes following the method described by Das and Devi [19] with slight modifications. The 3T3-L1 preadipocyte cell line was obtained from the National Centre for Cell Science (NCCS, Pune, India). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin, and maintained at 37°C in a humidified atmosphere containing 5% CO_2.

For adipocyte differentiation, 3T3-L1 cells were seeded into 24-well culture plates at a density of 1×10^4 cells/mL (0.5 mL per well) and allowed to reach complete confluence. Differentiation was initiated by replacing the growth medium with adipogenic induction medium consisting of DMEM supplemented with 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), and 1 µM dexamethasone. After 48 h of incubation, the induction medium was replaced with adipogenic progression medium containing DMEM supplemented with 10% FBS and 10 µg/mL insulin, followed by a further 48 h incubation under identical culture conditions. The cells were subsequently maintained in DMEM containing 10% FBS (maintenance medium) and treated with various concentrations of arjunetin or DEE, along with the appropriate control groups.

Following treatment, glucose uptake was determined in differentiated adipocytes according to the modified protocol of Das and Devi [19]. The extent of glucose uptake was quantified by measuring the residual glucose concentration in the culture medium, and the glucose uptake activity of the test samples was expressed relative to the untreated control and insulin-treated positive control groups.

2.5.3 Glucose uptake assay

Following complete differentiation, 3T3-L1 adipocytes were serum-starved overnight and washed with HEPES-containing Krebs–Ringer phosphate (KRP) buffer. The cells were subsequently incubated in KRP buffer supplemented with 0.1% bovine serum albumin (BSA) for 30 min at 37°C. Thereafter, the cells were treated with various concentrations of arjunetin or the direct ethanol extract (DEE) of *T. arjuna* bark, together with the appropriate control groups. D-Glucose was then added to each well, and the cells were incubated for an additional 30 min at 37°C to allow glucose uptake. The reaction was terminated by aspirating the incubation medium, followed by three

washes with ice-cold KRP buffer to remove residual extracellular glucose. Cells were lysed with 0.1 M NaOH, and aliquots of the lysates were collected for the determination of intracellular glucose uptake using a commercial Glucose Uptake Assay Kit (BioVision Inc., Milpitas, CA, USA), according to the manufacturer's instructions. Each experiment was performed independently three times, with duplicate measurements in each experiment. Glucose uptake was expressed as the percentage increase relative to the untreated control using the following equation

Three independent experimental values in duplicates were taken to determine the percentage enhancement of glucose uptake over controls [20].

% Glucose uptake= [(control OD - test OD)/ control OD]* 100.

2.6 Evaluation of anti-cancer activity of arjunetin and DEE against MDA-MB-B231 Cells by MTT assay

2.6.1 Cytotoxicity assay:

The human breast adenocarcinoma cell line MCF-7 and the human breast cancer cell line MDA-MB-231 were obtained from the National Centre for Cell Science (NCCS, Pune, India). MCF-7 cells are estrogen receptor (ER)-positive, progesterone receptor (PR)-positive, and glucocorticoid receptor (GR)-positive, whereas MDA-MB-231 cells are triple-negative (ER−, PR−, and HER2−). Both cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were maintained at 37°C in a humidified incubator containing 5% CO_2 and sub-cultured upon reaching approximately 80–90% confluence to maintain logarithmic growth [21].

2.6.2 Cytotoxicity effect

The cytotoxic effects of arjunetin and the direct ethanol extract (DEE) of *T. arjuna* bark were evaluated in MDA-MB-231 human breast cancer cells using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, as previously described by Mosmann[21]. Cells were seeded into 96-well culture plates at a density of 1×10^5 cells/well and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 48 h to achieve approximately 70–80% confluence.

The culture medium was then replaced with fresh medium containing different concentrations of arjunetin or DEE, and the cells were further incubated for 24 h. Morphological changes in treated and untreated (control) cells were examined using an inverted phase-contrast microscope at 40× magnification and documented by digital photomicrography [21].

Following treatment, the cells were washed gently with phosphate-buffered saline (PBS, pH 7.4), and 20 µL of MTT solution (5 mg/mL in PBS) was added to each well. The plates were incubated for 2 h at 37°C in the dark to allow the formation of intracellular formazan crystals. The supernatant was carefully removed, and the formazan crystals were dissolved in 100 µL dimethyl sulfoxide (DMSO). Absorbance was measured at 570 nm using a microplate reader. Cell viability was expressed as a percentage relative to untreated control cells, and the half-maximal inhibitory concentration (IC_{50}) values were calculated from dose–response curves generated using GraphPad Prism software.

Percentage of cell viability was calculated using the formula,

Cell viability (%) = (Absorbance of sample/Absorbance of control) X 100

2.7 Evaluation of cytotoxic potential on vero cells of arjunetin and DEE by MTT assay

2.7.1 Cytotoxicity assay

Cell Culture Maintenance: The Vero (African green monkey kidney epithelial) cell line was obtained from the National Centre for Cell Science (NCCS, Pune, India). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were maintained at 37°C in a humidified incubator with 5% CO_2 and subcultured at approximately 80–90% confluence to maintain exponential growth. All experiments were performed using cells in the logarithmic growth phase.

2.7.2 Cytotoxicity effect

The cytotoxic effects of arjunetin and the direct ethanol extract (DEE) of *T. arjuna* bark were evaluated in Vero cells using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, as described by Mosmann[21]. Vero cells were

seeded into 96-well culture plates at a density of 1×10^4 cells/well and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 48 h to achieve approximately 70–80% confluence.

The culture medium was then replaced with fresh medium containing different concentrations of arjunetin or DEE, and the cells were incubated for an additional 24 h. Morphological changes in treated and untreated (control) cells were examined using an inverted phase-contrast microscope at 20× magnification and documented by digital photomicrography.

Following treatment, the cells were washed twice with phosphate-buffered saline (PBS, pH 7.4), and 20 µL of MTT solution (5 mg/mL in PBS) was added to each well. The plates were incubated for 2 h at 37°C in the dark to allow the formation of intracellular formazan crystals. The supernatant was carefully removed, and the formazan crystals were dissolved in 100 µL dimethyl sulfoxide (DMSO). Absorbance was measured at 570 nm using a microplate reader. Cell viability was expressed as a percentage relative to untreated control cells, and the half-maximal inhibitory concentration (IC_{50}) values were determined from dose–response curves.

Percentage of cell viability was calculated using the formula,

Cell viability (%) = (Absorbance of sample/Absorbance of control) X 100.

2.8. Statistical Analysis

The experiments were performed in triplicate. The data are expressed as mean ± standard deviation. Significant differences between groups were determined by using Student t-test. p value of less than 0.05 was considered significant.

Results and Discussion

3.1 Isolation and Characterization of Arjunetin from *T. arjuna* bark

A scalable, economical, and efficient solvent-extraction method was developed for the isolation of arjunetin from the bark of *T. arjuna*. The process enabled the preparation of a single pure compound with a purity of approximately 95% without the use of column chromatography, relying exclusively on solvent extraction techniques (Fig. 2,

Fig. 3, and Fig. 4f). This approach provides a time-saving and cost-effective strategy for large-scale arjunetin production.

Arjunetin was obtained as a colorless solid from the ethanol fraction using 100% methanol as the eluent. The UV spectrum in methanol exhibited absorption maxima (λ_{max}) at 360, 310, and 234 nm (Fig. 4a). The IR spectrum displayed characteristic absorption bands corresponding to hydroxyl groups (3447 cm^{-1}), ester carbonyl functionality (1741 cm^{-1}), trisubstituted double bonds (1630 and 894 cm^{-1}), methyl groups (1452 cm^{-1}), gem-dimethyl groups (1391 cm^{-1}), and C–O stretching vibrations at 1260, 1161, and 1028 cm^{-1} (Fig. 4b).

Mass spectrometric analysis showed a molecular ion peak at *m/z* 673.39 $[M+Na]^+$, consistent with the molecular formula $C_{36}H_{58}O_{10}Na$. Additional fragment ions were observed at *m/z* 489 $[M-162+H]^+$, *m/z* 471 $[M-162-H_2O+H]^+$, and *m/z* 453 $[M-162-2H_2O+H]^+$, indicating the presence of one glycosidic moiety, three removable hydroxyl groups, and one ester functionality within the molecule (Fig. 4e). The ^1H NMR spectrum revealed an olefinic proton signal at δ_H 7.45 (1H, br s, H-12) and seven methyl singlets at δ_H 0.12, 0.16, 0.19, 0.33, 0.36, 0.40, and 0.57 (7 × CH_3, 21H). Three oxygenated methine protons were observed at δ_H 5.14 (1H, br s, H-19), 5.22 (1H, s, H-3α), and 5.69 (1H, d, J = 9.5 Hz, H-2β). A broad singlet at δ_H 3.45 (1H, br s, H-18) and signals in the range δ_H 3.22–3.76 corresponded to glucose protons. The ^{13}C NMR spectrum displayed 36 carbon signals, including seven methyl carbons at δ_C 16.6, 17.1, 17.5, 23.7, 25.3, 28.4, and 28.4. Signals corresponding to oxygenated carbons were observed at δ_C 76.8 (C-2), 78.1 (C-3), and 81.3 (C-19). The presence of a trisubstituted double bond was confirmed by resonances at δ_C 122.4 (C-12) and 143.8 (C-13), while the ester carbonyl carbon appeared at δ_C 176.6 (C-28). Glucose carbon signals were detected at δ_C 61.1, 63.6, 70.3, 72.8, 74.5, and 94.4. The anomeric proton signal at δ_H 6.91 (1H, H-1′) in the ^1H NMR spectrum and the corresponding anomeric carbon signal at δ_C 94.4 in the ^{13}C NMR spectrum confirmed the presence of a β-glucose unit esterified to the C-28 carbonyl group of the aglycone. These findings are consistent with previously reported spectroscopic data for arjunetin (Tripathi *et al*., 1996; Honda et al., 2001; Sunyana *et al*., 2009) [22, 23, 24]. Based on the combined UV, IR, mass spectrometric, and NMR spectroscopic analyses, the isolated compound was conclusively identified as arjunetin (Fig. 4a–f).

Figure 2. Extraction process of crude ethanolic extract from *T. arjuna* bark: (a) *T. arjuna* tree; (b) bark sample; (c) bark cut into small pieces; (d) pulverization of bark; (e) pulverized bark powder; (f) sequential solvent extraction using increasing solvent polarity (hexane, chloroform, and ethanol); (g) ethanolic extract; (h) solvent

evaporation using a rotary evaporator under reduced pressure; and (i) crude ethanolic extract powder obtained after solvent removal.

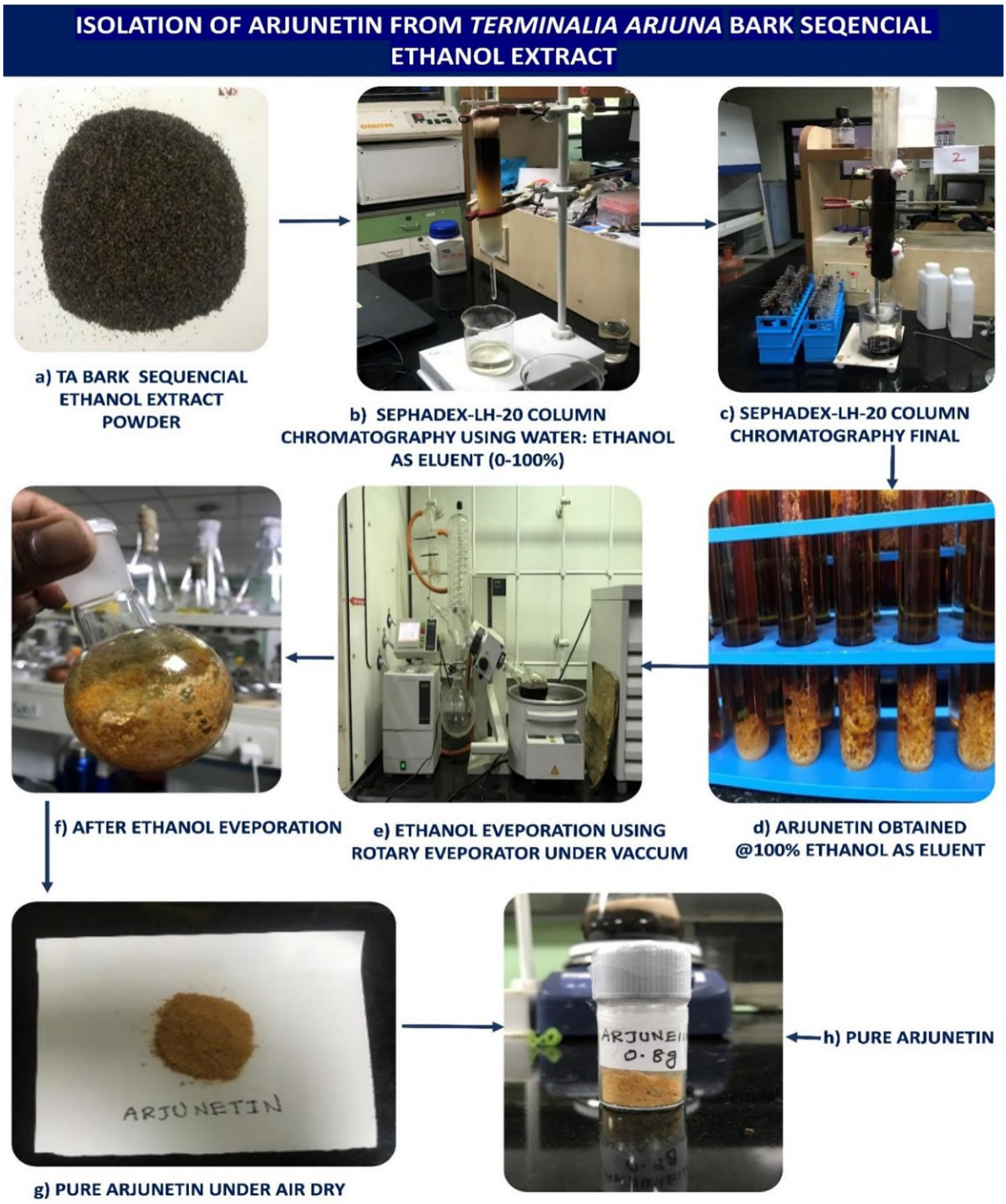


Figure 3. Isolation scheme of arjunetin from crude ethanolic extract of *T. arjuna* bark: (a) sequential ethanolic extraction of bark powder; (b) fractionation using

Sephadex LH-20 column chromatography with a water–ethanol gradient (0–100%); (c) final purification using Sephadex LH-20 chromatography; (d) elution of arjunetin with 100% ethanol; (e) air-dried isolated arjunetin; and (f) purified arjunetin.

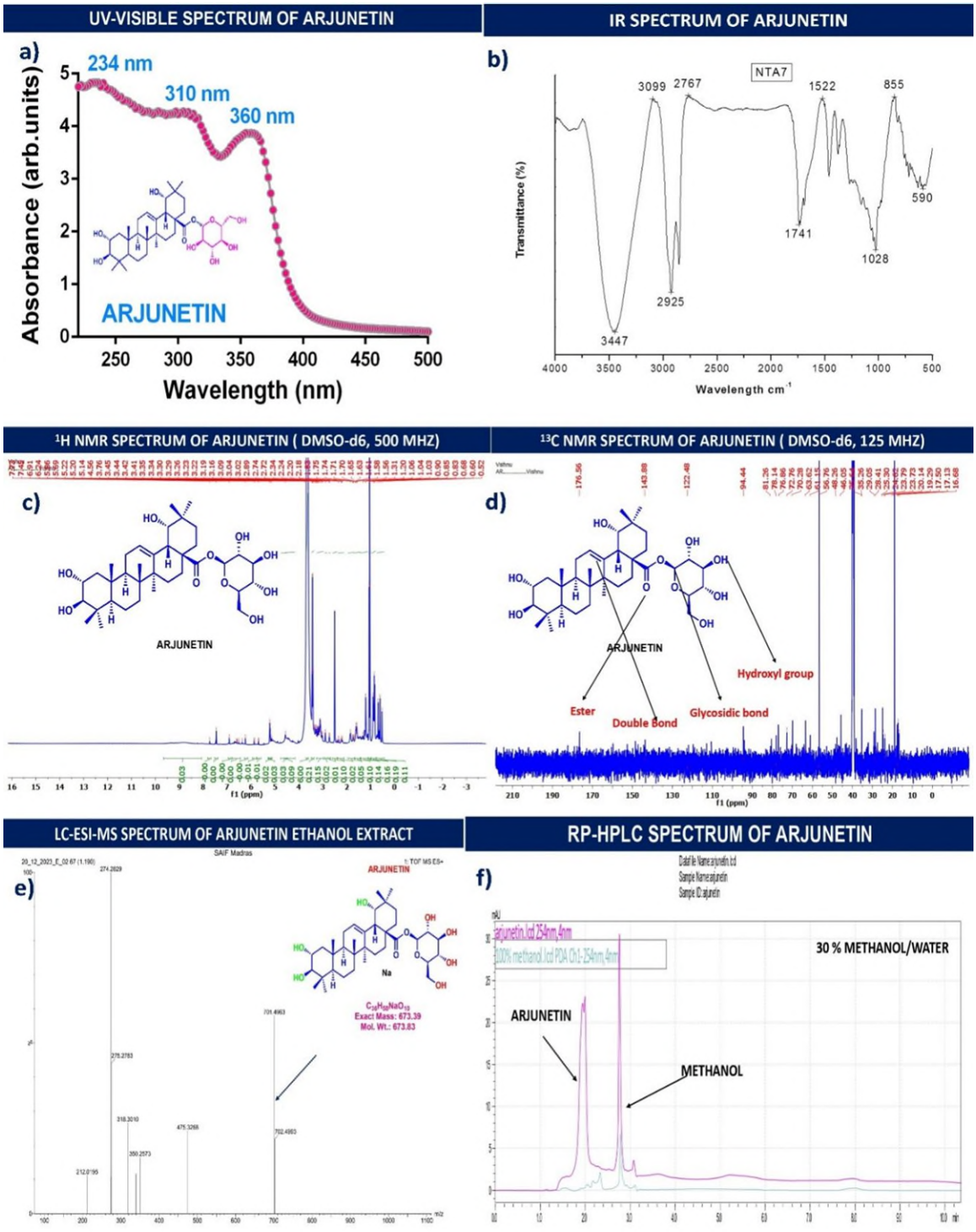


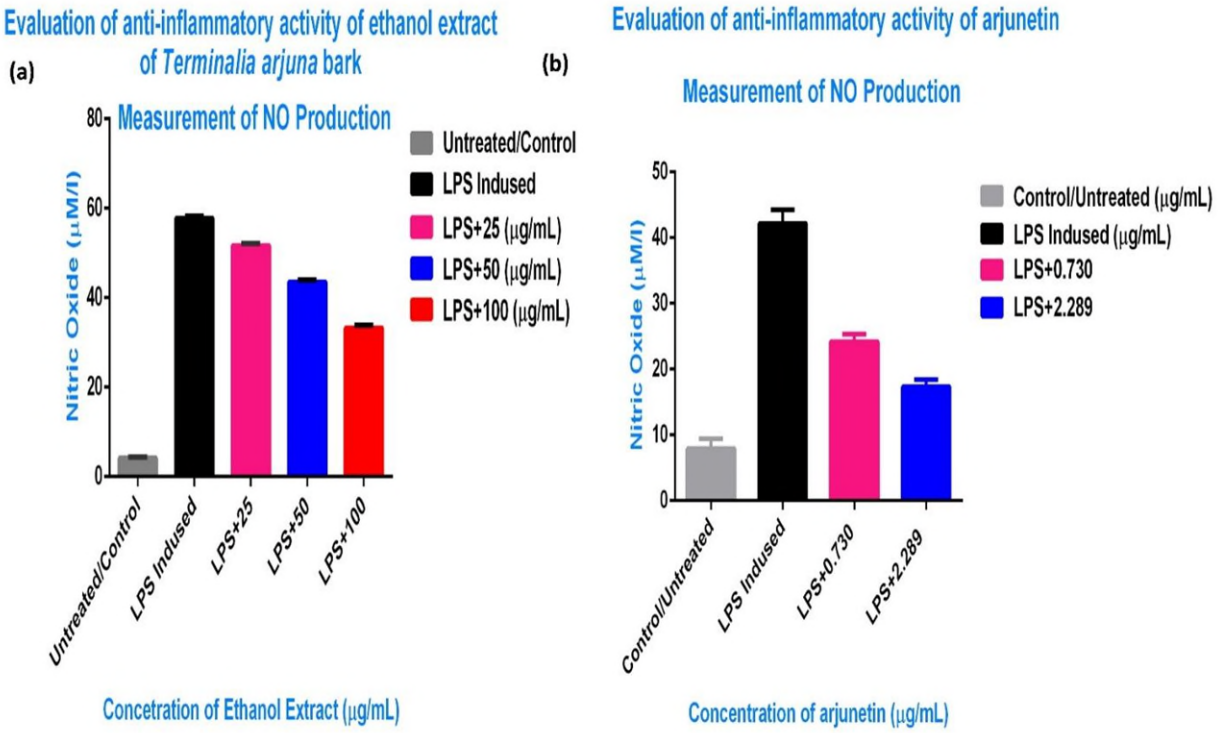
Figure 4. Spectroscopic and chromatographic characterization of isolated arjunetin: (a) UV–Visible spectrum in methanol; (b) Fourier-transform infrared

(FTIR) spectrum; (c) ^{1}H NMR spectrum in DMSO-d6; (d) ^{13}C NMR spectrum in DMSO-d6; (e) electrospray ionization mass spectrometry (ESI-MS) profile; and (f) reverse-phase high-performance liquid chromatography (RP-HPLC) chromatogram.

3.2 Evaluation of anti- inflammatory activity of arjunetin and DEE by assessing the NO production in RAW264.7 cells.

The *DEE and arjunetin* were investigated for its *in vitro* anti-inflammatory activity. The results presented in Fig. 5 demonstrate that both the direct ethanol extract (DEE) and the pure compound arjunetin effectively inhibited nitric oxide (NO) production in LPS-stimulated RAW 264.7 cells. The elevated NO production associated with LPS-induced inflammation was significantly reduced following treatment with the direct ethanol extract (DEE) and the pure compound. RAW 264.7 cells treated with LPS (LPS control) exhibited a markedly higher production of nitrite, equivalent to NO levels, as determined by the nitrite calibration curve. This increase in NO production in the LPS-treated group indicates that the RAW cells were inactive prior to LPS exposure and became activated post-treatment. Anti-inflammatory activity of arjunetin isolated from ethanol extract: Arjunetin at concentrations ranging from 0.730 to 2.289 µg/ml showed dose-dependent reduction of NO production compared with control. Under the conditions of the present study, the DEE demonstrated a concentration-dependent inhibition of nitric oxide production in LPS-stimulated RAW 264.7 macrophages. LPS stimulation significantly increased NO levels from 4.105 µM to 57.766 µM, confirming effective induction of an inflammatory response. Treatment with the DDE reduced NO production to 33.263 µM at 100 µg/mL, indicating a maximum inhibition of approximately 42.4%. This suggests that the DEE possesses moderate to significant anti-inflammatory activity, possibly through suppression of inducible nitric oxide synthase (iNOS)-mediated NO production. The substantial NO production induced by LPS was significantly diminished by the administration of the extract (DEE) and the pure compound arjunetin. This reduction was statistically significant at a 99% confidence interval across all doses of the direct ethanol extract (DEE) and the pure compound. The pure compound arjunetin notably inhibited NO production at a concentration of 100 µg/mL, whereas the direct ethanol extract (DEE) exhibited comparatively lower inhibition than the pure compound. Further studies are required to elucidate the underlying molecular mechanisms and confirm the safety profile [25].

The potent anti-inflammatory properties of *T. arjuna* extracts (DEE) and its compound arjunetin were found to suppress LPS-stimulated inflammatory responses in RAW



264.7 macrophage cells by significantly reducing nitric oxide production in a concentration-dependent manner. Consequently, it is established that the ethanol extract and its isolated compounds contribute significantly to its anti-inflammatory activity [10], [26] (Fig.6).

Figure 5. Evaluation of anti-inflammatory activity of *T. arjuna* bark ethanolic extract and isolated arjunetin: (a) anti-inflammatory activity of the ethanolic extract; (b) anti-inflammatory activity of arjunetin.

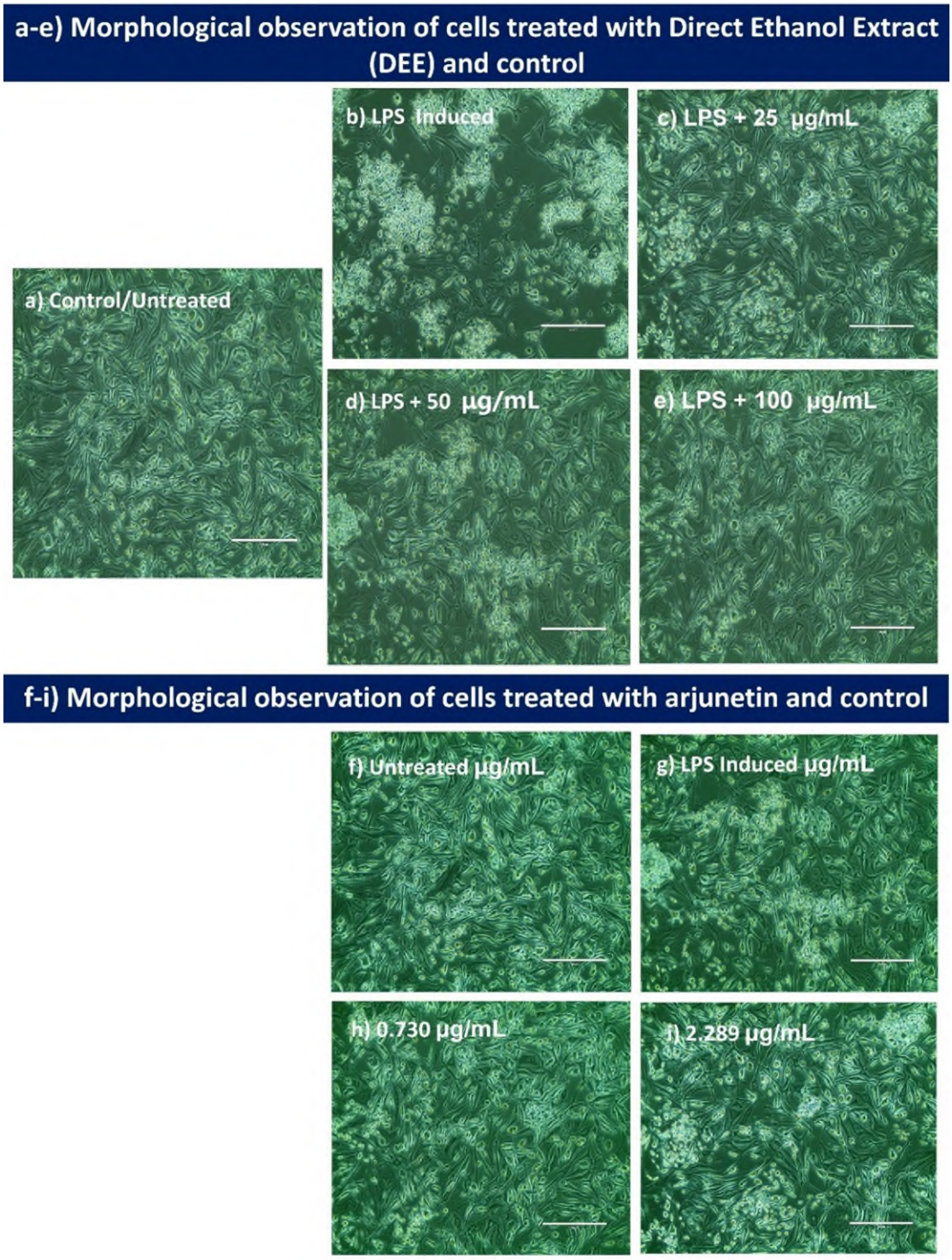


Figure 6. Morphological changes in LPS-stimulated cells following treatment with direct ethanolic extract (DEE) and arjunetin. (a–e) Cells treated with DEE: (a) untreated control; (b) LPS-induced inflammatory control; (c) LPS + 25 µg/mL DEE; (d) LPS + 50 µg/mL DEE; and (e) LPS + 100 µg/mL DEE. (f–i) Cells treated with arjunetin: (f) untreated control; (g) LPS-induced inflammatory control; (h) LPS + 0.730 µg/mL arjunetin; and (i) LPS + 2.289 µg/mL arjunetin.

3.3 Evaluation of anti-diabetic activity of arjunetin and extract using *In vitro* glucose uptake assay in 3T3 adipocytes in the presence of insulin.

The anti-diabetic properties of DEE and arjunetin were evaluated. Arjunetin at concentrations ranging from 0.730 to 2.289 µg/ml showed dose-dependent stimulation of glucose uptake. Under the conditions of the present study, the DEE demonstrated a significant and concentration-dependent enhancement of glucose uptake in differentiated 3T3-L1 adipocytes in the presence of insulin. The DEE increased glucose uptake from 56.81% (insulin alone) to 72.33% at 100 µg/mL, indicating a potential insulin-sensitizing effect (Fig 8). The progressive decrease in absorbance values further confirms enhanced cellular glucose utilization. Further mechanistic studies are warranted to elucidate the underlying pathways involved. Morphological observation of cells treated test item and control as shown in Fig 9. Previous studies have reported the oral administration of the ethanolic bark extract of *T.arjuna* at doses of 250 and 500 mg/kg body weight for 30 days significantly reduced blood glucose levels from 302.67 ± 22.35 mg/dL to 82.50 ± 4.72 mg/dL. It also decreased the activities of glucose-6-phosphatase, fructose-1,6-bisphosphatase, and aldolase, while increasing phosphoglucoisomerase and hexokinase activities in tissues, indicating improved glucose metabolism[11]. However, the present study assessed anti-diabetic effect of pure arjunetin has been reported. These studies could significantly contribute to the development of herbal medications aimed at regulating glucose levels in patients with type II diabetes [20, 27].

T. *arjuna* extracts (DEE) and pure arjunetin demonstrate significant antidiabetic activity in 3T3-L1 adipocytes by promoting glucose uptake, enhancing insulin sensitivity, and regulating adipogenesis (lipid accumulation) [28, 29, 30, 31.]

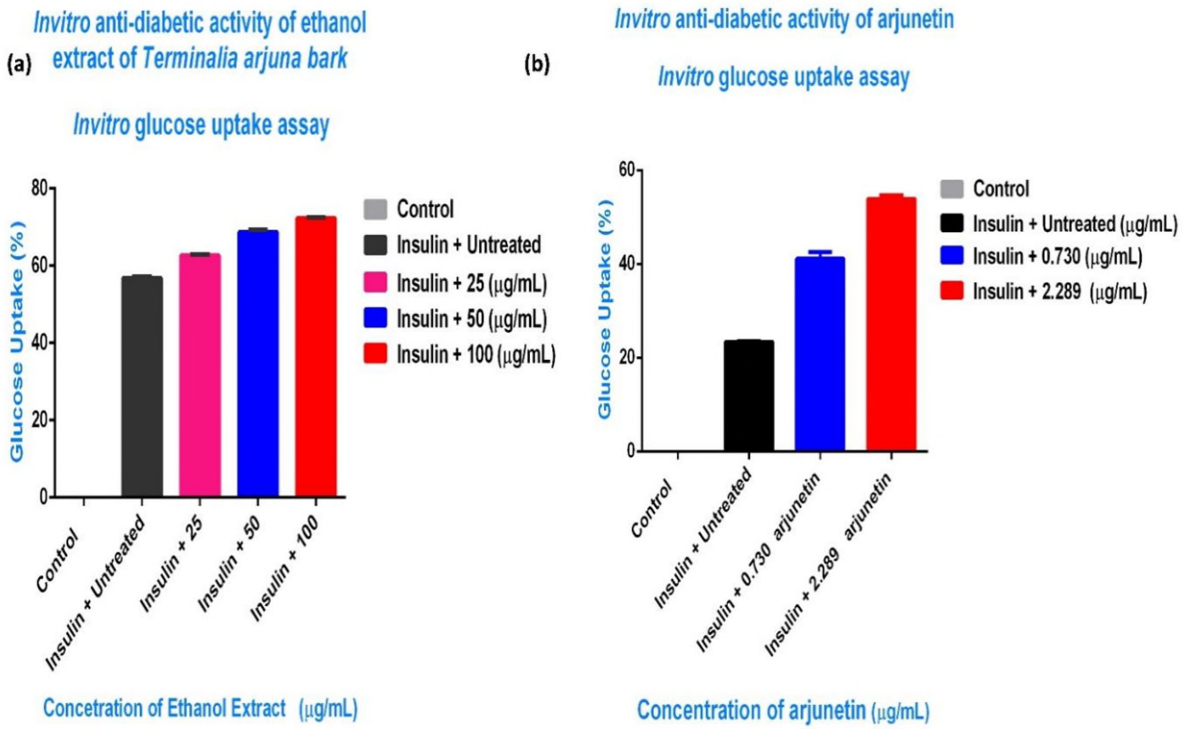
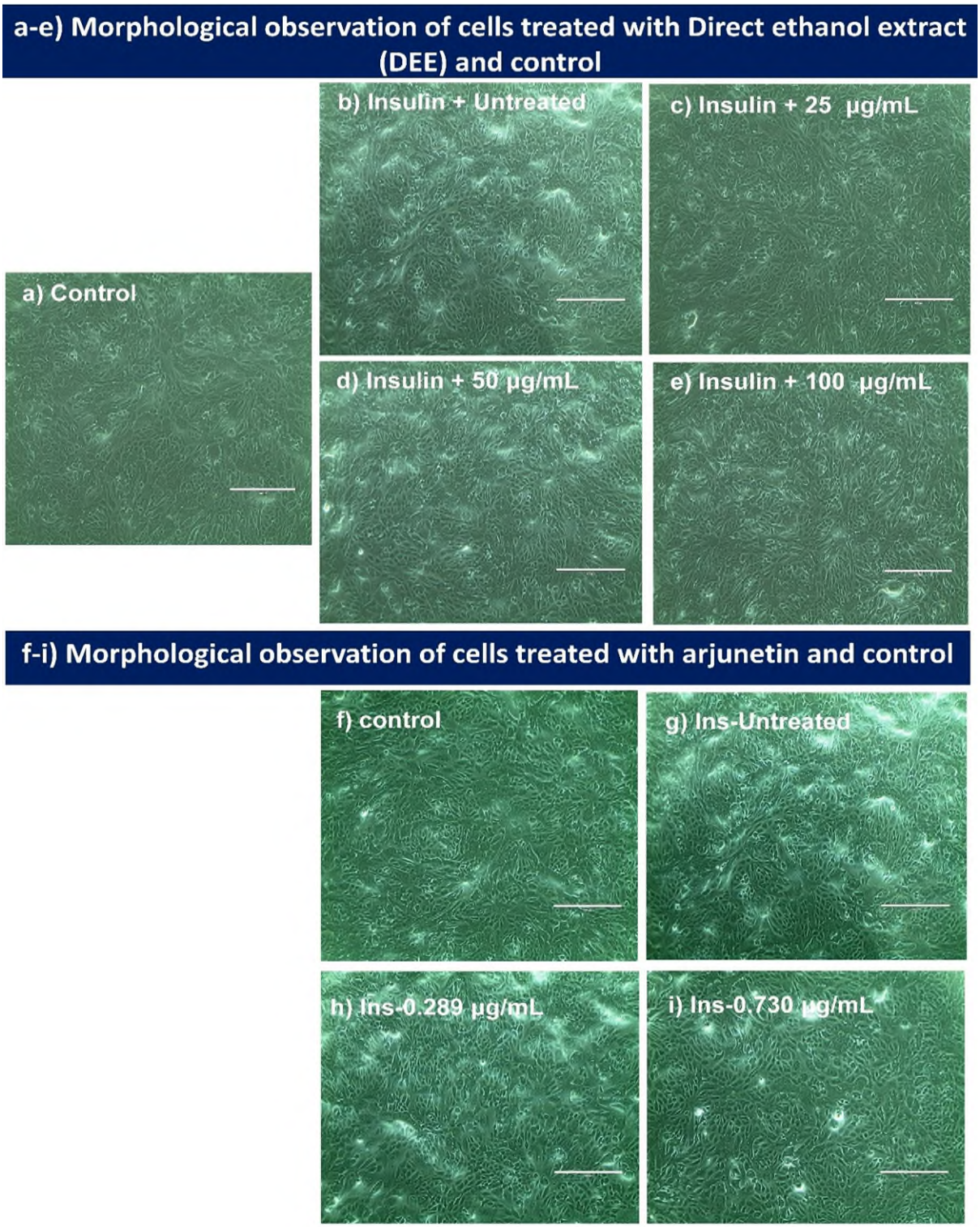


Figure 7. *In vitro* anti-diabetic activity assessed by glucose uptake assay. (a) Glucose uptake activity of the ethanolic extract of *T. arjuna* bark. (b) Glucose uptake activity of isolated arjunetin.



501 **Figure 8. Morphological changes in differentiated 3T3-L1 adipocytes following**
502 **insulin stimulation and treatment with DEE or arjunetin.** (a–e) Cells treated with
503 DEE: (a) untreated control; (b) insulin-treated control; (c) insulin + 25 µg/mL DEE; (d)
504 insulin + 50 µg/mL DEE; and (e) insulin + 100 µg/mL DEE. (f–i) Cells treated with
505 arjunetin: (f) untreated control; (g) insulin-treated control; (h) insulin + 0.289 µg/mL
506 arjunetin; and (i) insulin + 0.730 µg/mL arjunetin.

3.4 Evaluation of anti-cancer activity of arjunetin and DEE against MDAB231 Cells by MTT assay

The anti-cancer properties of DEE and arjunetin were also evaluated.The IC_{50} value of arjunetin on MCF-7 cell line was found to be 10.807 µg/ml. Cell morphology observation shows that increasing cytotoxicity and decreasing cell density of MCF-7 with increase in test concentration treatment. The IC_{50} value of DEE on MDAMB231 cell line was found to be 84.316 µg/ml. Cell morphology observation shows that increasing cytotoxicity and decreasing cell density of MCF-7 with increase in test concentration treatment. The arjunetin and DEE exhibited IC_{50} values of 10.807 ± 0.21, 84.316 µg/ml against MCF-7 and MDAMB231 cancer cells, respectively. The cytotoxicity of the arjunetin is approximately 2.3 times more potent than the IC_{50} value of the DEE (Fig. 9) [32].

The study's findings revealed that the extract of *T. arjuna* and its pure compound arjunetin exhibited significant anti-cancer activity, particularly against the MCF7 and triple-negative cancer cell lines. The evaluation involved cell counting and calculating the percentage of cell inhibition, which demonstrated a marked reduction in cell count when treated with the plant extract compared to the negative control (DMSO). A comparison between the 50% and 100% DEE and the negative control showed a distinct decline in the number of cancer cells. The anti-cancer activity was more pronounced at higher concentrations (100 µL) than at lower concentrations (50 µL). Notably, there was no significant difference in activity against the MCF7 cell line between the 100% and 50% DEE, as illustrated in Fig. 9a–e of the study. This indicates that both concentrations of the DEE possess similar efficacy in inhibiting the growth of MCF7 cancer cells [33].

Additionally, *T. arjuna* demonstrates significant anti-cancer properties in human triple-negative cancer (MDA-MB-231) and breast cancer (MCF7) cell lines, highlighting its potential as a natural source of anti-cancer compounds.

The viability of the MCF-7 and MDAMB231 cell lines was assessed using the MTT assay following treatment with 100 µg/ml of DEE and pure compounds arjunetin as illustrated in Fig. 9, the pure compound arjunetin exhibited the most significant cytotoxic effect, resulting in a 50.94% inhibition of MCF-7 cell viability. In contrast, the pure compound arjunetin demonstrated only a 1.3% inhibition of normal vero cell line cells, suggesting that it possesses selective cytotoxicity towards cancer cells while

sparing normal cells. This suggests that the extracts from *T. arjuna* may have potential therapeutic effects against breast cancer (Fig. 10) [34,35].

Plant extracts exhibit substantial in vitro anti-cancer activity by inducing apoptosis, inhibiting proliferation, and reducing migration in various cancer cell lines. Conversely, the extract demonstrated only a 1.3% inhibition rate on normal cells (BJ-1), indicating selective cytotoxicity towards cancer cells while sparing normal cells [8, 9]

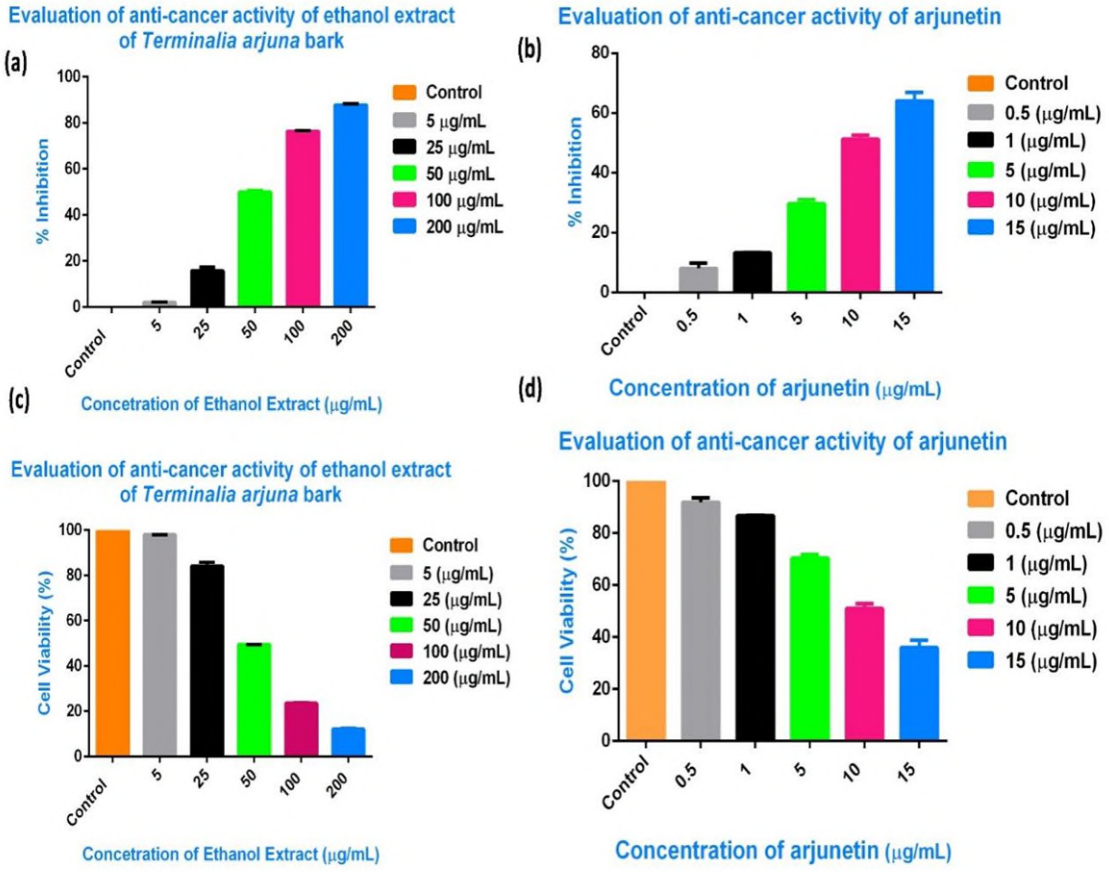
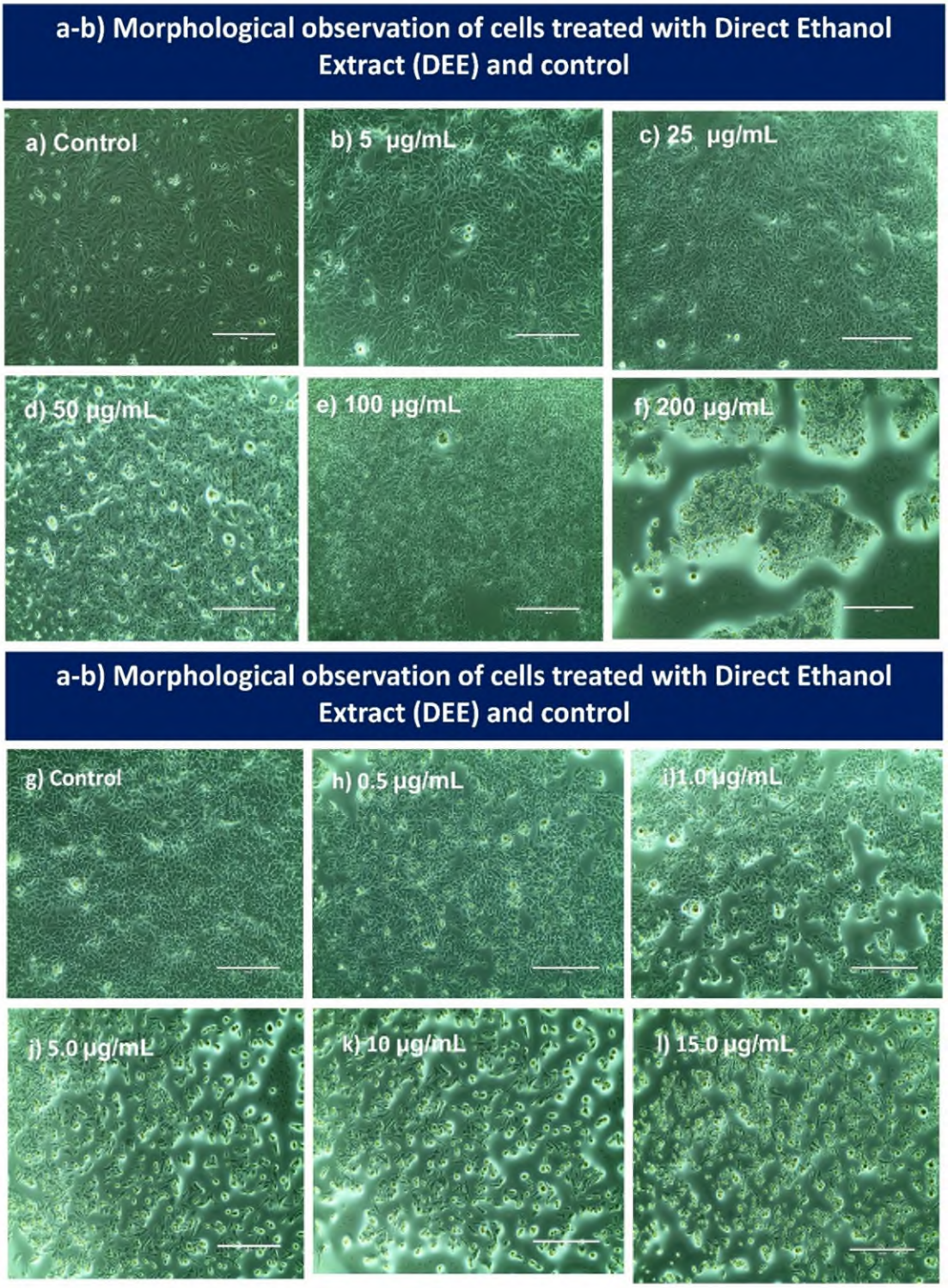


Figure 9. Evaluation of *in vitro* anti-cancer activity of DEE and arjunetin. (a) Dose–response curve showing percentage inhibition by direct ethanolic extract of *T. arjuna* bark. (b) Dose–response curve showing percentage inhibition by arjunetin. (c) Cell viability (%) following treatment with DEE. (d) Cell viability (%) following treatment with arjunetin. Data are presented as mean ± SD from three independent experiments.



555

556 **Figure 10. Morphological changes in cancer cells following treatment with DEE**
557 **and arjunetin.** (a–f) Cells treated with DEE: (a) untreated control; (b) 5 µg/mL; (c) 25
558 µg/mL; (d) 50 µg/mL; (e) 100 µg/mL; and (f) 200 µg/mL. (g–l) Cells treated with
559 arjunetin: (g) untreated control; (h) 0.5 µg/mL; (i) 1.0 µg/mL; (j) 5.0 µg/mL; (k) 10
560 µg/mL; and (l) 15 µg/mL.

3.5 Evaluation of Cytotoxic potential on vero cells of arjunetin and DEE by MTT assay

Cytotoxicity studies indicate that both the DEE and pure compound arjunetin demonstrate dose- and time-dependent effects on Vero cell viability. The compounds maintain safe concentrations, defined as more than 90% cell viability, at levels below 14.761 µg/mL at 24 hours, while the DEE extract achieves this threshold at concentrations below 237.46 µg/mL.

The study aimed to evaluate the cytotoxic activity of *T. arjuna* extract (DEE) and its pure compound arjunetin on the Vero cell line (African green monkey kidney normal cells). These extracts (DEE) and pure compounds arjunetin were assessed for their inhibitory effects on the Vero cell line across a range of concentrations. The cytotoxicity was determined using the MTT assay to establish the IC_{50} (50% growth inhibition). Results indicated that the percentage of cell viability increased as the concentration of the test compounds decreased. The findings suggest that the selected plant extracts and pure compounds exhibit minimal cytotoxic effects on the normal Vero cell line, indicating no significant cytotoxicity (Fig. 11) [36].

The cell viability of the ethanol extract (DEE) and the pure compound arjunetin was assessed to determine the toxic concentration using the MTT assay. The results indicated that the pure compound was safe at concentrations up to 14.761 µg/mL in the growth media, cell morphology observation shows that increasing cytotoxicity and decreasing cell density of Vero with increase in test concentration treatment. The GI50 value of direct ethanol extract (DEE) on Vero cell line was found to be 237.464 µg/ml, while the direct ethanol extract (DEE) was non-toxic even at a higher concentration of 237.464 µg/mL. Cell morphology observation shows that increasing cytotoxicity and decreasing cell density of Vero with increase in test concentration treatment. The current study indicates that the arjunetin and direct ethanolic extract (DEE) does not induce any adverse effects, thereby suggesting its nontoxic and safe profile. These findings affirm that the ethanol extract can be consumed safely (Fig. 12) [36].

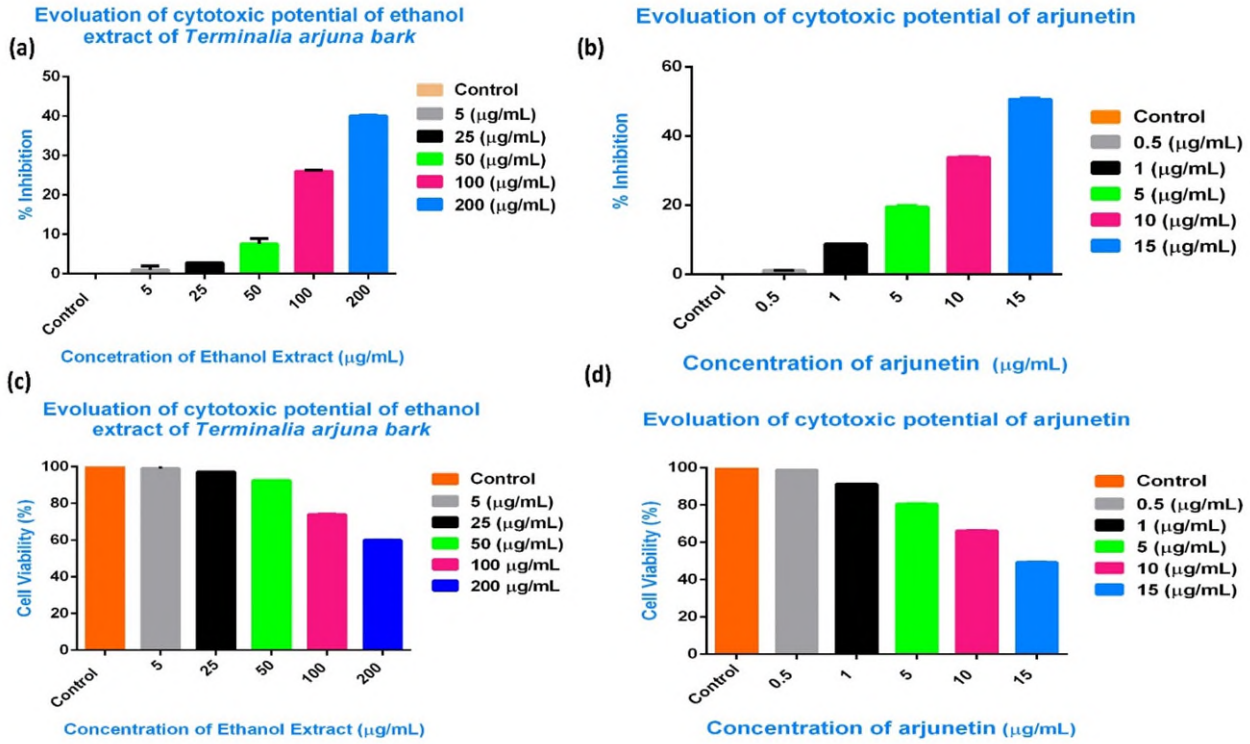


Figure 11. Evaluation of in vitro cytotoxic potential of DEE and arjunetin. (a) Dose–response curve showing percentage inhibition by DEE. (b) Dose–response curve showing percentage inhibition by arjunetin. (c) Cell viability (%) following treatment with DEE. (d) Cell viability (%) following treatment with arjunetin. Data are presented as mean ± SD from three independent experiments.

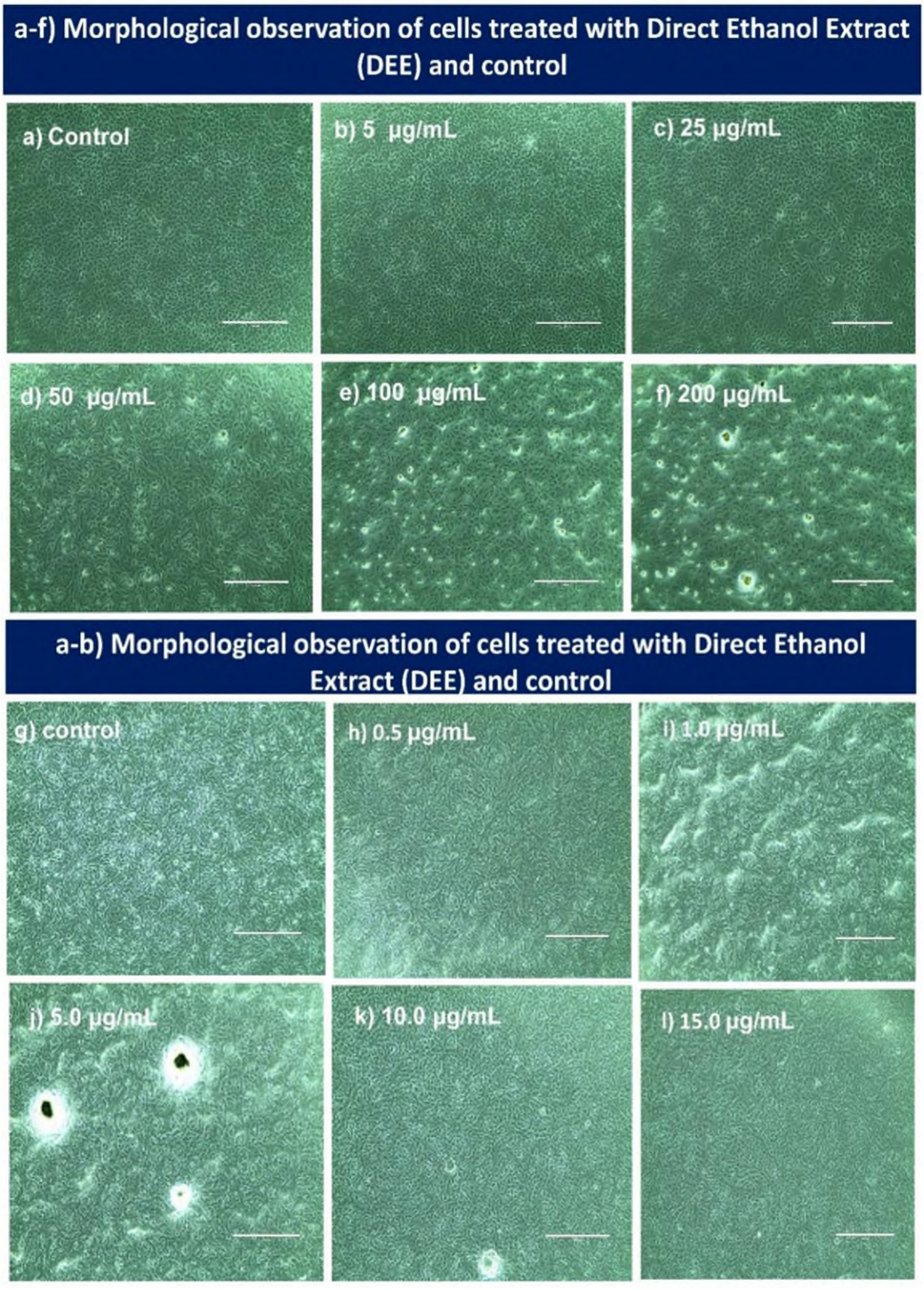


Figure 12. Morphological changes in Vero cells during cytotoxicity assessment following treatment with DEE and arjunetin. (a–f) Cells treated with DEE: (a) untreated control; (b) 5 µg/mL; (c) 25 µg/mL; (d) 50 µg/mL; (e) 100 µg/mL; and (f) 200 µg/mL. (g–l) Cells treated with arjunetin: (g) untreated control; (h) 0.5 µg/mL; (i) 1.0 µg/mL; (j) 5.0 µg/mL; (k) 10 µg/mL; and (l) 15 µg/mL.

3. Conclusion

This invention discloses a novel method for the isolation of arjunetin from the bark of *T. arjuna.* The proposed method provides a simple, cost-effective, and scalable approach for the extraction and purification of arjunetin using solvent extraction techniques. Notably, the method enables the production of an ethanolic extract containing arjunetin with approximately 95% purity, without the requirement for column chromatography, thereby reducing processing complexity, time, and overall cost. Arjunetin exhibits considerable therapeutic potential, particularly in anti-diabetic, anti-inflammatory, and anticancer applications. Both purified arjunetin and direct ethanolic extracts demonstrate significant anti-inflammatory effects through the modulation of key cellular signaling pathways. Although isolated pure compounds may show enhanced potency, crude extracts often provide synergistic therapeutic effects due to the combined action of multiple phytoconstituents. The direct ethanolic extract of *T. arjuna* bark possesses notable medicinal potential, exhibiting pronounced anti-inflammatory, anti-cancer, and anti-diabetic activities.

Declaration of Competing Interest

Declaration of Competing Interests: The authors affirm that there are no known financial interests or personal relationships that could have influenced the work presented in this paper.

Data availability

Data will be made available on request from the corresponding authors

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2024, and an international patent application PCT/IN2025/051792, filed on the same date, titled "A Method of Isolation of Arjunetin from *Terminalia Arjuna."*

CRediT authorship contribution statement

Gandarvakottai Arumugam Senthilkumar: Conceptualization, Investigation, Data curation, Validation, Methodology, Investigation, Formal analysis, Writing – original draft.

Gummadi Sathyanarayana: Writing – review & editing, Supervision, Methodology.

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Figure Legends

Figure 1. Schematic representation of the sequential extraction of various fractions from *T. arjuna* bark powder.

Figure 2. Extraction process of crude ethanolic extract from *T. arjuna* bark: (a) *T. arjuna* tree; (b) bark sample; (c) bark cut into small pieces; (d) pulverization of bark; (e) pulverized bark powder; (f) sequential solvent extraction using increasing solvent polarity (hexane, chloroform, and ethanol); (g) ethanolic extract; (h) solvent

evaporation using a rotary evaporator under reduced pressure; and (i) crude ethanolic extract powder obtained after solvent removal.

Figure 3. Isolation scheme of arjunetin from crude ethanolic extract of *T. arjuna* bark: (a) sequential ethanolic extraction of bark powder; (b) fractionation using Sephadex LH-20 column chromatography with a water–ethanol gradient (0–100%); (c) final purification using Sephadex LH-20 chromatography; (d) elution of arjunetin with 100% ethanol; (e) air-dried isolated arjunetin; and (f) purified arjunetin.

Figure 4. Spectroscopic and chromatographic characterization of isolated arjunetin: (a) UV–Visible spectrum in methanol; (b) Fourier-transform infrared (FTIR) spectrum; (c) ^{1}H NMR spectrum in DMSO-d6; (d) ^{13}C NMR spectrum in DMSO-d6; (e) electrospray ionization mass spectrometry (ESI-MS) profile; and (f) reverse-phase high-performance liquid chromatography (RP-HPLC) chromatogram.

Figure 5. Evaluation of anti-inflammatory activity of *T. arjuna* bark ethanolic extract and isolated arjunetin: (a) anti-inflammatory activity of the ethanolic extract; (b) anti-inflammatory activity of arjunetin.

Figure 6. Morphological changes in LPS-stimulated cells following treatment with direct ethanolic extract (DEE) and arjunetin. (a–e) Cells treated with DEE: (a) untreated control; (b) LPS-induced inflammatory control; (c) LPS + 25 µg/mL DEE; (d) LPS + 50 µg/mL DEE; and (e) LPS + 100 µg/mL DEE. (f–i) Cells treated with arjunetin: (f) untreated control; (g) LPS-induced inflammatory control; (h) LPS + 0.730 µg/mL arjunetin; and (i) LPS + 2.289 µg/mL arjunetin.

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Figure 10. Morphological changes in cancer cells following treatment with DEE and arjunetin. (a–f) Cells treated with DEE: (a) untreated control; (b) 5 µg/mL; (c) 25 µg/mL; (d) 50 µg/mL; (e) 100 µg/mL; and (f) 200 µg/mL. (g–l) Cells treated with arjunetin: (g) untreated control; (h) 0.5 µg/mL; (i) 1.0 µg/mL; (j) 5.0 µg/mL; (k) 10 µg/mL; and (l) 15 µg/mL.

Figure 11. Evaluation of in vitro cytotoxic potential of DEE and arjunetin. (a) Dose–response curve showing percentage inhibition by DEE. (b) Dose–response curve showing percentage inhibition by arjunetin. (c) Cell viability (%) following treatment with DEE. (d) Cell viability (%) following treatment with arjunetin. Data are presented as mean ± SD from three independent experiments.

Figure 12. Morphological changes in Vero cells during cytotoxicity assessment following treatment with DEE and arjunetin. (a–f) Cells treated with DEE: (a) untreated control; (b) 5 µg/mL; (c) 25 µg/mL; (d) 50 µg/mL; (e) 100 µg/mL; and (f) 200 µg/mL. (g–l) Cells treated with arjunetin: (g) untreated control; (h) 0.5 µg/mL; (i) 1.0 µg/mL; (j) 5.0 µg/mL; (k) 10 µg/mL; and (l) 15 µg/mL.