Upregulation of HDAC5 gene expression for treatment of osteoarthritis: Chondroprotective properties of regular and Nano-formulated Boswellia serrata Roxb

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Article

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Abstract

Osteoarthritis is a serious disorder that destroys cartilage and joints and inhibits collagen formation. Natural products are widely recognized for their wide variety of therapeutic uses and great safety records. Using human chondrocyte cells (C20A4) and MTT assays, the potential benefits of regular and nano-formulated *Boswellia serrata* (Bs) methanol extract for treating osteoarthritis by influencing the expression of the HDAC5, IL1β, IL6, PGE2, NF-kB genes were evaluated. HDAC5 gene expression was significantly upregulated and survival rate of C20A4 was strongly elevated. IL1β, IL6, PGE2, NF-kB inflammation biomarkers gene expression was significantly suppressed by both conventional and Nano-formulations. In comparison to conventional extracts, Nano-formulations exhibited greater activity revealing that they might represent a more effective treatment strategy. HPLC-MS/MS technology of the Bs extract against 28 standard phenolic compounds e.g. *p*-coumaric acid, ferulic acid, rutin, myricetin and quercetin, proved the absence of these compounds in Bs gum resin.

Introduction

Osteoarthritis (OA) is a chronic, progressive, degenerative condition that affects the entire joint, characterized by a reduction in joint space, the formation of osteophytes, subchondral sclerosis, and the presence of cysts[1]. The primary clinical manifestations of OA include joint pain and swelling, as well as a restricted range of motion[2]. OA collectively impair the quality of life for affected individuals[3]. This disease ranks among the most common diseases globally, and the disabilities it causes impose significant healthcare and socioeconomic challenges[4]. Due to its complex and multifactorial nature, halting the progression of OA is difficult; therefore, it is essential to adopt a strategy focuses on enhancing joint function, alleviating pain, and improving quality of life while minimizing adverse effects[5,6].

Currently, OA lacks a definitive cure, and earlier strategies primarily aimed at alleviating joint pain instead of preventing the disease's progression. As a result, various treatments, including oral and topical nonsteroidal anti-inflammatory drugs, opioids, and serotonin norepinephrine reuptake inhibitors, were utilized for symptom management and functional improvement, though their extended use poses significant health threats[7]. Recently, the emphasis has shifted towards slowing the progression of the disease by discovering disease-modifying drugs that interact with relevant biomarkers and therapeutic targets[8].

The inflammatory biomarkers Interleukin (IL)-6 and tumor necrosis factor-alpha (TNF-α) serve as significant indicators of severe OA, with their concentrations closely associated with reductions in joint space and cartilage degradation[9]. Furthermore, numerous studies have established a connection between the onset and progression of OA and the altered functions of histone deacetylases (HDACs) [10,11]. Among these, HDAC5, a member of the class IIa HDAC family, has been shown to inhibit chondrocyte hypertrophy by functioning as a co-activator of HDAC4, which operates via parathyroid hormone-related protein[12]. Additionally, reduced levels of HDAC5 have been observed in the cartilage of mice undergoing medial meniscus surgery and in patients with advanced OA. The silencing of HDAC5 was linked to increased degeneration of articular cartilage and chondrosenescence, while the activation of HDAC5 in chondrocytes through metformin effectively reversed OA[10].

Recently, there has been a growing interest in plant-based natural remedies due to their safety and beneficial effects on bone health, which are linked to their chondroprotective and osteoprotective properties[13,14,15]. One such remedy is *Boswellia serrata* (Bs) gum resin, derived from the Bs tree native to the Indian highlands. This resin has a long history of use in treating arthritis and chronic inflammatory disorders[16]. It is known for its potent anti-inflammatory

and antioxidant effects, attributed to the presence of six different boswellic acids reported in Fig. 1.[17,18,19,20] Furthermore, an ethanol extract of Bs gum resin, rich in boswellic acids (FJH-UBS), has been shown to reduce inflammation and prevent the degradation of the extracellular matrix, demonstrating an anti-osteoarthritis effect in fibroblast-like chondrocyte cells derived from a 72-year-old white female with chondrosarcoma (SW1353) stimulated by interleukin (IL)-1β, as well as in animal models of osteoarthritis[21]. Additionally, the Bs extract applied to primary chondrocytes from osteoarthritis patients exhibited antioxidant properties through the activation of Nrf1 and Peroxisome Proliferator Activated Receptor alpha (PPARα) pathways, alongside anti-inflammatory effects by promoting growth/differentiation factor 15 (GDF15), decreasing cholesterol uptake, and regulating genes involved in fatty acid metabolism, while also reducing Toll-like receptor activation[22].

Moreover, the administration of Bs gum resin extract in a rat model of osteoarthritis induced by monosodium iodoacetate showed significant therapeutic potential by alleviating knee joint swelling, cartilage damage, and tissue deformation, as well as enhancing the expression of collagen type II alpha 1 and aggrecan, while downregulating nitric oxide, inducible nitric oxide synthase, cyclooxygenase-2, 5-lipoxygenase, prostaglandin E2 (PGE2), leukotriene B4, IL-6, TNF-α, and matrix metalloproteinases 3 and 13[8].

A revolutionary method for improving the bioavailability and efficacy of medications, particularly in the treatment of conditions like osteoarthritis, is the application of novel nanotechnology techniques[23]. By increasing the solubility and stability of bioactive compounds, nanoscale transporters facilitate improved absorption and retention at the target sites[24]. Nanocarriers provide a significant solution to multiple hurdles, predominantly by promoting a sustained drug release and intensified internalization, thereby augmenting the therapeutic potency[25]. In addition, recent nanotechnology-driven formulations have demonstrated promising outcomes in the reduction of key inflammatory factors, comprising IL-1β and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) thereby aiding in the mitigation of osteoarthritis symptoms and disease pathology[26]. These progressions underscore the vital role of nanotechnology in enhancing the efficacy of naturally derived therapeutic regimens for improved clinical outcomes[27].

So, the aim of the present study is to investigate the potential effects of Bs extract and nano-emulsion on the human chondrocyte cell line (C20A4) in an *in-vitro* model of OA by evaluating the gene expression of histone deacetylase 5 (HDAC5) and inflammation biomarkers IL1β, IL6, PGE2, NF-kB, and the survival rate of C20A4.

Materials and Methods

Materials

Plant materials

In April 2024, the gum resin of *Boswellia serrata* was purchased from ATOS Pharma, Egypt. The plant material was formally verified for authenticity by Prof. Abdulhalim Abdelmogeli Mohammed, specialist in flora and vegetation at the Agricultural Research and Extension Authority, Cairo. A voucher specimen representing the authenticated sample was archived at the Department of Pharmacognosy, Faculty of Pharmacy, Heliopolis University. The obtained resin was preserved in an airtight container under proper storage conditions until further experimental use.

Chemicals and Reagents for cytotoxicity

To perform the cytotoxicity assessment through the MTT method, RPMI-1640 growth medium, phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO), and the C20A4 cell line were acquired from Sigma-Aldrich, Germany. The MTT dye solution was procured separately from Bio Basic Inc., Canada.

Chemicals and Reagents for HPLC-ESI-MS/MS

Delphinidin-3,5-diglucoside chloride and kaempferol-3-glucoside were obtained from PhytoLab, Vestenbergsgreuth, Germany, whereas the other analytical reference standards were sourced from Sigma-Aldrich, Milan, Italy. Stock solutions for each compound (1000 mg/L) were prepared by dissolving the respective pure standards in methanol of HPLC grade. All solvents employed in the study were of HPLC quality. Prior to chromatographic injection, the samples were passed through a Phenex™ RC syringeless filter (4 mm, 0.2 µm; Phenomenex, Castel Maggiore, BO, Italy).

Chemicals and reagents for Nano emulsion (NanoEm)

Sigma-Aldrich provided the chemicals required for NanoEm formulation, which included Tris, glycine, SDS, Tween 80, polyethylene glycol 600 (PEG 600), dimethyl sulfoxide (DMSO), phosphate-buffered saline tablets, and castor oil. All reagents were of analytical quality.

Methods

Extraction

A portion of 10 g from the collected sample was subjected to ultrasonic-assisted extraction with 90% methanol (HPLC grade) until complete exhaustion. The resulting extract was filtered, and the solvent was completely removed under reduced pressure using a rotary evaporator maintained at 50°C. The obtained dry residue was weighed, and the required portion was forwarded for further analysis.

Chondroprotective and osteoprotective activity - Cytotoxic activity (MTT assay)[28].

The MTT assay was employed to investigate the cytotoxic activity of Bs extracts (conventional and nano-formulated) in comparison with the standard Blank (Blk). Cells were seeded in 96-well plates at a density of 1×10^5 cells/mL (100 µL per well) and incubated at 37°C for 24 h, allowing a uniform monolayer to form. Upon reaching confluence, the growth medium was removed, and the plates were rinsed twice with washing medium. Test extracts were serially diluted in RPMI supplemented with 2% maintenance medium to prepare eight concentrations (10000, 5000, 2500, 1250, 625, 312.5, 156.25, and 78.12 µg/mL). A 0.1 mL portion of each dilution was dispensed into separate wells, while three wells containing only maintenance medium served as controls. Plates were incubated again at 37°C, and cells were monitored for toxic morphological changes, including monolayer disruption, rounding, shrinkage, and granule formation. Afterward, 20 µL of MTT solution (5 mg/mL in PBS) was pipetted into each well and gently agitated on a shaker at 150 rpm for 5 min. The plates were incubated for 4 h at 37°C in a 5% CO_2 atmosphere to permit the enzymatic reduction of MTT to formazan. Supernatants were then discarded, and crystals were solubilized with 200 µL DMSO under shaking (150 rpm, 5 min). Optical density values were determined at 560 nm with correction against a reference wavelength of 620 nm. Cell viability was expressed as percentage relative to untreated controls.The relative cell-viability percentage was evaluated by using the following relation:

Relative cell-viability %= $\frac{\text{Absorbance of cells treated with the tested sample at 560 nm}}{\text{Absorbance of untreated cells at 560 nm}} \times 100$.

Preparation and characterization of NanoEm[29].

The nanoemulsion (NanoEm) was prepared using castor oil, phosphate-buffered saline (PBS, 10 mM, pH 7.4), and Tween 80 in a ratio of 10:5:2.5, respectively. To begin, 50 mg of Bs powder was dissolved in 5 mL of PBS and subjected to ultrasonic treatment with a probe sonicator (cycle 1, amplitude 80%). Subsequently, 2.5 mL of Tween 80 was incorporated as the surfactant. The resulting mixture was then transferred to a cold-water bath, where castor oil (10 mL) was added dropwise under continuous homogenization (Daihan Homogenizer, ZS version, Korea) operated at 750 rpm for 11 minutes. The obtained nanoemulsion was employed for further characterization and subsequent experimental applications.

Characterization of NanoEm included determination of droplet size, polydispersity index (PDI), and zeta potential using a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK) maintained at 25 ± 0.1°C. Samples were diluted with PBS (10 mM, pH 7.4) to achieve a particle count within the range of 50–300 KCPs. Morphological features were examined by transmission electron microscopy (TEM, model H-700, Hitachi Ltd., Japan). For TEM analysis, samples were diluted at a ratio of 1:50 with PBS (10 mM, pH 7.4), and a drop of the diluted suspension was placed on carbon-coated copper grids. After air drying for 5 minutes, the grids were negatively stained with 2% w/w phosphotungstic acid for 50 seconds, and excess stain was removed using filter paper.

Additionally, the physicochemical properties of the NanoEm were evaluated. The pH was measured at room temperature using an Ohaus Starter 3100 bench pH meter (USA), which had been calibrated with standard buffer solutions of pH 4, 7, and 10. Viscosity was determined using a Lamy Rheology viscometer (B-One Plus, Germany) with spindle 6 at a rotation speed of 50 rpm for 3 minutes.

Quantitative real-time polymerase chain reaction

Total RNA from C20A4 cells was isolated using the RNeasy® Mini Kit (Catalog No. 74904, QIAGEN, Aarhus, Denmark) in accordance with the manufacturer's protocol. At the conclusion of the extraction and purification steps, RNA quality and purity were verified by spectrophotometric measurement of the absorbance ratio at 260/280 nm using a Denovix spectrophotometer (AGBL, USA). The purified RNA (0.5–2 µg) was then subjected to reverse transcription into complementary DNA (cDNA) with the aid of the SuperScript IV One-Step RT-PCR kit (Catalog No. 204843, Qiagen QuantiTect RT kit), which is highly sensitive for such applications.

For subsequent quantitative real-time PCR (qPCR), an optimized kit containing Taq polymerase, qPCR reaction buffer, primers, SYBR® Green I dye, and nucleotides was employed. The primer sequences utilized during the annealing stage are listed in Table 1. Relative expression levels (RQ) of target genes, including HDAC5, NF-κB, prostaglandin EP2 receptor, IL-1β, and IL-6 were determined using the calculation algorithm provided in the Applied Biosystems software formula, ΔCt = Ct gene test - Ct housekeeping control and ΔΔCt = Ct sample1 - Ct calibrator, where (RQ) = $2^{-\Delta\Delta Ct}$. The outcomes were normalized to GADPH and presented as arbitrary units.

Table 1
The primers sequence of studied genes in quantitative real-time polymerase chain reaction.

Gene	Primer Sequence
HDAC5	Forward 5'- TCTTGTCGAAGTCAAAGGAGC – 3'
	Reverse 5'- GAGGGGAACTCTGGTCCAAAG – 3'
NF-κB	Forward 5'- GCAGCACTACTTCTTGACCACC-3'
	Reverse 5'- TCTGCTCCTGAGCATTGACGTC-3'
EP2	Forward 5'-CCACCTCATTCTCCTGGCTA-3
	Reverse 5'-CGACAACAGAGGACTGAACG-3
IL-1β	Forward 5'- CCACAGACCTTCCAGGAGAATG-3'
	Reverse 5'- GTGCAGTTCAGTGATCGTACAGG-3'
IL-6	Forward 5'- AGACAGCCACTCACCTCTTCAG-3'
	Reverse 5'- TTCTGCCAGTGCCTCTTTGCTG-3'

HPLC-ESI-MS/MS[30].

High-performance liquid chromatography coupled with tandem mass spectrometry (HPLC–MS/MS) was conducted using an Agilent 1290 Infinity system linked to a Triple Quadrupole 6420 (Agilent Technologies, Santa Clara, CA, USA). The system was fitted with an electrospray ionization (ESI) interface, which was operated alternately in positive and negative ionization modes. Polarity switching was performed within a single analytical run without complications. For each analyte, MS/MS parameters were tuned through flow injection analysis (FIA) by injecting 1 µL of a 10 mg L^{-1} individual standard solution, using the Agilent Optimizer software.

Chromatographic separation of the analytes was achieved on a Synergi Polar-RP C18 analytical column (250 mm × 4.6 mm, 4 µm; Phenomenex, Cheshire, UK), protected with a Polar RP guard cartridge (4 mm × 3 mm ID). The mobile phase consisted of two solvents: (A) water with 0.1% formic acid and (B) methanol with 0.1% formic acid. A gradient elution program at a flow rate of 0.8 mL/min was applied as follows: 0–1 min, isocratic at 20% B; 1–25 min, linear increase from 20–85% B; 25–26 min, isocratic at 85% B; and 26–32 min, return from 85–20% B. All solvents and prepared solutions were filtered prior to use through 0.2 µm polyamide membranes (Sartorius Stedim, Goettingen, Germany).

The injection volume for each run was 2 µL. The column was maintained at 30°C, while the drying gas temperature in the ion source was set at 350°C. Additional source conditions included a drying gas flow of 12 L/min, nebulizer pressure of 55 psi, and capillary voltage of 4000 V. Data acquisition was carried out in dynamic multiple reaction monitoring (dynamic-MRM) mode. For quantitation, the most intense product ion was selected, while additional transitions were employed for confirmation. A retention time window of ± 2 min was assigned for each analyte. Detailed ion transitions along with optimized MS parameters for the studied compounds are provided in Table 2 **and** Fig. 2.

Statistical Analysis

All results were presented as mean values accompanied by their corresponding standard deviations (SD). The Kolmogorov–Smirnov test was applied to determine whether the datasets followed a normal distribution. To assess differences among groups, a one-way analysis of variance (ANOVA) was conducted, and when significance was detected, Tukey's post hoc test was employed for multiple pairwise comparisons. Statistical evaluations and preparation of graphical outputs were carried out using GraphPad Prism software, version 9 (GraphPad Software, Inc., San Diego, CA, USA). A probability value of less than 0.05 ($p < 0.05$) was considered the threshold for statistical significance. Outlier identification was performed using the Dixon test, and only values that failed this test were excluded from analysis.

Table 2
HPLC–MS/MS acquisition parameters (dynamic-MRM mode) used for the analysis of the 28 marker compounds.

No.	Compounds	Precursor ion, *m/z*	Product ion, *m/z*	Fragm-entor, V	Collision energy, V	Polarity	Retention time (Rt, min)	Delta retention time (ΔRt)
1	Gallic acid	169	125.2*	97	12	Negative	6.96	2
2	Neochlorogenic acid	353	191.2*, 179	82	12, 12	Negative	9.52	2
3	(+)-Catechin	289	245.2*,109.2	131	8, 20	Negative	11.44	2
4	Procyanidin B2	576.99	576.99*, 321.2	160	0, 32	Negative	12.41	2
5	Chlorogenic acid	353	191.2*, 127.5	82	12, 20	Negative	12.42	2
6	*p*-Hydroxybenzoic acid	137	93.2*	92	16	Negative	12.86	2
7	(-)-Epicatechin	289	245.1*, 109.1	126	8, 20	Negative	13.03	2
8	3-Hydroxybenzoic acid	137	93.2*	88	8	Negative	13.59	2
9	Caffeic acid	179	135.2*, 134.1	92	12, 24	Negative	13.65	2
10	Vanillic acid	167	152.4*, 108.1	88	12, 20	Negative	14.32	2
11	Resveratrol	227	185*	131	12	Negative	14.40	2
12	Syringic acid	196.9	182.2*, 121.2	93	8, 12	Negative	15.28	2
13	Procyanidin A2	575	575*, 285	170	0, 20	Negative	16.18	2
14	*p*-Coumaric acid	163	119.2*, 93.2	83	12, 36	Negative	16.70	2
15	Ferulic acid	193	134.2*, 131.6	83	12, 8	Negative	17.10	2
16	3,5-Dicaffeoylquinic acid	514.9	353.1*, 191	117	8, 28	Negative	17.61	2
17	Rutin	609	300.2*, 271.2	170	32, 50	Negative	17.73	2
18	Isoquercitrin	463	271.2*, 300.2	155	44, 24	Negative	18.36	2

No.	Compounds	Precursor ion, *m/z*	Product ion, *m/z*	Fragm-entor, V	Collision energy, V	Polarity	Retention time (Rt, min)	Delta retention time (ΔRt)
19	Delphindin-3,5-diglucoside	462.9	300.1*	165	24	Negative	18.38	2
20	Phloridzin	435.39	273*, 167	155	8, 28	Negative	18.83	2
21	Quercitrin	446.99	300.2*, 301.2	160	24, 16	Negative	19.61	2
22	Myricetin	316.99	179.1*, 182	150	16, 24	Negative	19.61	2
23	Naringin	578.99	271.3*, 151.3	170	32, 44	Negative	19.62	2
24	Kaempferol-3-glucoside	447	284.2*, 255.2	170	24, 40	Negative	19.77	2
25	Ellagic acid	301	301*, 229	170	0, 24	Negative	21.41	2
26	Quercetin	300.99	151.2*, 179.2	145	16, 12	Negative	21.87	2
27	Phloretin	272.99	167*, 123	116	8, 20	Negative	22.30	2
28	Isorhamnetin	314.99	300.2*, 196.1	145	16, 4	Negative	24.57	2

* These product ions were used for quantification.

Results and discussion

Chondroprotective and osteoprotective activity

The data illustrated in Fig. 3 compare the survival rate in C20A4 of the *Bs* extract in both conventional and nano-formulation forms against the standard Blk., evaluated through the MTT assay on Human Chondrocyte Cell Line C20A4 cells. The extracts and the standard were tested at various concentrations (10000, 5000, 2500, 1250, 625, 3125, 156.25, 78.12 μg/mL). Notably, the conventional *Bs* extract achieved an IC50 value of 674.5, which corresponds to 18.89% of the Blk. group's effect. In contrast, the nano-formulated version yielded an IC50 of 4274, equating to 119.69% of the standard, indicating enhanced cell viability with the Nano-formulation.

Bs Extract Restores Epigenetic Balance and Suppresses Inflammatory Markers in C20A4 Cells: Enhanced Efficacy with Nano-formulation

As shown in Fig. 4, the C20A4 osteoarthritic chondrocyte model displayed a notable dysregulation in inflammatory and epigenetic markers. Specifically, HDAC5 (Histone Deacetylase 5), a key regulator of gene expression and anti-inflammatory response, was reduced by 10.07% relative to the normal control group. In contrast, there was a marked upregulation of NF-κB, a pro-inflammatory transcription factor, and the Prostaglandin EP2 receptor, which

mediates inflammation and pain signaling. These were elevated to 111.75% and 109.29%, respectively, compared to the normal control.

Upon treatment with *Bs* extract, both the conventional and Nano-formulated preparations produced significant modulatory effects. Notably, HDAC5 levels increased beyond the baseline of the normal control, reaching 124% and 131% in the conventional and Nano-formulated extract-treated groups, respectively. This suggests a potential epigenetic restoration or enhancement effect, particularly with the nanoformulation.

Simultaneously, both treatments effectively reduced the elevated inflammatory markers. The conventional extract reduced NF-κB and EP2 receptor levels to 89% and 87%, respectively, while the nanoformulation demonstrated slightly greater efficacy, lowering them to 88% and 82% of normal control values. These findings indicate that *Bs*, especially in Nano-formulated form, has anti-inflammatory potential through suppression of key inflammatory pathways.

When compared to the standard therapeutic agent Blk., the extract showed comparable effects. Blk. also elevated HDAC5 and reduced NF-κB and EP2 expression to similar levels. The only statistically notable difference was in EP2 receptor expression, where the Nano-formulated *Boswellia* achieved a further reduction to 82%, while Blk. reached only 89%.

These findings highlight the potential of *Bs*, particularly in its Nano-formulated version, to counteract inflammation and restore epigenetic balance in osteoarthritic conditions. The extract's ability to elevate HDAC5 and suppress NF-κB and EP2 receptor expression supports its therapeutic value, possibly offering a comparable or even superior alternative to conventional treatments like Blk. for managing osteoarthritis-related inflammation.

Nano-formulated Bs Exhibits Superior Suppression of Pro-inflammatory Cytokines IL-1β and IL-6 in Osteoarthritic Chondrocytes

As depicted in Fig. 5, the C20A4 osteoarthritic chondrocyte model demonstrated a pronounced pro-inflammatory response, with IL-1β and IL-6 levels increasing to 115% and 112%, respectively, relative to the normal control group. These cytokines are key mediators of inflammation, cartilage degradation, and joint damage in osteoarthritis. Treatment with the standard therapeutic agent, Blk., resulted in a modest reduction in IL-1β, lowering it to 92% of the level observed in C20A4 cells. However, it had no significant impact on IL-6, suggesting limited anti-inflammatory efficacy in this context.

In contrast, treatment with *Bs* extract, both in conventional and Nano-formulated forms, led to notable reductions in both cytokines. The conventional extract decreased IL-1β to 70% and IL-6 to 90%, while the Nano-formulated extract produced an even more pronounced effect, reducing IL-1β to 64% and IL-6 to 82% compared to the C20A4 group.

When benchmarked against the standard Blk., the conventional extract showed superior efficacy in reducing IL-1β, and the nanoformulation demonstrated superiority in suppressing both IL-1β and IL-6. Specifically, the nanoformulation reduced IL-1β and IL-6 to 70% and 85%, respectively, relative to the standard. These findings underscore the anti-inflammatory potential of *Bs*, particularly in its Nano-formulated form, in mitigating key inflammatory cytokines involved in osteoarthritis. The extract not only outperformed the standard treatment in reducing IL-1β but also showed added benefit in lowering IL-6 levels, highlighting its promise as a more effective therapeutic strategy.

HPLC-MS/MS analysis of the dried extract

Found to be strongly potent in gene expression downregulation of the inflammation biomarkers IL1β, IL6, PGE2, NF-kB, upregulation of HDAC5 gene expression and elevation of survival rate in C20A4, methanolic extract of *Bs* gum resin was analyzed by HPLC-ESI–MS/MS to examine the chemical profile. None of the standard 28 compounds was identified in *Bs* methanol extract, out of 28 bioactive polyphenol reference compounds Table 3. The 28 compounds were quantified after the acquisition settings of the dynamic MRM mode were optimized. Table 3 lists the chosen ion transitions and mass spectrometer parameters for each molecule. Twenty-eight reference compounds belonging to different phenolic classes were used and the results are depicted in Table 2. HPLC-MS/MS chromatogram of a standard mixture of 28 phenolic compounds plotted as overlapped multiple reaction monitoring (MRM) negative and positive transition of each analyte is shown in Fig. 2.

Table 3
Content (µg/g of dried extract) of phenolic compounds found in *Bs* methanol extract

Phenolic compound	Content µg/g
Gallic acid	n.d.
Neochlorogenic acid	n.d.
Catechin	n.d.
procyanidin B2	n.d.
Chlorogenic acid	n.d.
4-Hydroxy benzoic acid	n.d.
Epicatechin	n.d.
3-Hydroxy benzoic acid	n.d.
Caffeic acid	n.d.
Vanillic acid	n.d.
Syringic acid	n.d.
Procyanidin A2	n.d.
P-Coumaric acid	n.d.
Ferulic acid	n.d.
Rutin	n.d.
Isoquercitrin	n.d.
Delphindin 3,5 diglucoside	n.d.
Phloridzin	n.d.
Naringin	n.d.
Quercitrin	n.d.
Myricetin	n.d.
Kaempferol-3-glucoside	n.d.
Ellagic acid	n.d.
Quercetin	n.d.
Phloretin	n.d.
Isorhamnetin	n.d.
TOT	**0.00**

Conclusions

The present study demonstrates the promising therapeutic potential of *Bs* extract in modulating key molecular pathways involved in osteoarthritis, particularly through its ability to restore epigenetic balance and suppress pro-inflammatory markers. The C20A4 osteoarthritic chondrocyte model revealed significant disruptions in cellular homeostasis, marked by decreased expression of HDAC5, a crucial regulator of anti-inflammatory gene expression, and elevated levels of NF-κB and the Prostaglandin EP2 receptor, both of which play central roles in mediating inflammatory responses and cartilage degradation.

Treatment with *Bs* extract, in both conventional and Nano-formulated forms, successfully reversed these alterations. Notably, HDAC5 expression was not only restored but enhanced, indicating potential epigenetic reprogramming in favor of anti-inflammatory activity. This was accompanied by a reduction in NF-κB and EP2 receptor expression, further supporting the extract's role in attenuating inflammatory signaling. The Nano-formulated form of the extract consistently exhibited greater efficacy than its conventional counterpart, suggesting improved cellular uptake or bioavailability as a result of the nanotechnology-based delivery system.

Parallel analysis of the pro-inflammatory cytokines IL-1β and IL-6 provided additional evidence of the extract's anti-inflammatory properties. While the standard therapeutic agent modestly reduced IL-1β without affecting IL-6, *Bs* extract significantly suppressed both cytokines. The Nano-formulated preparation again showed superior performance, effectively reducing both IL-1β and IL-6 levels beyond the effects observed with the conventional extract or standard treatment.

Taken together, these findings suggest that *Bs* exerts its therapeutic effects in osteoarthritic chondrocytes by targeting both epigenetic regulators and inflammatory mediators. The consistent superiority of the Nano-formulated version highlights the added value of advanced drug delivery systems in enhancing the bioactivity of phytotherapeutic agents. Overall, *Bs*, particularly in Nano-formulated form, represents a promising candidate for further development as a disease-modifying agent in osteoarthritis, with potential advantages over existing conventional therapies.

In the C20A4 chondrocyte model, osteoarthritic downregulation of HDAC5 was not only restored but exceeded normal baseline following Bs treatment, with nano-formulations showing superior efficacy. This was accompanied by reduced NF-κB, EP2 receptor, IL-1β, and IL-6 expression, suggesting upstream transcriptional reprogramming rather than mere downstream cytokine blockade. The enhanced activity of the nano-formulated extract likely reflects improved bioavailability, positioning Bs as a promising disease-modifying therapy for osteoarthritis through epigenetic restoration and inflammation suppression.

Declarations

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Author Contribution

Zobaida A. Mohamed: Conceptualization; Methodology; Writing–original draft; Writing & editing. **Marwa Saeed, Laura Acquaticci, Muhammed A. Saad, Yasmin Abo-zeid, Asmaa M. Elbakry:** Conceptualization; Methodology; Supervision; Writing–review & editing. **Mohamed Rabeh, Saad Alshehri:** Funding Conceptualization; Methodology;

Supervision; Writing–original draft; Reviewing & editing.**Omar Sabry**and**Giovanni Caprioli:** Conceptualization; Methodology; Supervision; Writing–original draft; Reviewing & editing.

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Conflicts of interest

The authors confirm that they have no conflicts of interest.

Ethical approval

Ethics approval was not required for this research.

Data Availability Statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary material.

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Figures

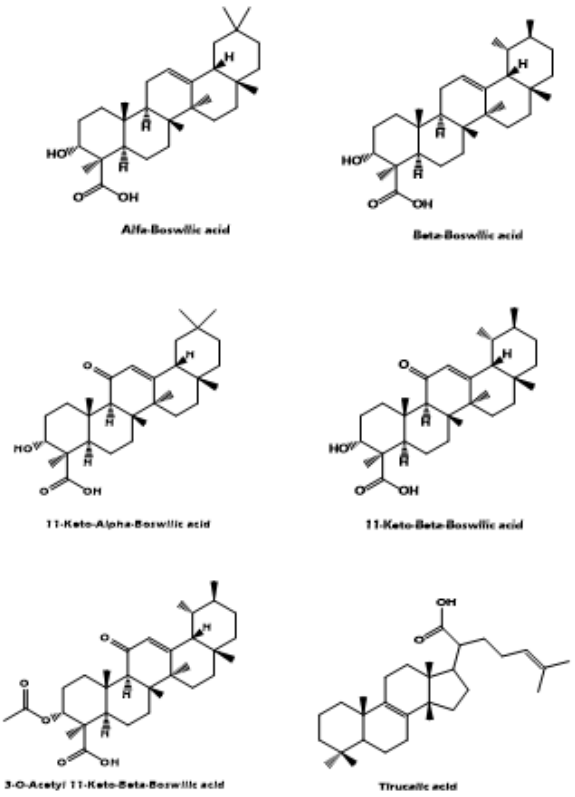

Figure 1

Representative examples of triterpene acids identified in *Boswellia serrata*

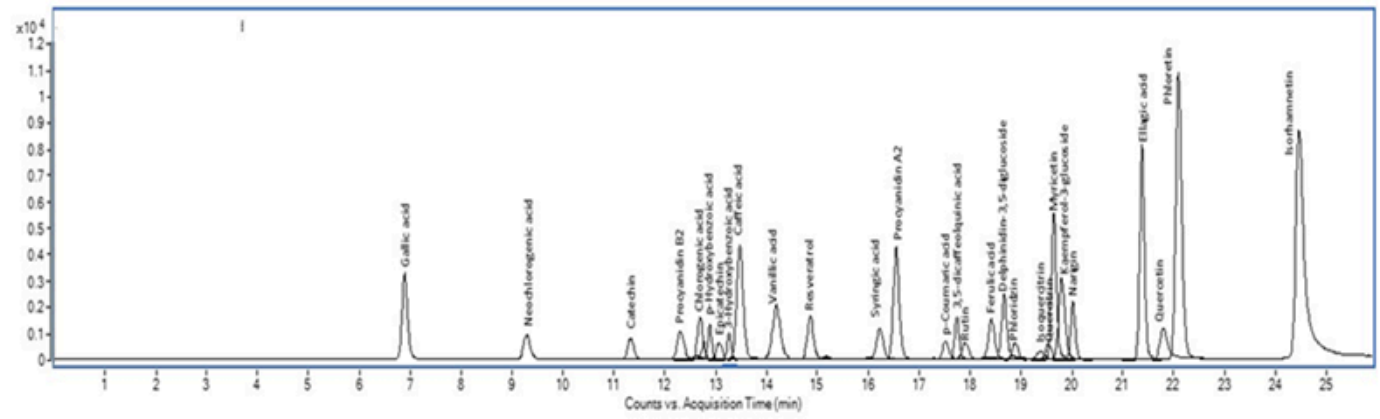

Figure 2

HPLC-MS/MS chromatogram of a standard mixture of 28 phenolic compounds plotted as overlapped multiple reaction monitoriative transition of each analyte.

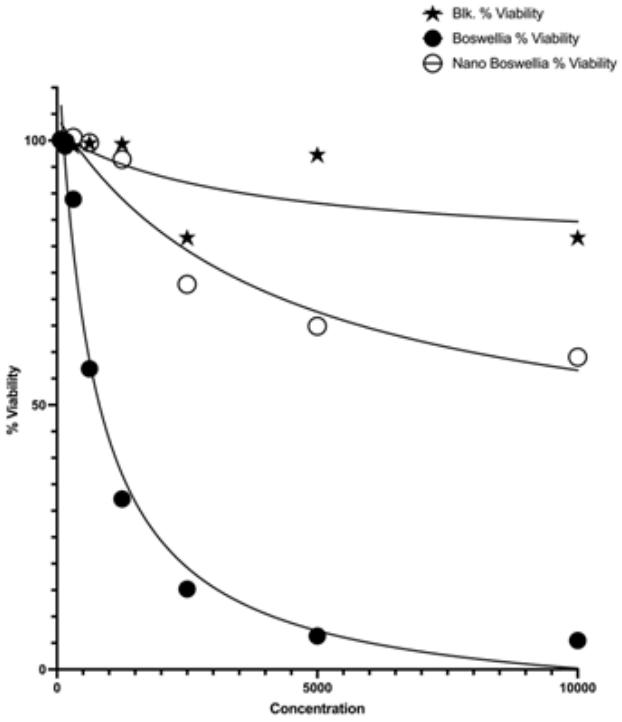

Figure 3

Represents the comparative study between the *Bs* extracts in regular and Nano-formulations versus the standard Blk. using MTT assay on the Human Chondrocyte Cell Line C20A4 cells.

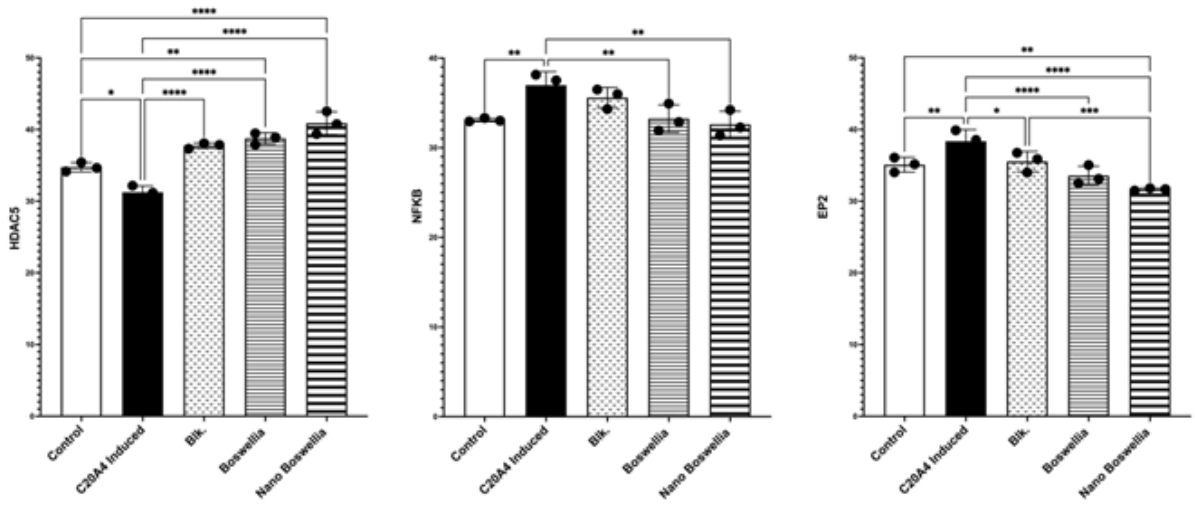

Figure 4

Effect of the Effect of *Bs* extract in regular and nano-formulations and the standard Blk on Histone deacetylase 5 (HDAC5) [F (20, 42) =15.33, p < 0.0001], nuclear factor kappa B (NF-kB) [F (20, 42) =8.575, p < 0.0001], and Prostaglandin EP2 receptor [F (20, 42) =16.28, p < 0.0001] using an in-vitro Human Chondrocyte Cell Line C20A4 cells. Each vertical-lined bar reflects the mean of the experiments ± S.D. Statistical analysis was performed by One-way ANOVA followed by Tukey's post-hoc test, with the criterion for statistical significance as follows: *p <0.05, **p <0.01, ***p <0.005, ****p <0.001, ns no significance.

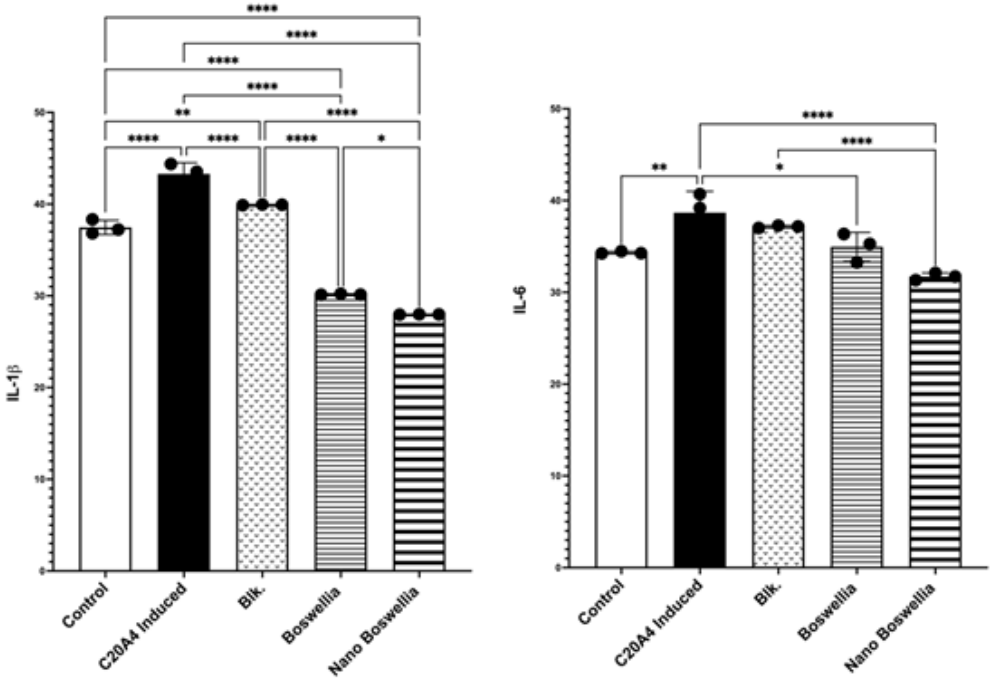

Figure 5

Effect of the *Bs* extract in regular and nano-formulations and the standard Blk on *Interleukin-1 beta (IL-1β)* [F (20, 42) =67.25, p < 0.0001], *and Interleukin 6 (IL-6)* [F (20, 42) =7.849, p < 0.0001] activities using an in-vitro Human

Chondrocyte Cell Line C20A4 cells. Each vertical-lined bar reflects the mean of the experiments ± S.D. Statistical analysis was performed by One-way ANOVA followed by Tukey's post-hoc test, with the criterion for statistical significance as follows: *p <0.05, **p <0.01, ***p <0.005, ****p <0.001, ns no significance.