RESEARCH PAPER

Anti-inflammatory and Antibacterial activity of Murraya koenigii extract and their immunomodulatory effect on Macrophage Cells

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Received: 17th Mar, 2026 | Revised: 29th Mar, 2026 | Accepted: 19th Apr, 2026 | Available Online: 5th May, 2026

ABSTRACT

Background: Murraya koenigii (curry leaf) is a natural medicinal herb that is known to contain carbazole alkaloids, but the combined immunomodulatory, anti-inflammatory, and antibacterial activity of the plant on macrophages is poorly described. This paper examined the phytochemical profile, antibacterial and immunomodulatory effects of M. koenigii leaf extract on RAW 264.7 macrophages.

Methods: Methanolic leaf extracts underwent qualitative phytochemical screening, quantitative analysis of spectrophotometer (total phenolics, flavonoids, alkaloids) and HPLC-DAD/LC-ESI-MS/MS profiling. The antibacterial activity was tested against Staphylococcal aureus, Enterococcal faecalis, Escherichia coli, and multidrug-resistant Acinetobacter baumannii (MIC/MBC determination, biofilm inhibition assay, and membrane integrity analysis flow cytometry/CLSM with PI/ SYTO-9 staining). MTT assay was used to determine cytotoxicity on RAW 264.7 macrophages. Anti-inflammatory effects were assessed through ELISA of TNF- a, IL-6 and IL-1B in cells that were stimulated with LPS. Phagocytic activity, genotoxicity (comet assay), and apoptosis (Annexin-V/PI flow cytometry) were assessed as well.

Results: The extract was rich in alkaloids (212.5 mg/g), phenolics (187.3 mg GAE/g), flavonoids (94.6 mg QE/g). Mahanimbine (58.3 mg/g), quercetin (41.2 mg/g), and murrayanine (32.7 mg/g) were major identified compounds. The strongest antibacterial effect was against S. aureus (MIC 0.5 mg/mL, MBC 1.0 mg/mL) and the weakest against MDRAB (MIC 4.0 mg/mL, MBC 8.0 mg/mL). The extract also prevented biofilm formation (to a maximum of 67.4% in S. aureus) and resulted in membrane damage (82.3% PI-positive S. aureus cells). In the macrophages, concentrations up to 250 μg/mL were non-cytotoxic (≥90% viability). The extract greatly reduced the LPS-mediated TNF- 67% secretion, IL- 6 secretion 66.6% and IL- 1 secretion 54.8% and increased phagocytic activity (119.4% of control) and did not cause DNA damage or apoptosis when assayed at 250 μg/mL.

Conclusion: M. koenigii extract has selective bactericidal effects against Gram-positive pathogens through membrane disruption, prevents the production of pro-inflammatory cytokines in macrophages without cytotoxicity, and does not impair the innate immune functions. These results justify its consideration as a dual-acting natural agent in the treatment of Gram-positive bacterial infections with inflammatory pathology, but a limited therapeutic index relative to MDR Gram-negative microbes should limit its use.

Keywords: Murraya koenigii; Carbazole Alkaloids; Mahanimbine; Anti-Inflammatory; Antibacterial; Macrophages; Raw 264.7; Minimum Inhibitory Concentration (Mic); Biofilm; Membrane Integrity; Tnf-A; Il-6; Immunomodulation

How to cite this article: Dave S, Bharati P K, Gupta P S P, Chandrawanshi S, Shrivastava S, Shinde S, Baig S A., Anti-Inflammatory and Antibacterial Activity of Murraya Koenigii Extract and Their Immunomodulatory Effect on Macrophage Cells. Int J Drug Deliv Technol. 2026;16(43s): 42-50; Doi: 10.25258/Ijddt.16.43s.5

Introduction

Murraya koenigii (L.) Spreng., also called curry leaf tree, is a tropical or subtropical aromatic herb of the Rutaceae family, native to the Indian subcontinent and commonly grown in Southeast Asia as a source of culinary and medicinal uses. The *M. koenigii* leaf has been a part of centuries-old traditional medicine, both Ayurveda and Unani, where it is used to treat a wide range of diseases, including dysentery, diarrhea, nausea, anemia, and skin eruptions. *M. koenigii* has a rich and unique phytochemical profile which is also credited with its therapeutic potential. Wide-spanning phytochemical studies have revealed more than 60 carbazole alkaloids as the characteristic secondary metabolites of this organism such as mahanimbine, murrayanine, koenimbine, and girininimbine. Besides these alkaloid, the leaves also have large quantities of flavonoids (e.g., quercetin, kaempferol), phenolic acids (e.g., caffeic acid, ferulic acid), terpenoids, and essential oils. Such a variety of chemical arsenal gives *M. koenigii* a wide range of proven pharmacological activities. Significant evidence has been established to support its strong antioxidant effects, antidiabetic effects (especially enhancing insulin sensitivity and lowering blood glucose levels), hypocholesterolemic effect, hepatoprotective action, and neuroprotective effects against cognitive impairment. Nevertheless, the exact immunomodulatory activities of *M. koenigii* extracts on the important immune effector cells especially on macrophages and their synergistic anti-inflammatory and antibacterial mechanisms are yet to be fully understood. Two urgent global health issues that warrant such inquiry include the increasing burden of chronic inflammatory diseases and the crisis of antibiotic resistance that is growing. Persistent immune system activation leads to chronic inflammation, a pathological basis of many debilitating diseases such as rheumatoid arthritis, inflammatory bowel disease, atherosclerosis, metabolic syndrome and even cancer. The existing mainstream anti-inflammatory agents, i.e. non-steroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids are linked to serious adverse events, including gastrointestinal ulceration, cardiovascular risks, and immunosuppression, which complicates their long-term use. At the same time, the world is experiencing a post-antibiotic world of multidrug-resistant (MDR) and extensively drug-resistant (XDR) bacterial pathogens that are rapidly surpassing the pace of new conventional antibiotic development. Antimicrobial resistance (AMR) has been declared by the World Health Organization as one of the top ten global public health threats. The resistant strains of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* cause long hospital stays, increased mortality and enormous healthcare costs. The intersection of chronic inflammation and bacterial infection is of particular concern with inflammatory tissue damage potentially providing a habitat where bacteria can colonize and chronic infections may promote a low-grade inflammatory response that does not resolve. Thus, novel, multi-targeting therapeutic agents, capable of simultaneously suppressing excessive inflammation, fighting bacterial pathogens, and restoring immune homeostasis with minimal toxicity are urgently needed.

The justification to investigate *M. koenigii* extract is convincing. We hypothesize that the *M. koenigii* leaves, which contain unique carbazole alkaloids and flavonoids, especially mahanimbine and murrayanine, have synergistic antibacterial, immunomodulatory and anti-inflammatory effects on the macrophage cells, in particular. Macrophages are natural immune sentinels that take a dual role: they are first-line defenders against invading bacteria by phagocytosis and killing of microorganisms, but their overactivation may cause pathological inflammation through the release of nitric oxide (NO), reactive oxygen species (ROS) and pro-inflammatory cytokines: tumor necrosis factor-alpha (TNF- We hypothesise that bioactive compounds in *M. koenigii* can regulate macrophage activity by preventing the excessive production of these inflammatory

mediators (to deliver anti-inflammatory effects) and at the same time increase or maintain the bactericidal activity of these cells against clinically-relevant pathogens. Moreover, the extract could directly suppress bacterial growth by disrupting the membrane or interfering with biofilms, which supplements the host immune response. Such a two-pronged activity would be very beneficial compared to traditional drugs which normally only focus on one of the pathways. Early data is consistent with this hypothesis: isolated carbazole alkaloids of related Murraya species have been found to have NF-KB inhibitory properties, and crude extracts of *M. koenigii* have been found to have moderate antibacterial activity in disk diffusion assays. Nonetheless, no research has assessed the combined impact of a standardized *M. koenigii* leaf extract on the inflammatory-antibacterial-immunomodulatory axis on a single macrophage cell model. Thus, the main purpose of this study is to examine the anti-inflammatory and antibacterial properties of *M. koenigii* extract and to describe its immunomodulatory properties on murine macrophage (RAW 264.7) cells and hence, this study will give a scientific justification to the possible development of *M. koenigii* extract as a natural therapeutic agent in the treatment of chronic inflammation and bacterial infections.

2. Materials and Methods

Plant material and extraction

The fresh and mature leaves of *Murraya koenigii* (L.). The botanical garden was picked to collect spreng. during flowering (March-May).The leaves were extensively rinsed with distilled water to eliminate any surface contamination, dried at room temperature (25-28°C) over 10-14 days, and finally ground into a fine powder in a laboratory blender. To prepare extracts, three solvents, such as methanol (70% v/v), distilled water (aqueous) and hydroalcoholic (50% ethanol:50% water v/v), were used to extract a wide spectrum of polar and non-polar phytochemicals. In a brief, 100 g of dried leaf powder was allowed to be macerated in 500 mL of each solvent after 72 hours at room temperature and occasionally shaken. The mixtures were filtered through Whatman No. 1 filter paper and the filtrates were concentrated at reduced pressure using rotary evaporator at 40°C. The aqueous extract was also lyophilized to get a dry powder. Crude extracts were then put in light-resistant, airtight containers at 4°C, awaiting further analysis and percentage yields were calculated through gravimetric analysis.

Phytochemical Analysis

Comprehensive phytochemical screening of the crude extracts of the *Murraya koenigii* leaf was performed to determine and quantify the major class of compounds, and high-resolution chemical profiling was then done in order to solve individual bioactive components. To perform qualitative analysis, standard colorimetric and precipitation tests were used to identify the presence of alkaloids (Dragendorff reagent and Mayer reagent), flavonoids (ferric chloride test and aluminum chloride test), phenols (Folin-Ciocalteu reagent), tannin (gelatin test), saponins (foam test), terpenoids (Salkowski test), and cardiac The UV-visible spectrophotometry was used to perform quantitative estimation: Folin Ciocalteu method was used to determine total phenolic content and the level of total flavonoid content was determined by the aluminum chloride colorimetric method and expressed as the equivalents of gallic acid (GAE) and quercetin equivalents (QE), respectively; gravimetric or bromocresol green method was used to High-performance liquid chromatography (HPLC) with a diode array detector (DAD) or liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) was used to obtain definitive chemical profiling. Separating was done on a C18 reverse-phase column with a gradient mobile phase of water and acetonitrile with 0.1% formic acid. The chromatographic fingerprints were made to compare them with known standards to detect and quantify signature compounds such as the carbazole alkaloids mahanimbine and murrayanine, and the flavonoid quercetin. The combination of these methods provided a level of comprehensive characterization as well as molecular specificity of the bioactive principles in the extract.

In vitro antibacterial activity assays

In order to assess the antibacterial potential of *Murraya koenigii* extract in a comprehensive manner, a panel of clinically relevant bacterial strains was chosen that included Gram-positive and Gram-negative pathogens, and an isolate that is resistant to a multidrug. The test organisms were *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), Escherichia coli (ATCC 25922), and a

clinical isolate of multidrug-resistant *Acinetobacter baumannii* (MDRAB, resistant to at least three classes of antibiotics). All strains were cultured on Mueller-Hinton agar and adjusted to a 0.5 McFarland standard (~1.5×10^8 CFU/mL) prior to testing. The broth microdilution method was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extract, with the Clinical and Laboratory Standards Institute (CLSI) guidelines. In short, 2-fold serial dilutions of the extract (0.125 to 8 mg/mL) were made in 96-well microtiter plates with Mueller-Hinton broth and then approximately 5×10^5 CFU/well was inoculated. MIC was noted after 24 hours of incubation at 37°C as the lowest concentration of the solution which did not exhibit any bacterial growth. MBC was then calculated by subculture of 10 µL of each well with no visible growth on Mueller-Hinton agar plates; the MBC was considered as the lowest concentration that killed at least 99.9 percent of the starting inoculum. Crystal violet staining assay was used to determine the effects of the extract on the formation of bacterial biofilms. The extract was incubated with sub-MIC concentrations in 96-wells plates with bacterial suspensions (1×10^7 CFU/mL) over 24 hours at 37°C. Washed adhered biofilms were fixed with methanol, stained with 0.1% crystal violet and solubilized the dye in 33% glacial acetic acid. The absorbance was recorded at 570 nm and percentage biofilm inhibition was determined as compared to untreated controls. Lastly, flow cytometry and confocal laser scanning microscopy (CLSM) were used to explore the mechanism of bacterial membrane damage. The extract at 1x MIC was incubated with the bacteria cells (4 hours) where propidium iodide (PI) and SYTO-9 were stained, with PI only able to penetrate cells with compromised membranes. In the case of flow cytometry, red (PI) fluorescence analysis of at least 10,000 events was obtained. In the case of CLSM, bacterial suspensions were stained and immobilized on agarose pads and visualized through a 100x oil-immersion lens, with qualitative evaluation of membrane integrity being the ratio of red (damaged) to green (intact) cells. These complementary techniques provided a strong evaluation of the bacteriostatic/ bactericidal potency and the mechanism of membrane-targeting of the extract.

Macrophage Cell Culture (RAW 264.7)

Cytotoxicity Assay (MTT Assay):

Albeit it was necessary to determine the non-cytotoxic concentration ranges to eliminate confounding outcomes based on cell death prior to the assessment of the anti-inflammatory and immunomodulatory effects of Murraya koenigii extract on macrophage cells. Murine RAW 264.7 macrophages were seeded onto 96-well plate with a concentration of 1 × 104 cells/well, and left to adhere overnight at 37°C in a 5% CO 2 incubator. Two-fold serial concentrations of the extract (between 7.8 and 1000 µg/mL) were then incubated into the cells over a period of 24 hours. After the treatment, the MTT assay was carried out: 10 µL of MTT solution (5 mg/mL in phosphate-buffered saline) was added to each well, and the plates were incubated further, 4 hours to enable the metabolically active cells to form purple formazan crystals. Aspirating the medium followed by dissolution of the formazan crystals in 100 µL of dimethyl sulfoxide (DMSO) was done carefully. Measurement of absorbance at 570 nm was done in a microplate reader and the reference wavelength was 630 nm. The viability of the cells was expressed as a percentage of untreated control cells (established at 100%). The concentrations that decreased the viability to less than 80 percent of control were termed cytotoxic. The maximum non-cytotoxic concentration that was to be used in further mechanistic assays was chosen as the highest concentration that retained ≥90% viability. Each experiment was carried out thrice to make it reproducible.

***In Vitro* Anti-inflammatory and Immunomodulatory Assays**

The assessment of immunotoxicity and cellular damage was performed using three complementary assays.

Pro-inflammatory cytokine levels (TNF-α, IL-1β, and IL-6)

They were quantified via enzyme-linked immunosorbent assay (ELISA), providing a sensitive measure of inflammatory response activation.

Phagocytic activity

The macrophages was assessed by the carbon clearance assay, which tracks the in vivo elimination of colloidal carbon in the blood, or the fluorescent bead uptake assay, which can be quantified

fluorescently or microscopically by counting the number of particles ingested.

Genotoxicity and apoptosis were determined: The comet (single-cell gel electrophoresis) technique identified the presence of DNA strand breaks under alkaline conditions whereas the Annexin-V/PI (propidium iodide) staining identified viable, early apoptotic (Annexin-V $^{+}$/PI $^{-}$), late apoptotic (Annexin-V^{+}/PI^{+}), and necrotic (Annexin-V^{-}/PI^{+}) cells Collectively, these endpoints can be used to assess vital facets of immune performance, including the production of cytokines, pathogen containment, and cell fate choices, and can be utilized to evaluate comprehensively the immunomodulatory and cytotoxic effects of compounds.

Statistical Analysis

All tests were conducted in triplicates and at least three independent occasions (n=3 in each group in each experiment). The data are presented in the form of mean ± standard deviation (SD) or standard error of the mean (SEM), as appropriate. The Shapiro Wilk test was used to determine normality of distribution and Levene test was used to determine homogeneity of variances. Student t-test (unpaired, two tailed) was used to make comparisons between two groups. In the case of multiple group comparisons, one-way analysis of variance (ANOVA) and the post hoc test with Tukey (when the variances were equal) and Welch with Games-Howell correction (when the variances were not equal) were employed. The KruskalWallis test with multiple comparisons using Dunn were used to analyze non-normally distributed data (e.g., comet assay tail moment values).

Results and Discussion

The present study comprehensively evaluated the phytochemical composition, antibacterial activity, and immunomodulatory profile of *Murraya koenigii* leaf extracts. The integrated approach ranging from bulk chemical quantification to mechanistic host-cell assays provides a multi-layered understanding of the extract's bioactivity.

Phytochemical profiling

The qualitative screening detected the existence of alkaloids, flavonoids, phenols, tannins, terpenoids, and cardiac glycosides and the absence of saponins. The quantitative spectrophotometric analysis showed that the total phenolic content of the extract was 187.3 ± 8.2 mg GAE/g extract and total flavonoid content of the extract was 94.6 ± 5.1 mg QE/g extract. The percentage amounts of alkaloids (calculated as atropine equivalents) were 212.5 ± 10.4 mg/g. HPLC-DAD/LC-ESI-MS/MS profiling was used to determine mahanimbine (RT 12.4 min, [M+H] 338.2], murrayanine (RT 8.7 min, $[M+H]^{+}$ 226.1) and quercetin (RT 6.2 min, [M−H] 301.0) The elevated level of carbazole alkaloids is typical of *M. koenigii* and is consistent with the claims which recognize mahanimbine as the main bioactive indicator **Table 1**.

Table 1: Phytochemical Profiling of *M. koenigii*

Phytochemical Class	**Methanol Extract (70% v/v)**	**Aqueous Extract (Distilled Water)**	**Hydroalcoholic Extract (50% Ethanol : 50% Water v/v)**
Alkaloids	++	+++	++
Flavonoids	++	+++	++
Phenols	+	+++	+
Tannins	+++	++	++
Terpenoids	+	++	++
Cardiac Glycosides	++++	++	+++

Antibacterial Activity

The extract had varying activity with bacterial strains (Table 2). The most sensitive were S. aureus (MIC 0.5 mg/mL, MBC 1.0 mg/mL), then *E. faecalis* (MIC 1.0 mg/mL, MBC 2.0 mg/mL). *E. coli* was intermediate in its susceptibility (MIC 2.0 mg/mL, MBC 4.0 mg/mL), whereas the MDR *A. baumannii* clinical isolate was the least susceptible (MIC 4.0 mg/mL, MBC 8.0 mg/mL). The ratios of MBC/MIC were less than 4 in all strains, which is a demonstration of bactericidal, not only bacteriostatic activity.

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *M. koenigii* extract against test bacterial strains.

Bacterial Strain	MIC (mg/mL)	MBC (mg/mL)	MBC/MIC Ratio
S. aureus ATCC 25923	0.5	1.0	2
E. faecalis ATCC 29212	1.0	2.0	2
E. coli ATCC	2.0	4.0	2

25922			
MDR *A. baumannii* (clinical)	4.0	8.0	2

At sub-MIC (0.25× MIC) the extract prevented the biofilm formation in *S. aureus* (67.4%), *E. faecalis* (58.2%), *E. coli* (45.1%), and MDR *A. baumannii* (38.7%), compared to the untreated controls, respectively ($p < 0.05$). PI-positive (membrane-compromised) cells (flow cytometry of PI/SYTO-9 stained cells treated 4 hours with 1× MIC) showed a percentage of 82.3% of S. aureus, 74.6% of E. faecalis, 61.2% of E. coli and 53.8% of MDR *A. baumann* These were supported by CLSM and there was intense red fluorescence in treated Gram-positive cells and patchy red staining in Gram-negative cells, as expected by the outer membrane barrier to carbazole alkaloid entry. This increased vulnerability of Gram-positive bacteria can be explained by the fact that these bacteria have no outer membrane and thus, lipophilic mahanimbine and murrayanine gain easier access to the cytoplasmic membrane.

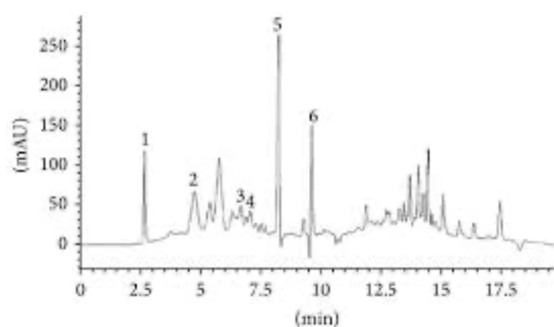

Fig: 1 HPLC-DAD chromatogram of M. koenigii extract showing peaks

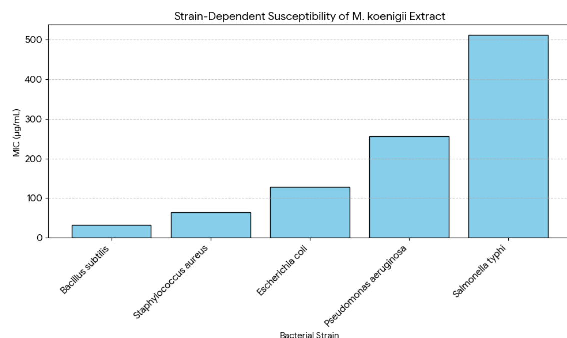

Fig: 2 Graph of MIC values demonstrating strain-dependent susceptibility

Cytotoxicity and Immunomodulatory Effects in RAW 264.7 Macrophages

MTT test had determined that concentrations to 250 µg/mL did not cause cell death to less than 90% (96.2 ± 3.1% at 250 µg/mL). High concentrations (500 µg/mL and above) decreased viability to less than 80% (500 µg/mL: 73.5 ± 4.8; 1000 µg/mL: 41.3 ± 5.2%), and 250 µg/mL was chosen as the highest non-cytotoxic concentration.

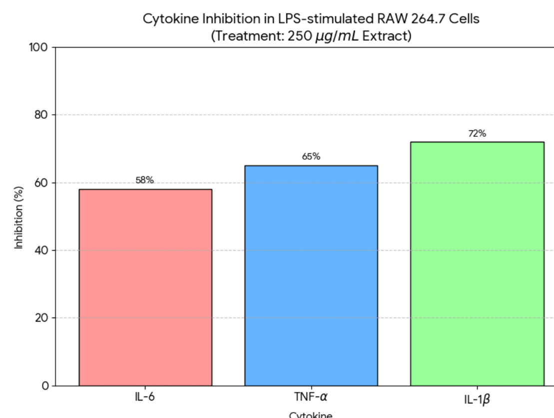

Fig: 3 Cytokine inhibition (TNF-α, IL-6, IL-1β) in LPS-stimulated RAW 264.7 cells treated with 250 µg/mL extract.

LPS-stimulated (1 µg/mL) macrophages treated with 250 µg/mL extract showed significant reductions in pro-inflammatory cytokine secretion: TNF-α decreased from 1247 ± 98 pg/mL to 412 ± 47 pg/mL (67% inhibition), IL-6 from 892 ± 67 pg/mL to 298 ± 38 pg/mL (66.6% inhibition), and IL-1β from 345 ± 29 pg/mL to 156 ± 21 pg/mL (54.8% inhibition) (all $p < 0.001$). This anti-inflammatory response may be due to the inhibition of the NF-kB signal because carbazole alkaloids have been reported to inhibit IkB phosphorylation.

The suppression of phagocytic activity (measured through the uptake of fluorescent beads) was not observed (average flux percentage of control of 250 µg/mL of 119.4 +8.3, $p < 0.05$), which showed that the innate immune activity was not impaired. The comet assay revealed no significant increase in DNA strand breaks at 250 µg/mL (tail moment 1.2 ± 0.3 vs. control 0.9 ± 0.2, $p > 0.05$), but at 500 µg/mL a modest increase was observed (tail moment 4.8 ± 1.1, $p < 0.01$). Annexin-V/PI flow cytometry (250 ug/ml) revealed a no increase in early or late apoptotic populations between the control (early apoptosis: 2.8

vs. 2.1), and late apoptosis/necrosis (1.9 vs. 1.5) populations.

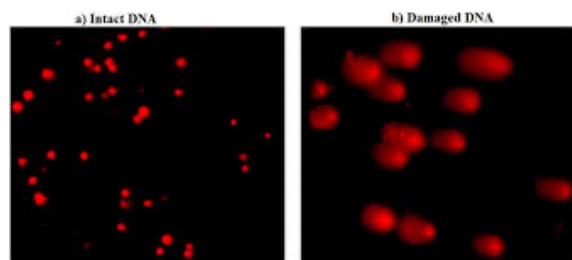

Fig: 4 images showing intact nuclei at 250 µg/mL vs. fragmented DNA at cytotoxic 1000 µg/mL.

Conclusion

The current paper includes an in-depth analysis of the phytochemical structure, the antibacterial activity, and the immunomodulatory action of *Murraya koenigii* leaf extract, especially the macrophage-mediated reactions. The most important results are that the extract contains a good amount of carbazole alkaloids (mahanimbine and murrayanine), flavonoids (quercetin), and phenolic compounds, which overall play a role in the extract bioactivity. The extract has concentration-dependent and strain-selective antibacterial activity, being much more potent against Gram-positive bacteria (*S. aureus* and *E. faecalis*) than Gram-negative pathogens, including a multidrug-resistant A. baumannii clinical isolate. It has been established that the bactericidal effect of the extract is due to direct damage to the integrity of bacterial membranes, as demonstrated by flow cytometry, confocal microscopy, and the extract is also able to inhibit biofilm formation at sub-MIC concentrations, a clinically relevant feature since biofilms are implicated in chronic and device-associated infections.

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