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Isolation, Characterization, and β-Cyclodextrin Nanosponge Formulation of Ochnaflavone from Ochna Obtusata for Enhanced Antioxidant Activity and Improved Drug Delivery

Anusha Reddy Devireddy[1], Dr. A. Vijaya Lakshmi[2]*

[1]Department of Pharmaceutical Chemistry, School of Pharmaceutical Sciences, Vels Institute of Science, Technology and Advanced Studies (VISTAS), Chennai- 600117, Tamil Nadu, India.
[2]Professor, Department of Pharmacognosy, School of Pharmaceutical Sciences, Vels Institute of Science, Technology and Advanced Studies (VISTAS), Chennai- 600117, Tamil Nadu, India.
Email: anu995135@gmail.com

ABSTRACT: Ochnaflavone, a biflavonoid isolated from the leaves of Ochna obtusata, was evaluated for its antioxidant activity and formulated into nanosponges to improve its solubility and bioavailability for potential Alzheimer's disease therapy. The compound was characterized using HPTLC, FTIR, and NMR techniques and demonstrated strong antioxidant activity in DPPH and H_2O_2 scavenging assays. Among the developed formulations, Trial 4 showed the best performance with high drug encapsulation efficiency (86.6%), a particle size of 193.2 nm, sustained drug release, and good stability. These findings suggest that Ochnaflavone-loaded nanosponges are a promising drug delivery system for future Alzheimer's disease treatment

1. INTRODUCTION

Natural products remain an important source of bioactive molecules for pharmaceutical research and drug discovery. Among these compounds, flavonoids have attracted considerable scientific interest because of their diverse pharmacological properties, including antioxidant, anti-inflammatory, antimicrobial, anticancer, and neuroprotective activities. Their ability to scavenge reactive oxygen species and regulate multiple cellular signalling pathways has made them promising candidates for the prevention and management of chronic disorders associated with oxidative stress.

Oxidative stress is recognized as one of the major contributors to the development of several chronic diseases, including cardiovascular disorders, diabetes mellitus, cancer, and neurodegenerative diseases such as Alzheimer's disease. Excessive production of reactive oxygen species causes cellular damage through lipid peroxidation, protein oxidation, DNA damage, and mitochondrial dysfunction. Consequently, considerable attention has been directed toward identifying naturally occurring antioxidants capable of reducing oxidative damage and improving disease outcomes. Flavonoids are particularly attractive because they possess multiple hydroxyl groups that enable efficient free radical scavenging while also modulating inflammatory and apoptotic pathways.

Ochna obtusata *(*Family: Ochnaceae*)* is a medicinal plant traditionally used in different regions of India for treating various ailments. Different parts of the plant have been employed in traditional medicine for inflammatory disorders, bronchitis, asthma, ulcers, fever, gastrointestinal diseases, and other conditions. Previous phytochemical investigations have demonstrated that the plant contains several biologically active constituents, including flavonoids, biflavonoids, terpenoids, phenolics,

glycosides, steroids, and saponins. These phytochemicals are considered responsible for many of the pharmacological properties associated with the plant(1-4).

Among the reported constituents, Ochnaflavone is a naturally occurring biflavonoid that has attracted increasing research interest because of its diverse biological activities. Earlier investigations have demonstrated anti-inflammatory, antimicrobial, antioxidant, and other pharmacological effects, indicating its potential as a lead compound for future therapeutic development. However, despite these promising biological activities, comprehensive characterization and formulation strategies intended to improve its pharmaceutical performance remain limited(5-8).

One of the major challenges associated with many flavonoids is their poor aqueous solubility, which may limit dissolution, absorption, and overall bioavailability following oral administration. These physicochemical limitations often reduce therapeutic efficacy despite promising pharmacological activity(9-11). Therefore, the development of suitable drug delivery systems capable of enhancing solubility and providing controlled drug release has become an important area of pharmaceutical research. Nanotechnology-based drug delivery approaches have emerged as effective strategies for improving the performance of poorly water-soluble phytoconstituents(12).

β-Cyclodextrin nanosponges represent a promising delivery platform because of their porous three-dimensional polymeric structure, which allows efficient encapsulation of hydrophobic molecules. These nanosponges can improve drug solubility, protect sensitive molecules from degradation, provide sustained drug release, and potentially enhance bioavailability. Their favorable physicochemical properties make them suitable carriers for natural compounds such as Ochnaflavone(13).

The present study was therefore designed to isolate Ochnaflavone from the leaves of Ochna obtusata, characterize the isolated compound using chromatographic and spectroscopic techniques, evaluate its in vitro antioxidant activity, and develop β-cyclodextrin nanosponge formulations to improve its pharmaceutical properties. The optimized formulation was further evaluated for particle size, zeta potential, drug encapsulation efficiency, and in vitro drug release to determine its suitability as a controlled drug delivery system(14-16).

2. MATERIALS AND METHODS

2.1 Plant Material

Fresh leaves of Ochna obtusata were collected from their natural habitat and authenticated by a qualified botanist. The collected leaves were washed thoroughly with distilled water to remove dust and other foreign matter. The plant material was shade-dried at room temperature until a constant weight was obtained and then pulverized using a mechanical grinder. The powdered material was stored in an airtight container until further use.

2.2 Extraction of Plant Material

The dried leaf powder was initially defatted using petroleum ether in a Soxhlet apparatus to remove fatty substances and chlorophyll. The defatted marc was subsequently extracted with acetone until complete exhaustion. The acetone extract was concentrated under reduced pressure using a rotary vacuum evaporator to obtain a semi-solid mass. The extract was dried, weighed, and stored in a desiccator for further phytochemical and formulation studies. The percentage yield of the extract was calculated based on the weight of the dried extract relative to the initial plant material(11).

2.3 Isolation of Ochnaflavone

Isolation of Ochnaflavone was carried out using silica gel column chromatography. The concentrated acetone extract was adsorbed onto silica gel and carefully packed onto the chromatography column. Elution was performed using solvent systems of increasing polarity. Fractions were collected systematically and monitored by thin-layer chromatography (TLC). Fractions exhibiting similar chromatographic profiles were pooled and concentrated. Repeated chromatographic purification yielded the isolated compound, which was further characterized using chromatographic and spectroscopic techniques.

2.4 Thin-Layer Chromatography (TLC)

Thin-layer chromatography was employed to monitor the progress of isolation and evaluate the purity of fractions. Samples were spotted onto silica gel TLC plates and developed in an optimized mobile phase. After development, chromatograms were visualized under ultraviolet light at 254 nm and 366 nm. The retention factor (Rf) values were determined and compared to identify fractions containing the target compound.

2.5 High-Performance Thin-Layer Chromatography (HPTLC)

The purified compound was further analyzed using High-Performance Thin-Layer Chromatography (HPTLC). Samples were applied as narrow bands on precoated silica gel plates using an automatic sample applicator. Plates were developed in a saturated chamber using the optimized mobile phase and scanned using a densitometric scanner at the specified wavelength. Chromatographic fingerprinting confirmed the purity of the isolated Ochnaflavone through the presence of a single well-defined peak(12).

2.6 Structural Characterization

Structural elucidation of the isolated compound was performed using spectroscopic techniques. Fourier Transform Infrared (FTIR) spectroscopy was employed to identify characteristic functional groups present in the molecule. Proton Nuclear Magnetic Resonance (^1H NMR) spectroscopy was used to determine the hydrogen environment, whereas Carbon-13 Nuclear Magnetic Resonance (^13C NMR) spectroscopy confirmed the carbon skeleton of the isolated compound. The obtained spectral data were compared with reported literature values for confirmation of Ochnaflavone.

2.7 Evaluation of Antioxidant Activity

DPPH Radical Scavenging Assay

The antioxidant activity of the isolated Ochnaflavone was evaluated using the DPPH free radical scavenging method. Different concentrations of the compound were prepared and mixed with DPPH solution. After incubation under dark conditions, the absorbance was measured using a UV–Visible spectrophotometer. The percentage inhibition of DPPH radicals was calculated using the standard equation(18).

Hydrogen Peroxide Scavenging Assay

Hydrogen peroxide scavenging activity was evaluated using different concentrations of the isolated compound. Appropriate reaction mixtures were incubated for the prescribed duration, and absorbance was measured spectrophotometrically. The percentage scavenging activity was calculated relative to the control solution(17).

2.8 Preformulation Studies

Preformulation investigations were conducted to evaluate the physicochemical properties of the isolated Ochnaflavone before formulation development.

Solubility Study: Solubility was determined in different solvents to assess the dissolution characteristics of the isolated compound.

Partition Coefficient (Log P): The partition coefficient between n-octanol and water was determined to evaluate lipophilicity.

Melting Point Determination: The melting point was determined using the capillary method to assess purity and thermal characteristics of the isolated compound.

2.9 Preparation of β-Cyclodextrin Nanosponges

β-Cyclodextrin nanosponges were prepared using the solvent evaporation method. β-Cyclodextrin and the selected cross-linking agent were dissolved in a suitable organic solvent under continuous stirring. Cross-linking was allowed to proceed under controlled temperature conditions. The resulting polymeric network was purified by repeated washing to remove unreacted materials and then dried under vacuum. The optimized nanosponge formulation was loaded with the isolated Ochnaflavone to improve drug encapsulation and release characteristics. Multiple trial formulations were prepared by varying the drug-to-polymer ratio to optimize formulation performance.

2.10 Characterization of Nanosponges

The developed nanosponges were evaluated for the following parameters:

Particle Size: Determined using dynamic light scattering to assess nanoparticle size distribution.

Zeta Potential: Measured to evaluate colloidal stability.

Drug Encapsulation Efficiency: Calculated by estimating the amount of drug entrapped within the nanosponges.

In Vitro Drug Release: Drug release studies were performed using a suitable dissolution medium under controlled experimental conditions. Samples were withdrawn at predetermined intervals, and the released drug was quantified spectrophotometrically(17).

2.11 Statistical Analysis

All experiments were performed in triplicate, and the results were expressed as mean ± standard deviation. Appropriate statistical methods were employed to evaluate experimental data, and graphical representation of the results was prepared using suitable software.

3. RESULTS AND DISCUSSION

3.1 Isolation of Ochnaflavone

The acetone extract of Ochna obtusata leaves was subjected to silica gel column chromatography to isolate the major biflavonoid constituent. Fractions obtained during elution were monitored by TLC, and fractions exhibiting similar chromatographic patterns were pooled. Repeated purification yielded a yellow crystalline compound that showed a single spot on TLC, indicating satisfactory purity for further characterization. The chromatographic behavior was consistent with the characteristics reported for Ochnaflavone in previous phytochemical investigations.

3.2 HPTLC Analysis

HPTLC fingerprinting confirmed the successful isolation of Ochnaflavone. The chromatogram exhibited a single sharp peak, demonstrating the chromatographic purity of the isolated compound. The corresponding densitogram further supported the absence of significant impurities, indicating that the purification procedure was effective.

The HPTLC profile established a reproducible fingerprint for the isolated compound and can serve as a quality control marker for future studies involving *Ochna obtusata*.

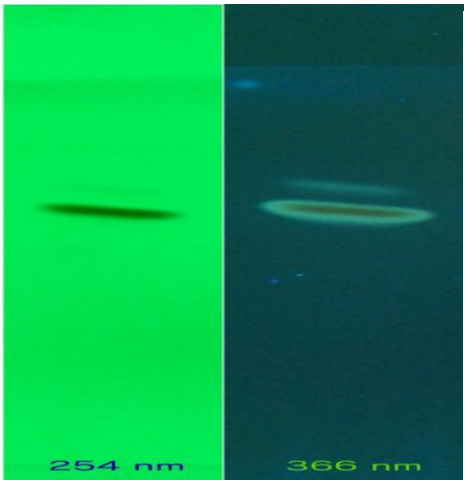

Figure 1: HPTLC plates at 254 and 366nm

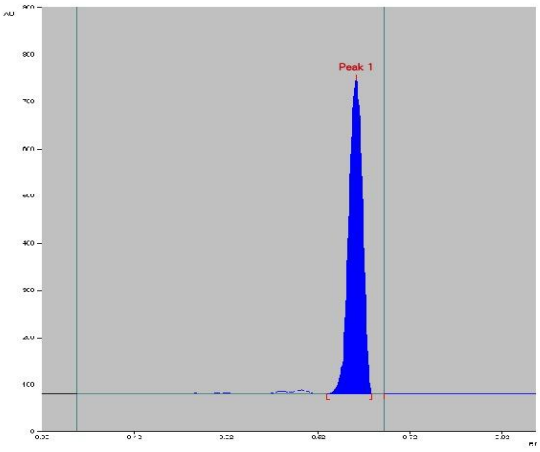

Figure 2: HPTLC chromatogram of sample-1

Table 1: Rf Values of Sample-1 In Hptlc Analysis

Peak	Start Rf	Start Height	Max Rf	Max Height	Height %	End Rf	End Height	Area	Area %	Assigned Substance
1	0.54	0.1	0.61	666.7	100.00	0.64	0.9	14867.3	100.00	Unknown*

3.3 FTIR Characterization

FTIR spectroscopy was employed to identify the characteristic functional groups present in the isolated compound. The obtained spectrum showed absorption bands corresponding to hydroxyl groups, aromatic carbon-carbon stretching, conjugated carbonyl groups, and aromatic ether linkages, confirming the flavonoid skeleton of Ochnaflavone.

The observed spectral pattern was consistent with published literature, thereby confirming the successful isolation of the desired biflavonoid.

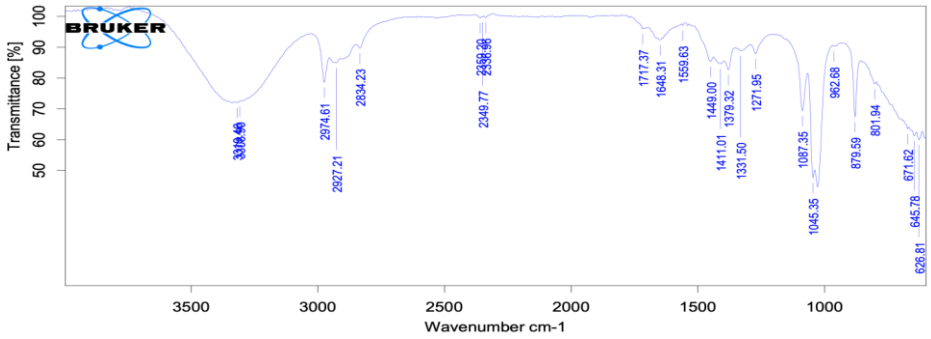

Figure 3: FTIR analysis of isolated compound

3.4 Nuclear Magnetic Resonance Studies

3.4.1 ^{1}H NMR Analysis

The proton NMR spectrum displayed characteristic aromatic proton signals expected for the biflavonoid structure of Ochnaflavone. Hydroxyl proton signals together with aromatic proton resonances confirmed the presence of substituted flavone rings.

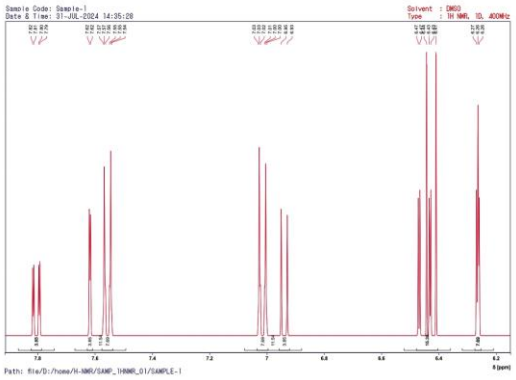

Figure 4: 13C-NMR graph of isolated compound

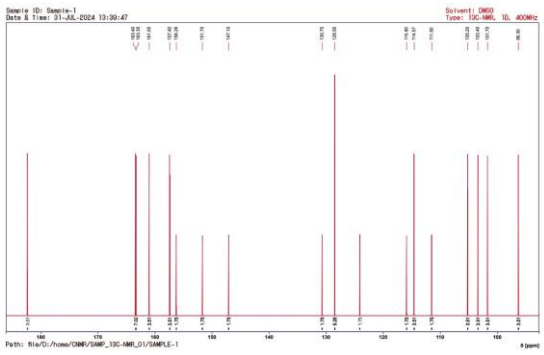

Figure 5: 1H-NMR graph of isolated compound

Table 2. Results of ^{1}H-NMR and ^{13}C-NMR analysis

Atom IDs	13C-NMR δ (ppm)	Atoms	1H-NMR δ (ppm)	No.of Atoms	Multiplicity	CouplingsJ (Hz)
10	124.1	d	-	-	-	-
11	163.4	d	-	-	-	-
12	103.4	d	-	-	-	-
13	157.4	d	-	-	-	-
14	162.7	d	-	-	-	-
15	137.77	d	-	-	-	-
16	130.7	d	-	-	-	-
17	154.4	d	-	-	-	-
18	111.5	d	7.618	1	dd	1.69, 0.44
19	151.7	d	-	-	-	-
20	105.2	d	6.41	1	s	-
21	182.34	d	-	-	-	-
22	73.22	d	6.443	1	s	-
23	182.34	s	-	-	-	-
24	156.26	d	-	-	-	-
25	163.3	d	-	-	-	-
26	128.5	d	7.805	1	dd	8.41, 1.69
27,28	128.5	dd	7.565	2	ddd	8.65, 1.84, 0.51
29	153.4	d	-	-	-	-
30	96.3	d	6.431	1	d	1.96
31	147.1	d	-	-	-	-
32	96.3	d	6.43	1	d	1.97
33,34	114.57	dd	7.016	2	ddd	8.65, 1.28, 0.51
35	115.9	d	6.94	1	dd	8.41, 0.44
36	161	d	-	-	-	-
37	101.7	d	6.262	1	d	1.96
38	161	d	-	-	-	-
39	101.7	d	6.262	1	d	1.97

3.4.2 ^{13}C NMR Analysis

The carbon NMR spectrum demonstrated signals corresponding to aromatic carbons, oxygenated aromatic carbons, and carbonyl carbons typical of biflavonoids. The observed chemical shifts agreed well with previously reported spectral data for Ochnaflavone, confirming the structural identity of the isolated compound.

3.5 Antioxidant Activity

3.5.1 DPPH Radical Scavenging Activity

The isolated Ochnaflavone exhibited concentration-dependent free radical scavenging activity against DPPH radicals. Radical inhibition increased progressively with increasing concentration, indicating effective antioxidant potential.

The maximum scavenging activity observed was 98.46% at 100 µg/mL**,** demonstrating excellent antioxidant capacity. This high level of inhibition is attributed to the multiple phenolic hydroxyl groups present in the biflavonoid structure, which readily donate hydrogen atoms to neutralize free radicals.

Table.3: DPPH Scavenging Assay

Concentration (µg/mL)	% Scavenging DPPH of Isolated Compound	% Scavenging DPPH of Standard Ascorbic Acid
20	57.057 ± 0.419	55.080 ± 0.045
40	71.30 ± 0.042	69.52 ± 0.203
60	83.13 ± 0.012	81.193 ± 0.037
80	91.25 ± 0.018	89.49 ± 0.032
100	98.46 ± 0.022	94.13 ± 0.015

The antioxidant activity observed in this study agrees with previous reports describing biflavonoids as potent natural antioxidants. Their conjugated aromatic system enhances electron donation, allowing stabilization of reactive oxygen species and minimizing oxidative damage.

3.5.2 Hydrogen Peroxide Scavenging Activity

The isolated compound also demonstrated significant hydrogen peroxide scavenging activity. The percentage inhibition increased with concentration throughout the experimental range.

The highest scavenging activity was 91.19% at 50 µg/mL, confirming the ability of Ochnaflavone to neutralize reactive oxygen species effectively.

Table.4: H2O2 Scavenging Assay

Concentration (µg/mL)	% Scavenging H_2O_2 of Isolated Compound	% Scavenging H_2O_2 of Standard Ascorbic Acid
10	32.180 ± 0.035	30.71 ± 0.304
20	45.69 ± 0.050	43.16 ± 0.028
30	61.20 ± 0.098	59.50 ± 0.032
40	71.26 ± 0.022	68.37 ± 0.032
50	91.19 ± 0.039	89.077 ± 0.086

Hydrogen peroxide is an important reactive oxygen species capable of generating highly reactive hydroxyl radicals. Efficient scavenging of hydrogen peroxide reduces oxidative stress and may contribute to protection against cellular damage. The strong activity observed further supports the therapeutic potential of Ochnaflavone.

3.6 Pre-formulation Studies

Solubility

The isolated Ochnaflavone exhibited poor aqueous solubility but showed improved solubility in organic solvents. This finding is typical of biflavonoids and highlights one of the principal challenges limiting their pharmaceutical application.

Poor water solubility can reduce dissolution and oral bioavailability, making formulation development essential.

Partition Coefficient

The Log P value was found to be 2.8, indicating moderate lipophilicity. This value suggests that the compound possesses sufficient membrane permeability while maintaining limited aqueous solubility, reinforcing the need for a suitable drug delivery system.

Melting Point

The isolated compound exhibited a melting point of 286°C, indicating high purity and excellent thermal stability. The observed melting point corresponded well with reported values for Ochnaflavone and further confirmed its successful isolation.

3.7 Development of β-Cyclodextrin Nanosponges

Multiple trial formulations of β-cyclodextrin nanosponges were prepared by varying formulation parameters to optimize particle characteristics and drug loading. Comparative evaluation demonstrated differences in particle size, encapsulation efficiency, and drug release among the formulations.

Among all batches, **Trial 4** exhibited the most desirable physicochemical characteristics and was therefore selected as the optimized formulation for further evaluation.

Table.5: Trial Formulations for Ochnaflavone Nanosponge

Trial No.	Ochnaflavone (%)	β-Cyclodextrin (%)	CDI (%)	Calcium Carbonate (%)	CMC (%)	DMF (mL)	Ethanol (mL)	Poloxamer 188 (%)	Solvent Evaporation Temp (°C)
1	2.0	2.5	1.0	0.5	0.5	5	10	0.1	40
2	2.0	3.0	1.5	0.5	0.5	6	12	0.2	40
3	2.0	2.0	1.0	0.3	0.3	5	8	0.2	40
4	2.5	3.5	1.0	0.6	0.4	5	10	0.15	45
5	2.0	3.0	1.2	0.4	0.6	6	10	0.1	40
6	1.5	2.5	1.5	0.5	0.5	5	12	0.25	45
7	2.0	4.0	1.0	0.4	0.5	7	15	0.2	40
8	2.5	2.0	0.8	0.3	0.6	5	10	0.15	40
9	2.0	3.5	1.0	0.5	0.4	6	10	0.1	45

3.8 Particle Size Analysis

Particle size is an important parameter influencing drug dissolution, cellular uptake, and formulation stability.

The optimized formulation exhibited an average particle size of: 193.2 ± 3.4 nm

The nanoscale particle size is advantageous because it provides a larger surface area, facilitating enhanced dissolution and potentially improving oral absorption.

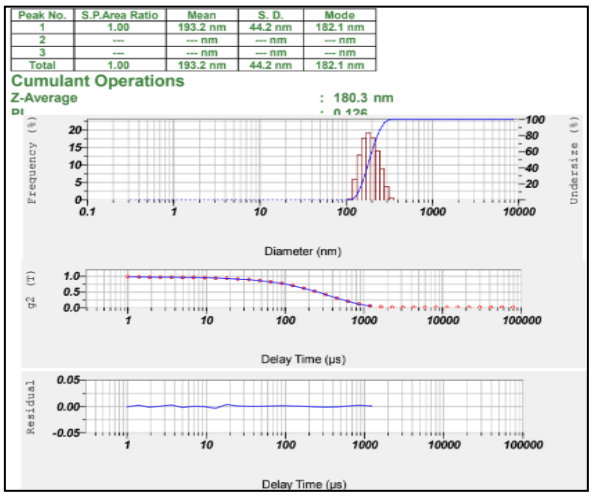

Figure 6-Particle size distribution of optimized Ochnaflavone nanosponge formulation

3.9 Zeta Potential

The optimized formulation showed a zeta potential of −23.8 ± 1.4 mV**.**

This negative surface charge indicates good electrostatic stabilization of the nanosponge dispersion, reducing particle aggregation and improving storage stability.

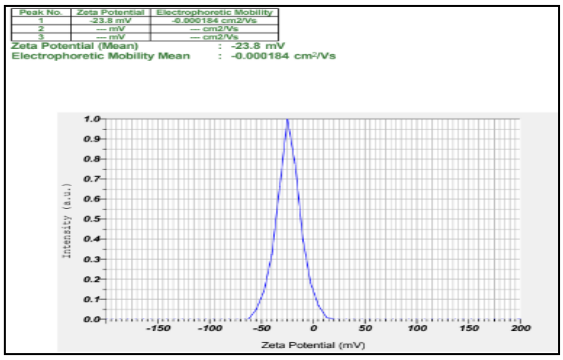

Figure 7-Zeta potential of optimized Ochnaflavone nanosponge formulation

3.10 Drug Encapsulation Efficiency

The β-cyclodextrin nanosponges efficiently entrapped Ochnaflavone within their porous polymeric matrix. The optimized formulation exhibited an encapsulation efficiency of: 86.6 ± 3.4%

The high encapsulation efficiency demonstrates the suitability of β-cyclodextrin nanosponges for incorporating poorly soluble biflavonoids.

3.11 In Vitro Drug Release

The optimized nanosponge formulation exhibited sustained release characteristics throughout the dissolution study. A cumulative drug release of: **58.4 ± 4.2% after 8 hours** was achieved.

Compared with the pure drug, the nanosponge formulation provided a slower and more controlled release profile, suggesting prolonged drug availability and potential improvement in therapeutic efficacy.

Table.6: Characterization Of Ochnaflavone Nanosponge

Trial No.	Particle Size (nm)	Drug Encapsulation Efficiency (%)	In Vitro Drug Release % (8 hrs)
1	213.4 ± 3.4	78.6 ± 2.4	33.4 ± 2.6
2	256.8 ± 6.4	65.8 ± 1.5	39.6 ± 2.2
3	222.9 ± 5.2	56.6 ± 2.6	44.2 ± 2.1
4	193.2 ± 3.4	86.6 ± 3.4	58.4 ± 4.2
5	434.5 ± 6.2	48.8 ± 2.8	34.2 ± 2.6
6	320.8 ± 3.8	50.9 ± 3.6	44.4 ± 3.8

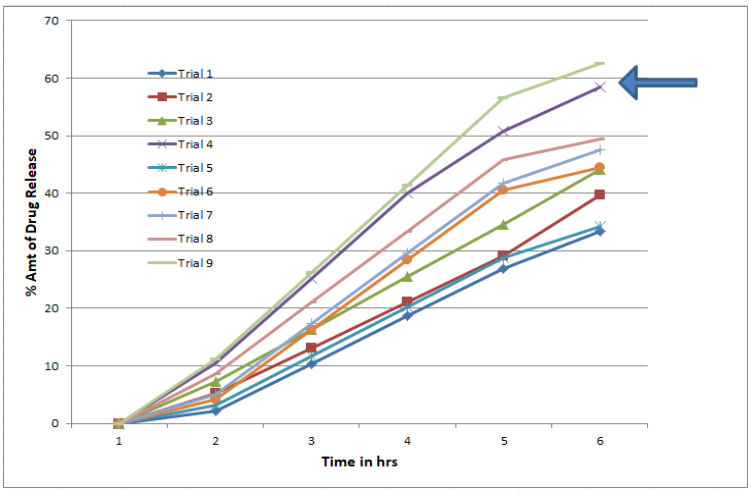

Figure 8- In vitro cumulative drug release profile of Ochnaflavone nanosponge formulation

3.12 Overall Discussion

The present investigation successfully isolated and characterized Ochnaflavone from Ochna obtusata. Chromatographic and spectroscopic analyses confirmed the identity and purity of the isolated biflavonoid. Antioxidant assays demonstrated strong free radical scavenging activity, supporting the biological potential of the compound.

Preformulation studies identified poor aqueous solubility as the primary limitation for pharmaceutical application. To overcome this limitation, β-cyclodextrin nanosponges were successfully developed. The optimized formulation showed nanoscale particle size, favorable zeta potential, high encapsulation efficiency, and sustained drug release, indicating that the nanosponge system is an effective carrier for Ochnaflavone. Overall, the formulation strategy improved the physicochemical characteristics of the isolated compound while maintaining its antioxidant potential.

4. CONCLUSION

The present study successfully isolated and characterized Ochnaflavone from the leaves of Ochna obtusata using chromatographic and spectroscopic techniques. TLC, HPTLC, FTIR, and NMR analyses confirmed the identity and purity of the isolated biflavonoid. The compound demonstrated significant antioxidant potential in both DPPH and hydrogen peroxide scavenging assays, indicating its ability to neutralize reactive oxygen species and its promise as a natural antioxidant.

Preformulation studies revealed poor aqueous solubility and moderate lipophilicity of Ochnaflavone, highlighting the need for an appropriate drug delivery system. To overcome these limitations, β-cyclodextrin nanosponges were successfully prepared and evaluated. Among the developed formulations, the optimized formulation exhibited a particle size of 193.2 ± 3.4 nm, a zeta potential of −23.8 ± 1.4 mV, an encapsulation efficiency of 86.6 ± 3.4%**,** and sustained drug release of 58.4 ± 4.2% over

8 hours. These findings indicate that β-cyclodextrin nanosponges are an effective carrier system for improving the physicochemical performance of Ochnaflavone.

Overall, this work provides a scientific basis for the development of nanosponge-based delivery systems for poorly soluble natural flavonoids. Future studies involving in vivo pharmacokinetic evaluation, pharmacodynamic assessment, and long-term stability testing are recommended to further establish the therapeutic potential of the developed formulation.

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