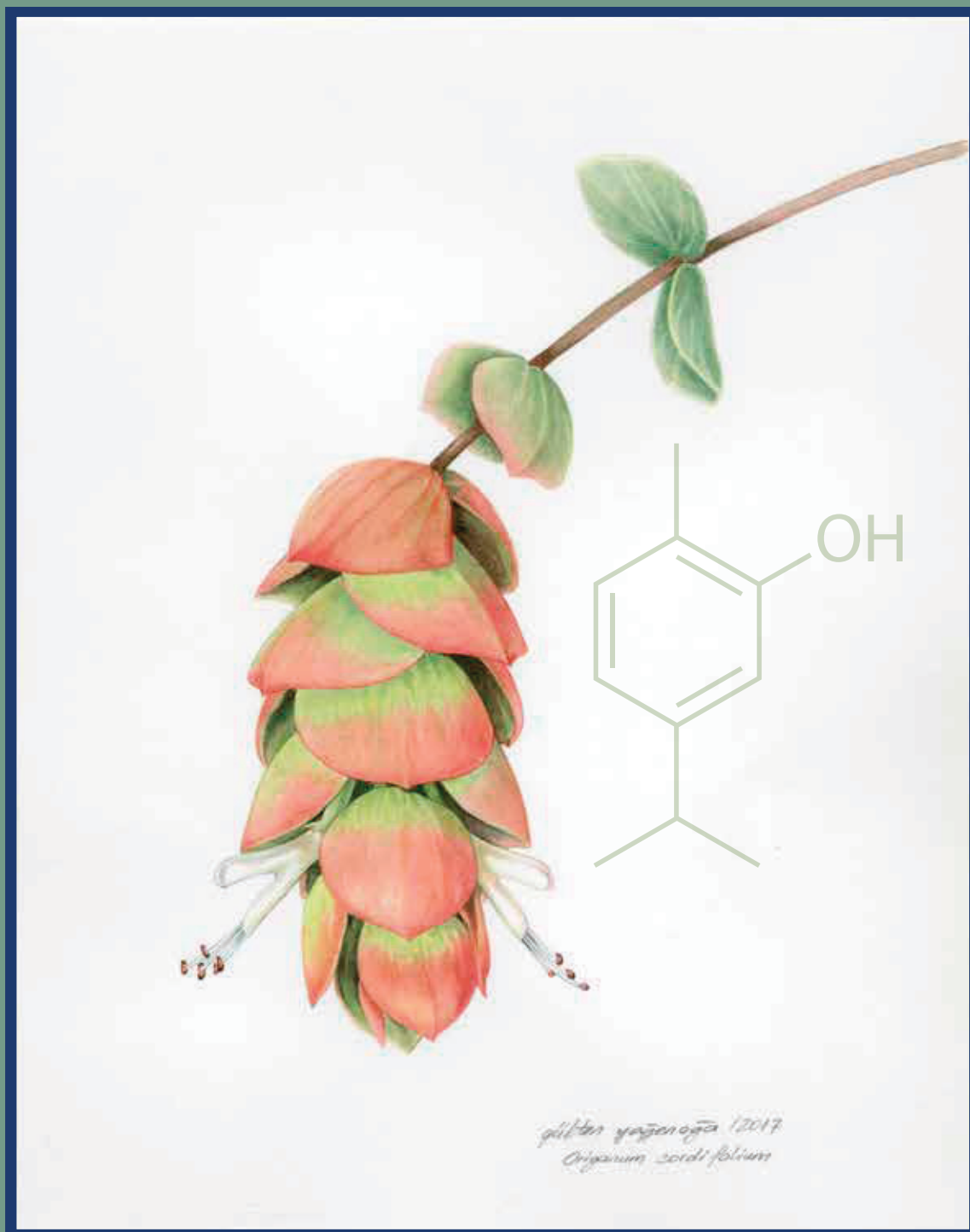


September 2026



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MESSAGE FROM THE EDITOR IN CHIEF

Dear Scientists,

It is our pleasure to introduce you to the 2nd issue of 2026 for EMU Journal of Pharmaceutical Sciences. We have had the joy of receiving scientific manuscript submissions from many parts of the world.

Mainly, a formulation study on vonoprazan tablet preparation, synthesis and biological activity investigation of novel N-(Aryl)-3-(N-benzylsulfamoyl)benzamides, hepatoprotective effects of linalool on hepatic injury, and a phytochemical Study on the fruits of *Vitex agnus-castus* L. have constituted this issue's research manuscripts. Moreover, a review on coumarin-piperazine hybrid drug design is expected to have attention of scientists in the relevant field. We extend our gratitude to the researchers who shared their valuable scientific work with us.

As a member of 'DergiPark' Akademik, an establishment under the Scientific and Technological Research Council of Türkiye (TÜBİTAK), EMU Journal of Pharmaceutical Sciences continues its journey with a transparent peer-review and publication process of scientific studies in diverse fields related to pharmaceutical sciences. The journal will continue to serve and promote global dissemination of pharmaceutical research, providing a platform for scientists worldwide. It is important to note that the journal is free of submission or acceptance fee.

Looking forward to your scientific contributions,

Best wishes,

Prof. Dr. H. Ozan Gülcan

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Formulation Development and In-Vitro-In-Vivo Correlation (IVIVC) Level A of Vonoprazan Tablets (20 mg) Using a Convolution-Based Method

Bijay Bhattarai¹, Uttam Budhathoki^{1*}, Panna Thapa¹

¹Kathmandu University, Department of Pharmacy, School of Science, Dhulikhel, Nepal.

Abstract

The present study aimed to develop a 20 mg Vonoprazan tablet formulation and a convolution-based Level A IVIVC model was developed using published in-vivo data. Various hits and trials were done to obtain desired release profiles by changing concentration of super disintegrants, hardness of tablets and dissolution Speeds. High-performance Liquid Chromatography (HPLC) was used to analyze drug concentration. Pharmacokinetic parameters such as Maximum Plasma Drug Concentration (C_{max}), Time to reach maximum concentration (T_{max}) and Area Under Curve (AUC) were predicted using convolution method based on in-vitro data. The predicted pharmacokinetic parameters: C_{max} = 16.61 ng/ml, T_{max} = 1.98 hr, and AUC_{0-∞} = 187.14 ng·h/ml showed close agreement with observed values C_{max} = 18.2 ng/ml, T_{max} = 1.98 hr, AUC₀₋₂₄ = 160.8 ng·h/ml, AUC_{0-∞} = 179.3 ng·h/ml. The %PE for C_{max} is 8.74%, AUC_{0-∞} is 4.37% and T_{max} is 1.0 %. These results demonstrate acceptable correlation for all pharmacokinetics parameters indicating that the developed model has good predictive performance. The developed formulation demonstrated a close correlation between in vitro dissolution and in-vivo absorption profiles, confirming the predictability of the model. This study supports the potential of convolution-based modeling as a reliable approach for formulation optimization and regulatory biowaiver justification.

Keywords

Convolution, dissolution, pharmacokinetics, IVIVC, vonoprazan fumarate.

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INTRODUCTION

The development of robust, biopharmaceutically sound oral dosage forms is a fundamental objective of pharmaceutical sciences, requiring not only the optimization of formulation and process variables but also a clear understanding of how in vitro performance relates to in vivo behavior.

Vonoprazan fumarate is a novel developed potassium-competitive acid blocker (P-CAB). It was developed as an alternative to a substitute to proton pump inhibitors (PPIs). Vonoprazan is used for treating conditions such as gastroesophageal reflux disease (GERD) and peptic ulcer disease, both of which are acid-related disorders. Unlike PPIs, which require an acidic environment for activation and irreversibly inhibit block the H^+/K^+ -ATPase, vonoprazan inhibits the gastric proton pump reversibly and competitively. This results in rapid, potent, and sustained acid suppression (Echizen, 2016). It acts rapidly and consistently to reduce gastric acid secretion. It has more predictable absorption and less variation than proton pump inhibitors (PPIs). Clinically practice, 20 mg of vonoprazan is commonly used for the treatment of gastroesophageal reflux disease and *Helicobacter pylori* infection (Vinayagam et al. 2024). The fumarate salt used in vonoprazan tablets improves the physicochemical stability of the drug and improves its dissolution properties—two key factors influencing formulation performance

and bioavailability. Vonoprazan is stable in an acidic environment, so an an enteric coating is unnecessary to protect the molecule in the acidic environment of the stomach (Sachs et al. 2007; Shin and Sachs, 2009). Binding to the proton pump, Vonoprazan does not require acid activation (Savarino et al. 2016).

Vonoprazan is a basic pyrrole derivative characterized by preferential accumulation in gastric parietal cells and stability in acidic environments (Echizen, 2016). It changes ionically and binds reversibly with the gastric proton pumps of stomach (Horiet et al. 2010; Oshima and Miwa, 2018). Studies have shown it has high membrane permeability but relatively low solubility in water (Liqui et al. 2017) indicating that dissolution may represent a rate-limiting step for absorption in specific formulations (Takeda, 2026) Vonoprazan is absorbed rapidly following oral administration and reaches peak concentration within 2 hours after once-daily administration (Savarino et al. 2016; Jenkins et al. 2015). The elimination half-life ($t_{1/2}$) for vonoprazan ranges from 7 to 8 hours. The steady-state plasma concentration is achieved within 3 to 4 days with once daily dosing (Echizen, 2016; Otake et al. 2016). Vonoprazan is primarily metabolized by cytochrome P450 (CYP) 3A4, CYP2B6, CYP2C19, and CYP2D6, that resulting in pharmacologically inactive metabolites, designated M-I, M II, and M-III (Echizen, 2016; Sugano, 2018).

Various manufacturing methods, including direct compression, wet granulation and dry granulation are employed in the manufacturing of tablets. The innovator tablet was a film-coated tablet with excipients as D-mannitol, microcrystalline cellulose, croscarmellose sodium, hydroxypropyl cellulose, fumaric acid, and magnesium stearate (Takeda, 2026). The finished product should comply with the Pharmacopeial specifications like including disintegration time, friability, weight variation, assay and dissolution.

According to the 1997 FDA IVIVC Guidance from 1997, dissolution testing should be performed in biorelevant media that replicate gastrointestinal (GI) conditions, specifically at pH 1.2 (gastric), pH 4.5 (acetate buffer), and phosphate buffer pH 6.8 (Taha and Emara, 2022).

An in vitro – in vivo correlation (IVIVC) is defined by the U.S Food and Drug Administration (FDA) as a predictive mathematical model describing the relationship between the In-vitro property of an oral dosage form and relevant In-vivo response (Taha and Emara, 2022). The FDA has defined four levels of IVIVC: Level A, Level B, Level C, and multiple Level C (Taha and Emara, 2022). Among these, Level A correlation is considered the most robust and regulatory-relevant. It allows for direct prediction of plasma concentration-time profiles, thus making it the preferred level in both industrial development and academic

research (Nainar et al. 2012). The drug development process of developing drugs is time-consuming and costly. A significant reason for failures during the clinical trials of drug development is insufficient pharmacokinetic information data sufficient for the drug candidates; thus, the ability to predict the pharmacokinetic properties of these drug candidates in advance would be beneficial. One key challenge in making such predictions is the difficulty in accurately scaling in-vitro data to the in-vivo context. Outcomes from in-vitro in-vivo correlation (IVIVC) studies have been utilized to select appropriate excipients and optimize the manufacturing process for quality assurance, as well as for analyzing the release characteristics of newly developed formulations dosage forms with the reference products (Kaur et al. 2015). For drugs administered orally, plasma concentration profiles are used as surrogates for drug available at the target site. The pharmacokinetic parameters – absorption, distribution, metabolism, and elimination govern the systemic availability of a drug, with absorption being the most formulation-dependent factor. IVIVC can be used as an alternative to human bioequivalence (BE) studies and has been shown to decrease the reduce the number of bioequivalence studies required during the initial or initial approval process, for some scale-up and post-approval modifications (Kaur et al. 2015). IVIVC can support regulatory biowaiver applications aid

in the support of biowaivers, allowing sponsors to forgo in-vivo bioavailability/bioequivalence studies. When seeking biowaivers due to changes in manufacturing processes or facilities, IVIVC can take the place of certain in-vivo studies that would typically be required, as long as adequate model data regarding safety and efficacy are available. A robust in-vitro in-vivo correlation can reduce the need for animal studies, promoting ethical research practices and expediting development timelines. Deconvolution and convolution are the two main approaches used to establish Level A IVIVC models. Convolution is in vitro to in vivo process whereas deconvolution is an in vivo to in-vitro process. In Convolution when we get in-vitro (dissolution) data, adaptation of in vitro data is used as the model input and then adapted in-vitro data will be processed using pharmacokinetic disposition equations of the data. The convolution-based IVIVC model is a one-stage modeling technique where the IVIVC model directly links the time course of plasma concentration to the in-vitro release profile. Convolution method needs plasma drug concentration data from the administration of an immediate-release formulation form. The model directly correlates the in vitro release profile of a drug to the plasma concentration–time profile. In technical terms, it refers to models where the

in-vitro in-vivo relationship is described using a convolution integral (Gillespie, 1997).

Among the various modeling techniques, convolution-based approaches stand out as particularly attractive because they directly estimate plasma drug concentration–time profiles using in-vitro dissolution data and pharmacokinetic parameters from previous studies, without requiring the need for an intravenous reference formulation (Gillespie, 1997). The successful validation of convolution-based Level A IVIVC model has been shown to provide an accurate prediction of key pharmacokinetic parameters like C_{max} and AUC (Ghosh, 2008).

Absorption of vonoprazan is frequently limited by its dissolution rate, developing an IVIVC is scientifically relevant. Therefore, establishing a convolution-based Level A IVIVC for vonoprazan fumarate tablets can assist in facilitate formulation optimization formulations and reduce the need for extensive bioequivalence studies. Consequently, this research aims on aims to develop and to establish a Level A IVIVC for 20 mg vonoprazan tablets using a convolution-based approach, by integrating in vitro dissolution profiles of vonoprazan to predict in vivo pharmacokinetic parameters and reducing the need for costly BE studies.

MATERIALS AND METHODS

Methodology

The steps involved in the preparation of Vonoprazan tablets and Level A IVIVC were:

1. Selection of Excipients
2. Formulation of Trial batches
3. Evaluation of Trial batches
4. Performing Level A IVIVC
5. Interpretation of the findings

Selection of excipients

All excipients, including Mannitol, Microcrystalline Cellulose (MCC),

Croscarmellose Sodium, Hydroxy Propyl Cellulose-Low substituted (HPC-LF), Fumaric Acid, Magnesium Stearate, Hydroxy Propyl Methyl Cellulose K 4 M (HPMC K4M) and Vonoprazan Fumarate were of pharmaceutical grade and received from M.D.H. Pharmaceuticals Pvt. Ltd, Changunarayan-3, Bhaktapur, Nepal.

Formulation of trial batches

Total 8 formulations were developed (Table 1).

Table 1: Formulation for tablets.

Formulation	Vonoprazan Fumarate	Mannitol	Mcc	Ccs	Hpc-Lf	Fumaric Acid	Magnesium Stearate	Total Weight
F1	26.80	56.00	96.20	10.00	6.00	2.00	3.00	200.00
F2	26.80	56.00	100.20	6.00	6.00	2.00	3.00	200.00
F3	26.80	56.00	104.20	2.00	6.00	2.00	3.00	200.00
F4	26.80	56.00	104.20	2.00	6.00	2.00	3.00	200.00
F5	26.80	56.00	104.20	2.00	6.00	2.00	3.00	200.00
F6	26.80	56.00	104.20	2.00	6.00	2.00	3.00	200.00
F7	26.80	56.00	100.20	HPMC K 4 M (6 mg)	6.00	2.00	3.00	200.00
F8	26.80	56.00	90.20	16.00	6.00	2.00	3.00	200.00

Tablets were prepared by the wet granulation method using HPC LF as a binder and were evaluated for hardness, friability, weight variation, and assay. The granules were evaluated for different rheological properties like bulk density, tapped density, compressibility index, and angle of repose before compression.

A 27-station rotary tablet compression machine (ACCURA; Fluid Pack Machinery Pvt. Ltd.) with round punch of size of 8.0 mm was used to compress the lubricated granules.

A manual coating machine (SAMARIKA) was

used for coating. WINCOAT WT (Wincoat) was used as film-coating agent. Non-aqueous coating was performed using with Iso-Propyl Alcohol and Methylene Chloride. Following coating, the dissolution profiles of the coated tablets was evaluated for correlation purposes. USP Type II dissolution equipped with an autosampler from Campbell Electronics was used for performing dissolution. Different dissolution media like Phosphate Buffer pH 6.8, Acetate Buffer pH 4.5 and 0.1 N HCl with different dissolution RPM was used for the analysis of the tablets. Nexera Series HPLC was

used for the analysis of drug content.

Establishment of level a IVIVC (convolution approach)

The convolution technique was employed to establish a Level A IVIVC by correlating the in vitro drug release profile of vonoprazan with its in vivo plasma concentration–time data. Dissolution studies were performed at $37 \pm 0.5^\circ\text{C}$ using a paddle apparatus at 20–75 rpm, with sampling intervals adjusted for fast-, medium- and slow-release tablets. The obtained dissolution profiles served as input functions for convolution analysis to predict plasma concentrations. Published pharmacokinetic parameters of vonoprazan were used as constants to simulate the reference plasma concentration–time profile, enabling the prediction of in vivo drug behavior from in vitro dissolution data (Mulford, 2021).

Adaptation of in-vitro and in-vivo data

Due to the differences in the physiological timescales of dissolution and absorption, a time-scaling factor of 1/1156.94 was utilized to align both profiles. This adjustment factor was empirically derived by matching corresponding points, such as, like the concentration at 25 min, between the in-vitro and in-vivo profiles. A linear relationship was assumed that there exists a linear relationship between the timescales of in-vitro and in-vivo processes, and a consistent time-scaling factor adjustment was implemented throughout the dataset to ensure accurate alignment and improve the correlation precision.

Time scaling/ levy plot

Levy plots illustrate the relationship between in vivo absorption and in vitro dissolution by comparing the corresponding time points comparisons at corresponding values for both processes. The plots are constructed by linear regression of the data points that intersect at the origin, accompanied by the correlation coefficient (R^2) and the slope of the fitted line. To construct a Levy plot, it is requires to have two distinct datasets: one representing the in vivo time points and the other representing the corresponding in vitro time points.

Convolution process

The convolution process was was performed to transform the in-vitro concentration data into predicted in vivo plasma concentrations. Each in-vitro concentration point was numerically integrated over time with the system's unit impulse response, which was derived from the exponential pharmacokinetic equation:

$$C_p(t) = C_{p0} e^{-(k_{el} \times t)}$$

Where:

- $C_p(t)$ = predicted plasma concentration at time 't'
- C_{p0} = Plasma drug concentration at time '0'
- K_{el} = elimination rate constant
- t = time difference

The convolution was implemented using Microsoft Excel 2010 with using incremental time steps corresponding to the in vitro sampling intervals. This approach generated a continuous predicted plasma concentration–time profile based on the in vitro data.

Calculation of pharmacokinetic parameters

Pharmacokinetic metrics, including maximum concentration (C_{max}), time to peak concentration (T_{max}), and area under the curve (AUC), were derived for both the observed and predicted plasma drug profiles. C_{max} and T_{max} values were directly obtained from the concentration-time data. The AUC was calculated using the utilizing the trapezoidal rule across the full concentration-time range. These metrics served as a quantitative basis for evaluating the predictive performance of the IVIVC model.

Method sensitivity test

To evaluate the sensitivity of the developed IVIVC model, two formulations were compared: a fast-release formulation and a modified slow-release formulation on developed in this study. The objective was to determine whether the established IVIVC model could detect and accurately predict the expected pharmacokinetic differences between formulations with distinct release rates. The in-vitro dissolution data of both formulations were used as inputs for the convolution process to generate their respective predicted plasma concentration-time profiles. The model's sensitivity was

assessed by evaluating changes in key pharmacokinetic parameters including – C_{max}, T_{max}, and AUC. A sensitive and valid IVIVC model should demonstrate a consistent changes in these pharmacokinetic parameters that corresponds to the formulation's release characteristics; that is, a slower in vitro release should reflect a delayed T_{max}, reduced C_{max}, and comparable or slightly reduced AUC in the predicted profile and rapid T_{max} and higher C_{max} for fast release formulation

Prediction error analysis

The internal predictability of the established IVIVC model was evaluated using the percentage prediction error (%PE) for C_{max}, T_{max} and AUC, calculated using the following equation:

$$PE (\%) = (|Predicted - Observed| / Observed) \times 100$$

According to the U.S. FDA (1997) IVIVC guideline, an average absolute prediction error of less than 10% is considered acceptable for internal validation, and less than 15% for external validation.

Statistical analysis

Statistical analysis was performed by Microsoft EXCEL 2010 version.

RESULTS AND DISCUSSION

Vonoprazan tablets were formulated using the wet granulation method. All pre- and post-compressional parameters were analyzed. A total of eight formulations were included in the

study. Among these formulations, six were conventional formulations, one was a fast-release formulation, and the remaining formulation was a slow-release formulation.

All critical formulation and manufacturing parameters were evaluated.

Dissolution profiles of all formulations were evaluated and correlated with the published plasma concentration–time profile. Dissolution was controlled by changing the concentration of super disintegrants, hardness of tablets and dissolution RPM. The results obtained from these formulations are discussed below.

Analysis of lubricated granules, compressed tablets and coated tablets

Pre-compression results (Table 2) demonstrated acceptable flow properties for formulations F2–F7 and good flowability for F1 and F8, which may be attributed to the wet granulation process. The analysis of compressed tablets is presented in Tables 3 and 4. All evaluated parameters were within pharmacopoeial limits. Dissolution profiles obtained under different media and rotation speeds are presented in Table 5. In vitro dissolution studies of formulations F1–F8 demonstrated considerable differences in drug release under different buffer conditions,

disintegration time, and RPM. Formulations F1–F4 with disintegrating time of 4 minutes (DT 4 min) exhibited rapid drug release (>95% within 20–30 min), indicating rapid-release behavior that was unsuitable for IVIVC. To obtain dissolution profiles suitable for IVIVC development, the superdisintegrant concentration was reduced, tablet hardness was increased, and dissolution RPM was lowered. Formulations F5 with disintegrating time of 12 minutes (DT 12 min) and F6 produced release profiles closely matching in-vivo plasma profile, supporting their suitability for IVIVC modeling. F7, containing HPMC K4M, showed the slowest and incomplete release (68.72% at 60 min), while F8 containing 8% croscarmellose sodium (CCS) showed complete drug release within 5 min. These results demonstrate that formulation variables demonstrate that formulation variables such as superdisintegrant level, hardness, and dissolution variables like paddle RPM significantly affect dissolution kinetics, with F5 and F6 providing the most desirable profiles.

Table 2: Pre-compression analysis of Vonoprazan Fumarate granules.

Formulation	Angle Of Repose	Compressibility Index	Hausner Ratio
F1	31.00	23.41	1.32
F2	29.80	14.76	1.17
F3	29.70	14.05	1.16
F4	29.33	13.72	1.16
F5	28.61	14.28	1.17
F6	28.75	14.28	1.21
F7	28.38	14.03	1.16
F8	30.25	17.64	1.21

Table 3: Analysis of compressed tablets.

Formulation	Average Wt.	Assay %	Hardness	Friability	Disintegration (min)	Thickness
F1	200.00± 1.33	103.76	3.98 ± 0.05	0.05	1 min 48 sec	4.00±0.02
F2	200.10 ± 2.21	100.42	3.86 ± 0.10	0.20	2 min 30 sec	3.98±0.02
F3	200.70 ± 1.06	100.81	3.86 ± 0.18	0.30	4 min 12 sec	3.98±0.02
F4	200.1 ± 1.15	100.19	18.49 ± 0.93	0.27	12 min	3.50±0.02
F5	200.20 ± 0.79	100.48	20.93 ± 0.97	0.30	12 min	3.40±0.02
F6	201.00 ± 0.94	99.19	19.62 ± 1.28	0.25	12 min	3.40±0.02
F7	200.20 ± 1.03	101.56	7.91 ± 1.36	0.28	10 min	3.80±0.02
F8	200.00 ± 1.15	100.63	4.53± 0.22	0.29	50 sec	4.00±0.02

Table 4: Analysis of coated tablets.

Formulation	Average Wt.	Thickness	Disintegration Time
F1	205.40 ±084	4.00±0.02	2 min
F2	204.40±1.26	3.98±0.02	3 min 5 sec
F3	205.10±1.29	3.98±0.02	5 min
F4	205.10±1.20	3.50±0.02	12 min
F5	204.80±1.23	3.40±0.02	12 min
F6	204.60±1.17	3.40±0.02	12 min
F7	204.60±1.96	3.80±0.02	10 min
F8	204.30±1.25	4.00±0.02	50 sec

Table 5: Dissolution profile of coated tablets.

Formulation	5min	10 Min	15 Min	20 Min	30 Min	45 Min	60 Min	Buffer/ Rpm
F1	96.6	100.8	102.4	101.6	100.6	95.5	100.7	Phosphate Buffer pH 6.8, RPM 75 0.1N HCL pH 1.2, RPM 75 Acetate Buffer pH 4.5, RPM 75
	95.5	101.4	103.0	101.9	102.6	97.2	102.6	
	90.4	100.5	101.3	101.5	100.6	96.0	102.6	
F2	80.87	99.06	94.75	102.10	79.71	100.18	98.89	Phosphate Buffer pH 6.8, RPM 75
F3	20.31	53.10	82.71	96.40	101.86	99.02	95.81	Phosphate Buffer pH 6.8, RPM 75
F4	DT 4 52.75	92.83	99.13	98.30	97.78	98.29	103.00	Phosphate Buffer pH 6.8, RPM 50
	DT 8 15.2	60.94	92.61	94.13	95.71	97.21	98.81	
	DT 12 14.69	19.52	43.76	65.99	102.29	103.10	101.86	
F5	DT 4 60.72	99.57	102.19	102.27	100.15	101.50	95.87	Phosphate Buffer pH 6.8, RPM 20
	DT 8 6.35	17.03	60.01	82.06	89.41	92.75	93.05	
	DT 12 6.82	10.38	18.20	32.24	75.63	89.92	96.26	
F6	8.12	10.75	18.53	53.37	83.86	90.91	97.45	Phosphate Buffer pH 6.8, RPM 20
F7		38.84		56.02	62.17		68.72	Phosphate Buffer pH 6.8, RPM 20
F8	5 min	10 min	15 min	20 min	25 min			PHOSPHATE BUFFER PH 6.8, RPM 20
	101.63	102.17	100.34	99.21	97.73			

Time scaling and levy plot

The Levy plot illustrates the relationship between the in vitro dissolution times and the in vivo absorption times of the drug (Table 6; figure 1). The regression coefficient (R^2) was

Table 6: Time scaling.

In vitro Time (hr)	In vivo Time (hr)
0	0
0.25	0.25
0.75	1.0
1.0	2.0

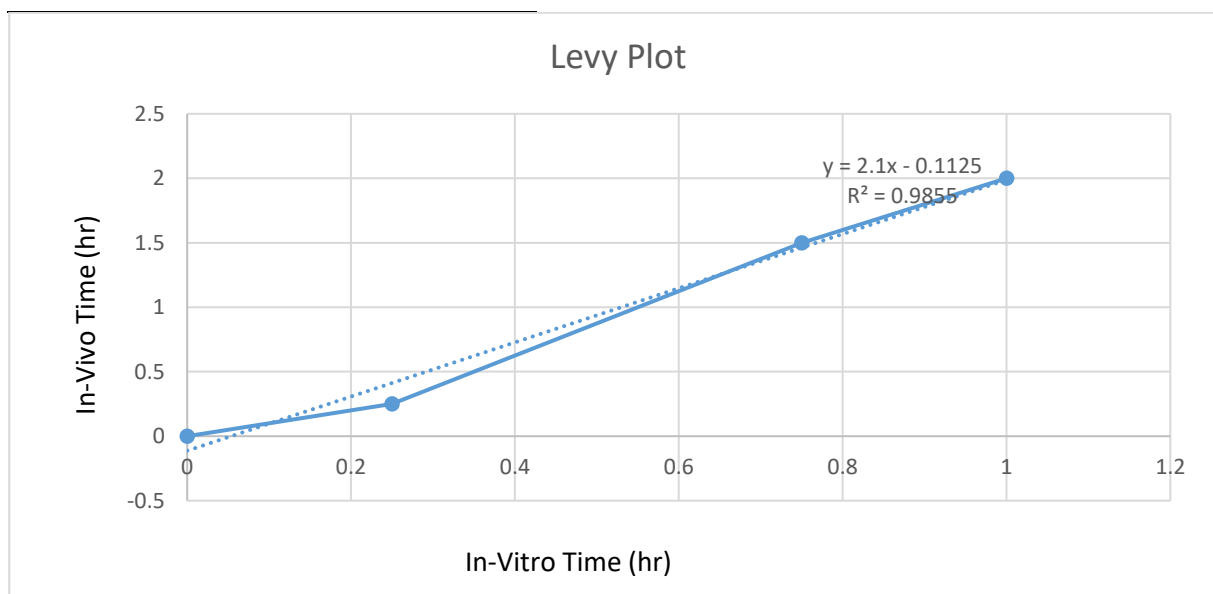


Figure 1: Levy plot.

In-Vitro and In-Vivo drug profile comparison

The in vitro (predicted) and in vivo drug profile (Observed). The comparison between the predicted in vitro dissolution profile and

0.9855, indicating a strong linear correlation between these two timeframes. This suggests that the dissolution of the drug in gastrointestinal fluids is the rate-limiting factor for its absorption into systemic circulation.

observed in vivo drug absorption profile is presented in Figure 2. The x-axis shows in vitro drug profile and the y-axis shows in vivo drug absorption profile.

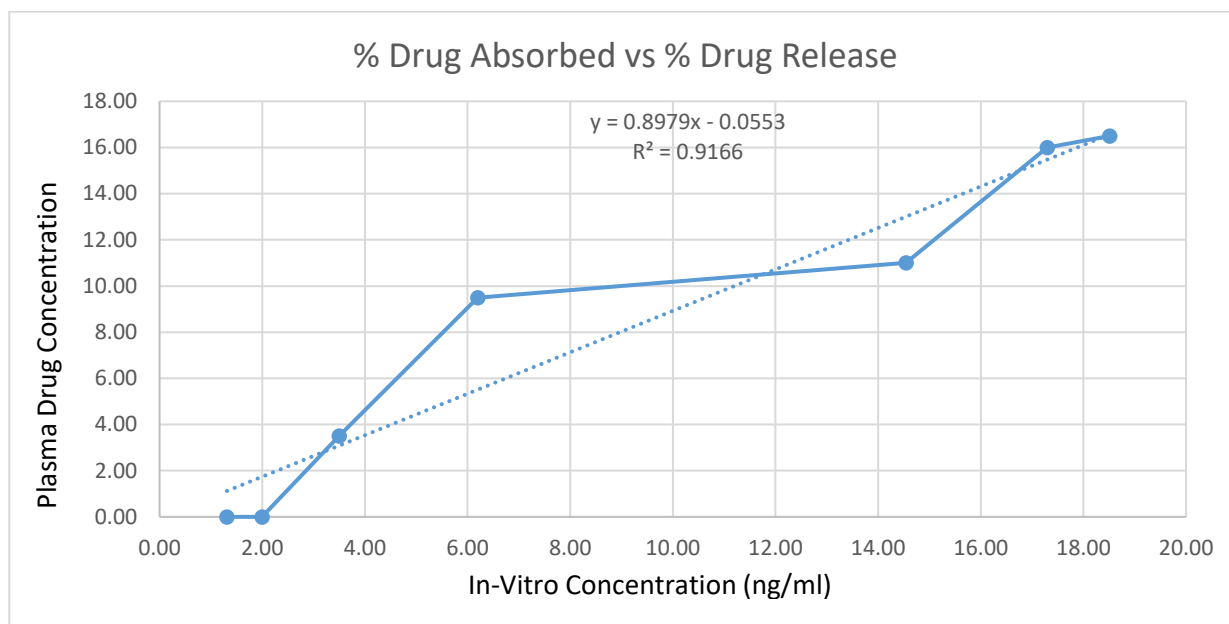


Figure 2: In-Vitro and in-vivo drug profile comparison.

A significant correlation was observed between in-vitro and in-vivo data for the developed formulation (F6). The graph demonstrates a strong linear relationship between the percentage of drug released during in vitro dissolution and the percentage absorbed in-vivo. This correlation is quantitatively supported by high R^2 value of 0.916, suggesting that more than 91% of the variability observed in in vivo absorption can be attributed to the in vitro release figures. The regression equation ($y = 0.8979x - 0.0553$) further illustrates an almost close to a 1:1 relationship, indicating that the rate of dissolution in vitro primarily governs the absorption rate observed in- vivo.

Plasma drug profile comparison and method sensitivity test

Vonoprazan fumarate is a highly permeable molecule with bioavailability >85%. The drug exhibits rapid absorption, therefore the drugs follow Rapid IV Bolus one-compartment

model after dissolution and systemic absorption in the body. So, using IV Bolus one compartment model, Plasma concentrations at different time points were predicted. The predicted and observed plasma drug profile is shown in (Figure 3).

The Medium release formulation predicted plasma profile was found to be similar to the in-vivo observed plasma drug profile with C_{max} of 16.61 ng/ml at 1.98 hr, AUC (0-24) of 170.97 hr.ng/ml and AUC (0-∞) of 187.14 hr.ng/ml. The in-vivo observed plasma drug profile with C_{max} of 18.2 ng/ml at 2 hr, AUC (0-24) of 160.8 hr.ng/ml and AUC (0-∞) of 179.3 hr.ng/ml was in good agreement with the predicted values.

The Predicted fast release formulation profile (yellow curve) shows a higher initial peak compared to the predicted slow release formulation profile (blue curve). The blue curve exhibits a slower rise and prolonged decline, consistent with controlled or sustained

drug release characteristics with $AUC(0-24)$ and $AUC(0-\infty)$ values of 127.57 ng $\times$ ml/hr and 139.36 ng $\times$ ml/hr respectively indicating slow rise in plasma concentration. The yellow curve shows the rapid rise in plasma concentration and exhibits a sharp and high initial peak (C_{max} = 19.54 ng/ml) achieved rapidly at

T_{max} = 5 minutes, indicating rapid drug absorption from the formulation.

The significant change in the value of pharmacokinetic parameters between medium, slow and fast release formulation indicates that the developed IVIVC method is sensitive.

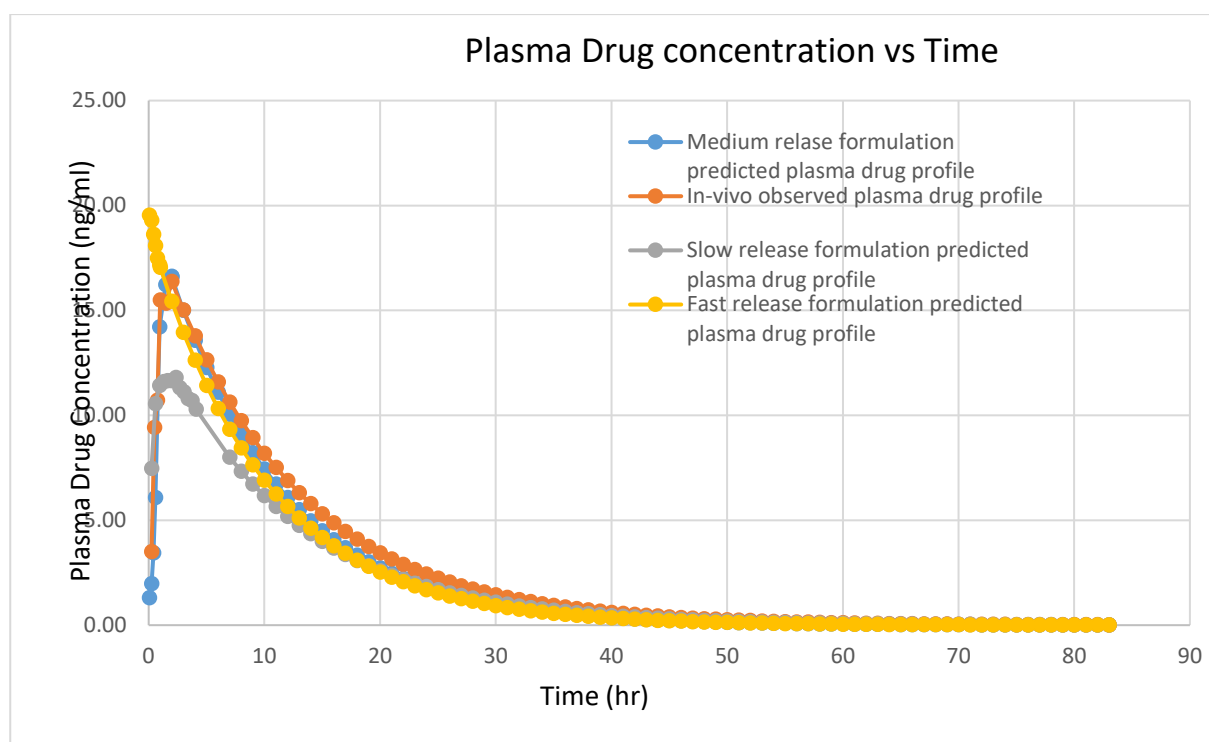


Figure 3: Predicted medium release, slow release, fast release and in-vivo plasma drug profile.

Percent prediction error (%PE)

The prediction errors for the medium-release formulation are presented in Table 7.

Table 7: Percent prediction error for medium release formulation.

Formulation (test)	Predicted	Observed	%Error
C_{max} (ng/ml)	16.61	18.2	8.74
AUC_{0-24} (ng $\times$ hr/ml)	170.97	160.8	6.32
$AUC_{0-\infty}$ (ng $\times$ hr/ml)	187.14	179.3	4.37
T_{max} (hr)	1.98	2	1.0

The percentage prediction errors (%PEs) for the medium-release formulation indicate good predictive performance between predicted and observed pharmacokinetic parameters. The %PE for C_{max} was 8.74%, AUC_{0-24} was 6.23%, $AUC_{0-\infty}$ is 4.37% and T_{max} was 1.0

% which is well within the acceptable limit of $\pm 10\%$.

The developed in vitro–in vivo correlation (IVIVC) model demonstrated good predictive performance though further model refinement and may improve AUC prediction accuracy and strengthen the level A IVIVC relationship.

CONCLUSIONS

An in vitro–in vivo correlation (IVIVC) model was developed for Vonoprazan Fumarate, a BCS Class II potassium-competitive acid blocker used in reflux esophagitis. Formulations were developed using excipients comparable to those of the innovator product and dissolution profiles were modified to disintegrant levels, tablet hardness, and dissolution RPM. Formulations F5 and F6 showed dissolution patterns closely matching in-vivo plasma profiles. A one-compartment IV bolus model was applied to predict plasma concentrations, yielding strong correlations. The Levy plot showed demonstrated good agreement between in vitro

dissolution time points and in vivo absorption time points with a regression coefficient (R^2) of 0.9855. The predicted and the observed plasma drug profile also shows a good correlation with regression coefficient (R^2) of 0.916. Predicted pharmacokinetic values (C_{max} 16.61 ng/ml, T_{max} 1.98 hr, $AUC_{0-\infty}$ 187.14 hr $\times$ ng/ml) closely matched with observed values (C_{max} 18.2 ng/ml, T_{max} 2 hr, $AUC_{0-\infty}$ 179.3 hr $\times$ ng/ml). The % prediction errors for C_{max} (8.74%), AUC_{0-24} (6.23%), $AUC_{0-\infty}$ (4.37%), and T_{max} (1.0%) were within $\pm 10\%$, confirming an excellent Level A IVIVC.

LIMITATION OF THE STUDY

This study has two main limitations:

1. The in vivo pharmacokinetic data were obtained from published literature rather than generated experimentally in this study.
2. The in vivo and in vitro data were generated from different formulations.

While going through the literature survey, a systematic review of IVIVC studies demonstrated that many published Level A IVIVC models were developed using two or three formulations with different release rates, consistent with FDA recommendations (Davanço et al. (2020). FDA considers IVIVC models to be formulation-specific. Consequently, the use of dissolution data from one formulation and pharmacokinetic data from another formulation is may not be appropriate for regulatory Level A IVIVC. However, such an approach may still be useful in exploratory scientific studies when the formulations are scientifically comparable and the limitations are clearly acknowledged (Kaur et al. 2015; FDA, 1997). The authors emphasize that an IVIVC links the dissolution behavior of a dosage form to the in vivo absorption of that same dosage form. Therefore, changes in formulation composition or manufacturing process can alter the dissolution profile and the pharmacokinetic behavior, making the correlation formulation dependent (Cardot et al. 2007). In-vitro and in-vivo data from the same product are required for regulatory application but if the research is for academic and product development purpose (exploratory), in-vivo data and in-vitro data from different formulation provided that your formulation composition must be similar with product from which in-vivo data was generated. Our case was matching with second condition, as the research was not for regulatory purpose (biowaiver) and our formulation was pharmaceutically equivalent with the product from which in-vivo data was generated. Thus, in-vivo data obtained from

literature and in-vivo and in-vitro data from two different formulations are valid in our case and they are no more limitations of our research which is supported by literature as well.

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CONFLICT OF INTEREST

All the authors declared that there is no conflict of interest.

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Identification of Novel *N*-(Aryl)-3-(*N*-benzylsulfamoyl)benzamides as NAMPT Activators

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Abstract

Nicotinamide adenine dinucleotide (NAD⁺) plays a central role in numerous physiological processes, and maintaining or increasing cellular NAD⁺ levels has emerged as a promising strategy for promoting healthy aging. Nicotinamide phosphoribosyltransferase (NAMPT) is the rate-limiting enzyme in the NAD⁺ salvage pathway, which makes it an attractive target for the treatment of many diseases associated with NAD⁺ depletion, such as inflammation, neurodegenerative, and metabolic diseases. Herein, we report the synthesis and *in vitro* biological evaluation of **STA1-STA3**, which increased NAMPT activation by 2- to 38-fold. In addition, molecular docking studies were conducted within both the catalytic and allosteric sites of NAMPT, and the resulting binding patterns were analyzed to correlate structural differences with the observed *in vitro* NAMPT activity, highlighting the significance of molecular interactions.

Keywords

Activator, molecular docking, NAMPT.

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INTRODUCTION

Nicotinamide adenine dinucleotide (NAD⁺) serves as an essential coenzyme in a wide range of redox and non-redox processes. In energy metabolism, it functions as a universal electron carrier during key metabolic stages, including glycolysis, the tricarboxylic acid cycle, and oxidative phosphorylation, where a hydride is transferred from a substrate to oxidized NAD⁺ to form NADH (Yang and Sauve, 2016; Luengo et al. 2021). NAD⁺ also acts as a substrate for NAD⁺-consuming enzymes, such as sirtuins (SIRT1-7), poly(ADP-ribose) polymerases (PARPs), mono(ADP-ribosyl) transferases (ARTs), cyclic ADP-ribose synthases (cADPRS), sterile alpha and TIR motif-containing 1 (SARM1), and cyclic ADP ribose hydrolases (CD38 and CD157) which modulate signaling pathways across diverse cellular processes et al. 2021). Through these activities, NAD⁺ regulates a broad spectrum of cellular functions such as energy metabolism, DNA repair, regulation of immune response, and inflammation (Canto et al. 2015; Croteau et al. 2017; Audrito et al. 2019; Navarro et al. 2022). Consequently, NAD⁺ deficiency in normal cells has been associated with a variety of age-related diseases, including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, atherosclerosis, retinal degeneration, diabetes, osteoarthritis,

impaired immunity, and lipid metabolism disorders (Figure 1) (Okabe et al. 2019; Jadeja et al. 2020; Ralto et al. 2020; Obrador et al. 2021; Wang et al. 2021; Navarro et al. 2022; Mischley et al. 2023; Wu et al. 2025). Thus, restoring cellular NAD⁺ levels through precursor supplementation or biosynthetic pathway modulation represents a promising therapeutic strategy for age-related diseases the prognosis of age-related diseases (Iqbal and Nakagawa, 2024; Yang et al. 2024).

In eukaryotic cells, there are three major NAD⁺ biosynthesis pathways: the salvage pathway, the Preiss-Handler pathway, and the *de novo* pathway (Ozgencil et al. 2024). Among these, mammals primarily rely on the salvage pathway, in which nicotinamide riboside (NR) and nicotinamide (NAM) serve as precursors, wherein NR is phosphorylated by riboside kinase (NRK), whereas the rate-limiting enzyme nicotinamide phosphoribosyltransferase (NAMPT) catalyzes the reversible transfer of the ribose group from 5-phosphoribosyl-1-pyrophosphate (PRPP) to NAM, generating nicotinamide mononucleotide (NMN). Then, NMN is converted into NAD⁺ by nicotinamide mononucleotide adenylyltransferase (NMNAT) (Figure 1) (Katsyuba et al. 2020; Conlon and Ford, 2022).

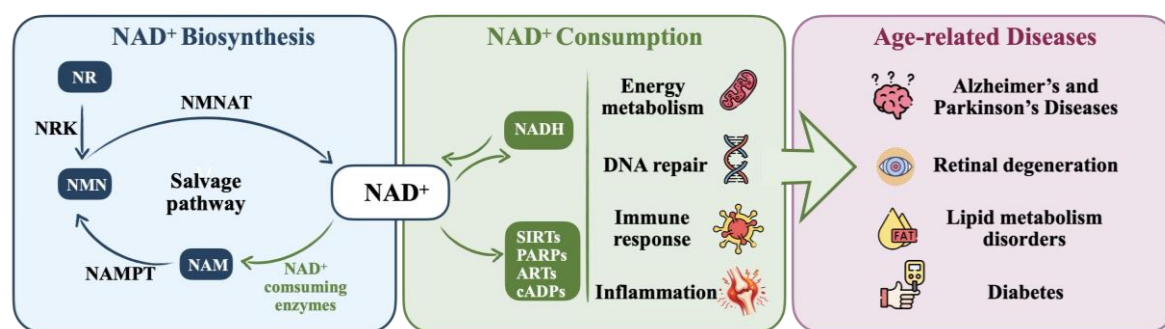


Figure 1: Schematic overview of the main NAD⁺ biosynthesis pathway and its association with age-related diseases.

Recent studies on NAMPT activators (Figure 2) have emphasized the therapeutic promise of boosting cellular NAD⁺ concentrations in the context of neurological and metabolic disorders, with reported effects including neuroprotection, increased insulin secretion, improved muscle performance, and antilipidemic effects (Wang et al. 2022; Iwatani et al. 2026; Jiang et al. 2026; Li et al. 2026). The carbazole derivative P7C3 represented the first class of small molecules reported to increase intracellular NAD⁺ levels, yet several follow-up studies have questioned its role as a direct NAMPT activator (Wang et al. 2014; Gardell et al. 2019). The 4-pyridine derivative SBI-797812 (EC₅₀=0.37 mmol/L) represents a well-characterized class of NAMPT activators that which exhibits structural similarity to classical 3-pyridyl NAMPT inhibitors (Gardell et al. 2019). More recently, allosteric NAMPT

activators (N-PAMs), NAT (EC₅₀=6 μM) and NP-A3-B2 (EC₅₀=4.06 μM) have been reported to enhance NAMPT activity and elevate intracellular NAD⁺ levels by binding to the allosteric site located in the rear channel of NAMPT (Yao et al. 2022; Ratia et al. 2023; Gordon-Blake et al. 2024). Despite these advances, there remains a strong need to develop more potent and drug-like NAMPT activators, as their pharmacological properties and mechanism of action are still largely unexplored. In this study, we report the synthesis and *in vitro* biological activity results of **STA1**, **STA2**, and **STA3** as NAMPT activators, which were identified during a previous inhibitor optimization workflow. The potential binding modes of these compounds and their specific interactions at catalytic and allosteric activator binding sites were subsequently investigated using molecular docking studies.

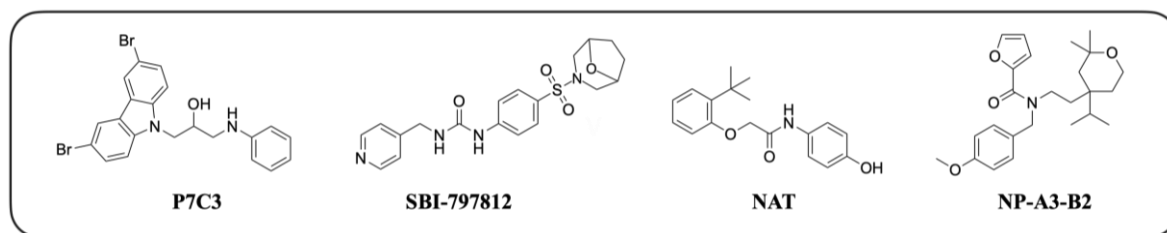


Figure 2: Previously reported NAMPT activators.

MATERIALS AND METHODS

Chemistry

All the chemicals were obtained from commercial suppliers (Abcr, BLDpharm, Sigma-Aldrich) and used without further purification. Reaction progress was monitored by thin-layer chromatography (TLC) using alumina plates coated with Merck silica gel 60 F₂₅₄, visualized under UV light at 254 nm or 366 nm. NMR analyses (¹H-NMR and ¹³C-NMR) were performed on an Avance Neo 500 MHz high-performance digital FT-NMR spectrometer, employing tetramethylsilane (TMS) as the internal standard. Chemical shifts are reported as δ values ppm relative to TMS, and all coupling constants (*J*) are given in Hertz (Hz), with DMSO-*d*₆ as the solvent. High-resolution mass spectrometry (HRMS) analyses were performed using a Waters LCT Premier XE UPLC/MS-TOF system in positive electrospray ionization mode (ESI⁺). Chromatographic separations were carried out on an ACQUITY BEH C18 column (2.1 x 50 mm, 1.7 μ m, Waters Corporation), using a water: acetonitrile gradient (80:20 to 10:90) at a flow rate of

0.25 mL/min. The melting points of the compounds were measured using a Stuart SMP50 automatic melting point apparatus, without correction

Synthesis

General synthetic procedure of 1a and 2a

Benzene-1,2-diamine or 2-aminophenol (1 equiv.) and cyanogen bromide (1.5 equiv.) were dissolved in a methanol:water (1:1) mixture, and the reaction mixture was heated at 50 °C for 1 h. Upon completion, the reaction mixture was cooled to room temperature, and methanol was removed *in vacuo*. The resulting aqueous mixture was adjusted to pH 8 using 1 N NaOH solution and extracted with ethyl acetate (3x10), followed by brine (3x10 ml). The combined organic layers were dried over anhydrous Na₂SO₄ and concentrated *in vacuo*. The crude product was purified by consistently from an appropriate organic solvent.

1H-Benzo[d]imidazol-2-amine (1a)

The compound was synthesized according to the general procedure from benzene-1,2-diamine (332 mg; 2.5 mmol) and cyanogen bromide (397 mg; 3.8 mmol). The crude

product was purified by recrystallisation from methanol to afford pure **1a** as a brown solid (300 mg, 90%). MP 221.3-221.9 °C. CAS no: 934-32-7. HRMS (ESI⁺) calculated for C₇H₈N₃ [M+H]⁺ (*m/z*) 134.0718; Found 134.0723.

Benzo[d]oxazol-2-amine (1b)

The compound was synthesized according to the general procedure from 2-aminophenol (272 mg; 2.5 mmol) and cyanogen bromide (397 mg; 3.8 mmol). The crude product was purified by recrystallisation from methanol to afford pure **1b** as a white solid (66 mg, 20%). MP 129.7-130.5 °C. CAS# 4570-41-6. HRMS (ESI⁺) calculated for C₇H₇N₂O [M+H]⁺ (*m/z*) 135.0558; Found 135.0556.

Synthesis of 3-(chlorosulfonyl)benzoic acid

Chlorosulfonic acid (4 ml) was added dropwise to benzoic acid (611 mg; 5 mmol) at 0 °C, and the reaction mixture was gradually heated to 80 °C and stirred for 5 h. Upon completion, the reaction mixture was cooled and poured into ice-cold water. The resulting precipitate was collected by filtration and used in the next step without further purification (666 mg, 61%). MP 110-110.3 °C. CAS# 25-64-3. HRMS (ESI⁺) calculated for C₇H₄ClO₄S [M-H]⁺ (*m/z*) 218.9519; Found 218.9516.

Synthesis of 3-(N-benzylsulfamoyl)benzoic acid

3-(Chlorosulfonyl)benzoic acid (1066 mg; 4.84 mmol) and benzylamine (794 µl; 7.26 mmol) were dissolved in dichloromethane, and the reaction mixture was stirred in the presence of triethylamine (1.5 equiv) at room temperature for 5 h. Upon completion, the reaction mixture was concentrated *in vacuo*, and the resulting residue was dissolved in ethyl acetate and extracted with 0.1 M HCl (3 x 10 ml), and the combined organic layers were then washed with 5% NaOH (3x 10 ml). The combined aqueous phases were then adjusted to pH 3-4 using conc. HCl. The resulting precipitate was collected by filtration and obtained as a pure white solid (807 mg, 57%). MP 226.3-226.8 °C. CAS# 7326-77-4. HRMS (ESI⁺) calculated for C₁₄H₁₂NO₄S [M-H]⁺ (*m/z*) 290.0487; Found 290.0486.

General synthetic method of STA1-STA3

A mixture of 3-(N-benzylsulfamoyl)benzoic acid (1.2 equiv.), HATU (1.2 equiv.), HOBt (1.2 equiv.), and DIPEA (3 equiv.) were dissolved in tetrahydrofuran and stirred at room temperature for 10 min. The corresponding amine derivative (**1a**, **2a** or naphthalen-2-amine) (1 equiv.) was then added, and the reaction mixture was stirred for 24-48h at room temperature. Upon completion, the reaction mixture was concentrated *in vacuo* and diluted with dichloromethane. The organic phase was sequentially washed with 2N HCl (3x10 ml), 1% NaHCO₃, and 3%

NaCl (3x10 ml) solutions. The combined organic layers were dried over anhydrous Na₂SO₄ and concentrated *in vacuo*. The crude product was purified by consistently or washing with an appropriate solvent.

N-(1*H*-Benzo[*d*]imidazol-2-yl)-3-(*N*-benzylsulfamoyl)benzamide (STA1)

The compound was synthesized according to the general procedure from **1a** (55 mg; 0.42 mmol) and 3-(*N*-benzylsulfamoyl)benzoic acid (150 mg; 0.51 mmol). The crude product was purified by recrystallizing from methanol to afford the desired compound as a white solid (72 mg, 42%). MP 238.7-240.3 °C. CAS# 1390132-73-6. HRMS (ESI⁺) calculated for C₂₁H₁₉N₄O₃S [M+H]⁺ (*m/z*) 407.1178; Found 407.1163. ¹H-NMR (DMSO-*d*₆, 500 MHz) δ ppm: 4.04 (2H, s, -CH₂-); 7.20-7.29 (5H, m, Ar-H); 7.32-7.39 (2H, m, Ar-H); 7.51-7.75 (3H, m, Ar-H); 8.03 (1H, s, Ar-H); 8.25-8.62 (3H, m, Ar-H and -SO₂NH-); 12.35 (1H, s, Ar-H); 13.20 (1H, br s, -NHCO-).

N-(Benzo[*d*]oxazol-2-yl)-3-(*N*-benzylsulfamoyl)benzamide (STA2)

The compound was synthesized according to the general procedure from **1b** (54 mg, 0.40 mmol) and 3-(*N*-benzylsulfamoyl)benzoic acid (142 mg, 0.48 mmol). The crude product was purified by washing with ethanol and acetone to afford the desired compound as a white solid (105 mg, 64%). MP 210.0-210.7 °C.

HRMS (ESI⁺) calculated for C₂₁H₁₈N₃O₄S [M+H]⁺ (*m/z*) 408.1018; Found 408.1001. ¹H-NMR (DMSO-*d*₆, 500 MHz) δ ppm: 4.03 (2H, s, -CH₂-); 7.18-7.29 (7H, m, Ar-H); 7.43-7.47 (2H, m, Ar-H); 7.70 (1H, t, *J*=7.8 Hz, Ar-H); 7.95 (1H, d, *J*=7.8 Hz, Ar-H); 8.26 (1H, t, *J*=6.6 Hz, -SO₂NH-); 8.36 (1H, d, *J*=7.8 Hz, Ar-H); 8.61 (1H, s, Ar-H); 12.51 (1H, br s, -NHCO-).

3-(*N*-Benzylsulfamoyl)-*N*-(naphthalen-2-yl)benzamide (STA3)

The compound was synthesized according to the general procedure from naphthalen-2-amine (73 mg; 0.45 mmol) and 3-(*N*-benzylsulfamoyl)benzoic acid (160 mg; 0.54 mmol). The crude product was purified by recrystallizing from ethanol to afford the desired compound as a white solid (65 mg, 35%). MP 168.5-169.6 °C. HRMS (ESI⁺) calculated for C₂₄H₂₁N₂O₃S [M+H]⁺ (*m/z*) 417.1273; Found 417.1291. ¹H-NMR (DMSO-*d*₆, 500 MHz) δ ppm: 4.03 (2H, d, *J*=6.4 Hz -CH₂-); 7.20-7.30 (5H, m, Ar-H); 7.45 (1H, t, *J*=7.5 Hz, Ar-H); 7.51 (1H, t, *J*=8.2 Hz, Ar-H); 7.77 (1H, t, *J*=7.8 Hz, Ar-H); 7.86 (1H, dd, *J*=8.9 Hz and 2.1 Hz, Ar-H); 7.88-7.90 (2H, m, Ar-H), 7.94 (1H, d, *J*=8.9 Hz, Ar-H), 8.02 (1H, dt, *J*=8.9 Hz and 1.5 Hz, Ar-H), 8.26 (1H, t, *J*=1.5 Hz, Ar-H), 8.33 (1H, t, *J*=6.4 Hz, -SO₂NH-); 8.42 (1H, s, *J*=1.9 Hz, Ar-H); 8.45 (1H, d, *J*=2.3 Hz, Ar-H); 10.70 (1H, br s, -NHCO-).

***In vitro* NAMPT activity assay**

In vitro NAMPT inhibition assay was performed using a NAMPT Inhibitor Screening Assay Kit (BPS Bioscience, Catalog no: 71276) according to the manufacturer's protocol, (Ozgencil et. al., 2020), in which test compounds and the NAMPT inhibitor FK866, used as a positive control for inhibition, were evaluated at 100 μ M and 50 μ M screening concentrations. In the first step, test compounds (10 μ l/well) and human NAMPT enzyme (10 μ l/well) (diluted in NAMPT dilution buffer, enzyme concentration 30 ng/ μ l) were added to all wells except for the blank and positive control wells and incubated at room temperature for 30 min. Subsequently, a substrate mixture (12.5 μ l NAMPT test buffer + 2.5 μ l ATP + 2.5 μ l nicotinamide + 2.5 μ l PRPP + 2.5 μ l 30% ethanol + 12.5 μ l water) was added to all wells (35 μ l/well) and incubated at 30 °C for 2 h. The resulting fluorescence was recorded at excitation/emission wavelengths of 360/460 nm using a Molecular Devices SpectraMax i3x microplate reader. Each experiment was performed in triplicate. Fluorescence values were normalized to those of the blank wells, and fold-change activity relative to the control was calculated for each compound.

Molecular docking

The molecular docking studies were performed using the Glide module

implemented in the Schrödinger Small-Molecule Drug Discovery Suite (Small-Molecule Drug Discovery Suite 2024-4, Schrödinger, LLC, New York, NY, 2024). The X-ray crystal structures of human NAMPT in complex with urea-based NAMPT activator (PDB: 7Q8T) (Tang et. al., 2023) and with allosteric NAMPT activator (NP-A3-B2) (PDB: 8TM7) (Gordon-Blake et. al., 2024) were retrieved from the Protein Data Bank and prepared using the Protein Preparation Wizard tool. During the preparation stage, atom types and charges were assigned, missing hydrogen atoms were added, and all water molecules except (HOH607, HOH620, HOH635, HOH637, HOH719, HOH727, HOH752, HOH793, HOH841, HOH852, HOH874, and HOH889 for 7Q8T; HOH812 and HOH856 for 8TM7) were removed. Finally, energy minimization and refinement were done up to an RMSD cutoff of 0.30 Å by applying an OPLS4 force field. Each grid box, containing the active site coordinates of the proteins, was defined with a 20 Å radius around the coligand using the Receptor Grid Generation tool, applying default parameters and OPLS4 force field. The docking protocols for both proteins were validated by redocking the corresponding coligand and calculating RMSD values between their docked and bioactive conformations, which were 1.0225 Å for

urea-based NAMPT activator and 0.3672 Å for NP-A3-B2, demonstrating the accuracy of pose predictions. The 3D structures of all compounds were prepared using the LigPrep module and docked into NAMPT active site using the XP mode of Glide, with

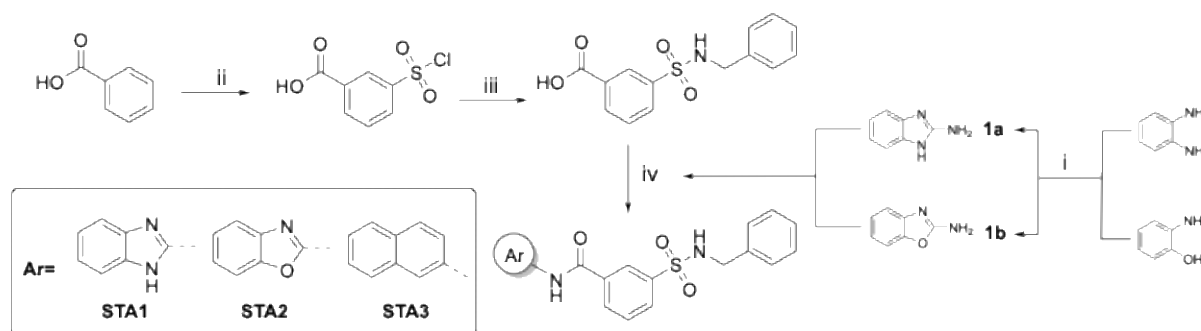
a Van der Waals radius scaling factor of 0.80 and partial charge cutoffs of 0.15, respectively (Friesner et. al., 2006). The conformations obtained for each compound were then analyzed.

RESULTS AND DISCUSSION

Synthesis

As depicted in Scheme 1, the synthesis of the target compounds commenced with the reaction benzene-1,2-diamine or 2-aminophenol with cyanogen bromide to yield the corresponding amine derivatives (**1a** and **1b**). In parallel, benzoic acid was reacted with chlorosulfonic acid to give 3-(chlorosulfonyl)benzoic acid, which was then subjected to nucleophilic substitution with benzylamine in the presence of

triethylamine to afford 3-(*N*-benzylsulfamoyl)benzoic acid. Finally, amide coupling of **1a** and **1b** with 3-(*N*-benzylsulfamoyl)benzoic acid using HATU, HOBt, and DIPEA yielded the target compounds (**STA1-STA3**). The structures and purity of the synthesized compounds were confirmed by ¹H-NMR, HRMS spectra and UPLC-UV chromatogram (see the Supporting Information).



Scheme 1: Synthetic route for **STA1-STA3**. Reagents and conditions: (i) cyanogen bromide, methanol and water (1:1), 50 °C, 1 h; (ii) chlorosulfonic acid, 0 °C → 80 °C, 5 h; (iii) benzylamine, triethylamine, dichloromethane, rt, 5 h; (iv) **1a** or **1b**, HATU, HOBt, DIPEA, dichloromethane, rt, 24-28 h.

Biological evaluation

The synthesized 3-(*N*-Benzylsulfamoyl)-*N*-(naphthalen-2-yl)benzamide derivatives (**STA1-STA3**) were evaluated for *in vitro* NAMPT activity using a fluorescence-based assay at screening concentrations of

100 and 50 μM (Figure 3). According to the results, all compounds increased NAMPT enzyme activity, with fold increases ranging from 2 to 38 relative to the control. Among the tested compounds, **STA1** bearing 1*H*-benzimidazole, exhibited the highest

NAMPT activation (38-fold: 100 μ M, 18-fold: 50 μ M), whereas substituting the nitrogen atom in the benzimidazole with oxygen (STA2) decreased the activity to 23-fold (100 μ M) and 8.6-fold (50 μ M). Furthermore, replacement this moiety to the heteroatom-deficient naphthalene ring

(STA3) resulted in the weakest activity (16-fold: 100 μ M, 2-fold: 50 μ M), suggesting that the electron-rich, heteroatom-bearing aromatic systems contributed positively to binding and favored interactions that enhance NAMPT activation.

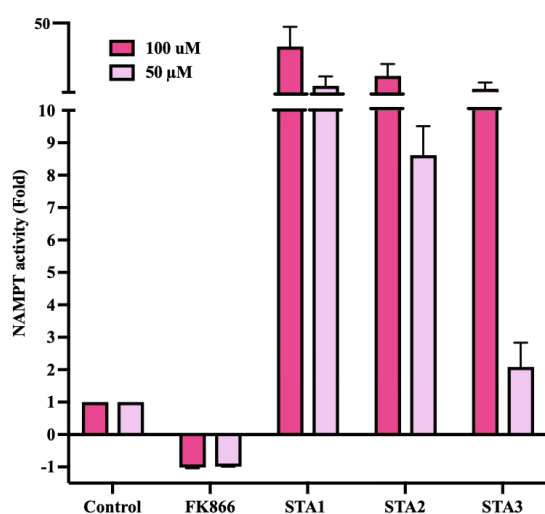


Figure 3: Fold-change in NAMPT activity of the compounds.

Molecular docking simulations

To elucidate the molecular interactions that may contribute to the the observed NAMPT activation, molecular docking simulations of STA1-STA3 were carried out within the catalytic (PDB: 7Q8T) and allosteric sites (PDB: 8TM7) of NAMPT, and the resulting binding poses were evaluated in comparison with co-crystallized ligands.

The NAMPT dimeric interface contains a long, narrow tunnel-shaped active site extending to the solvent-exposed region through a funnel-shaped rear channel, comprising hydrophobic residues. (Kim et. al., 2006). The catalytic site comprises the

ID	Structure	NAMPT Fold activity*	
		100 μ M	50 μ M
STA1		38.17 $\pm$ 9.91	18.33 $\pm$ 4.75
STA2		23.35 $\pm$ 5.97	8.62 $\pm$ 0.89
STA3		16.80 $\pm$ 3.19	2.09 $\pm$ 0.75
FK866		-1.02 $\pm$ 0.02	-0.99 $\pm$ 0.003

*Values are presented as mean $\pm$ standard deviation ($n = 3$)

PRPP-binding region at the tunnel entrance and the nucleobase pocket where NAM binds. This is connected to the rear channel through a narrow tunnel known as the doorsill (Velma et. al., 2024).

As depicted in Figure 4, unlike NAMPT inhibitors, which typically form π - π stacking interactions by being sandwiched between F193 and Y18', the co-crystallized NAMPT activator (PDD: 7Q8T) formed a π -cation interaction with R311 and π - π interaction only with Y18' through its nicotinamide-like phenyl ring (Tang et. al., 2023). Within the nucleobase pocket and the adjacent doorsill region, the coligand

established direct or water-mediated H-bonds with D219, S275, and R311, *via* its urea and ribose groups. Furthermore, it formed direct or water-mediated H-bond networks with H247, K400, R196, R392, and G353 in the catalytic site, further complemented by H-bonding interactions with H191 and S379 in the rear channel.

Analysis of the binding modes of the compounds revealed that **STA1** and **STA2** were oriented toward the catalytic pocket through their benzylsulfamoyl moiety and central phenyl ring, forming π -cation interactions with R311, whereas **STA1** additionally engaged R392 *via* a π -cation interaction *via* its terminal phenyl ring (Figure 4). Moreover, although both **STA1** and **STA2** exhibited π - π stacking interactions with F193 or Y18', their central phenyl ring was not sandwiched between F193 and Y18', similar to the coligand. Both **STA1** and **STA2** established H-bonds with D313, S275, and R311 *via* sulfonamide NH

and the amide carbonyl, and notably, **STA1** formed an additional water-mediated H-bond through its benzimidazole NH with D219 and participated in π - π stacking interactions with H191. This distinct binding pattern may contribute to have accounted for the higher NAMPT activation observed for **STA1** relative to **STA2**. In contrast, although **STA3** formed a π -cation interaction with R311 *via* the naphthalene ring and an H-bond with G353, it was unable to interact with specific residues in the catalytic pocket and the doorsill (Figure 4). The limited interactions of **STA3** are likely responsible for its lower NAMPT activation compared to **STA1** and **STA2**. In addition, the docking scores of **STA1** (-7.19 kcal/mol), **STA2** (-6.50 kcal/mol), and **STA3** (-5.28 kcal/mol) followed the same trend as their experimentally determined activities, while the coligand NAMPT activator showed a comparable score of -7.61 kcal/mol.

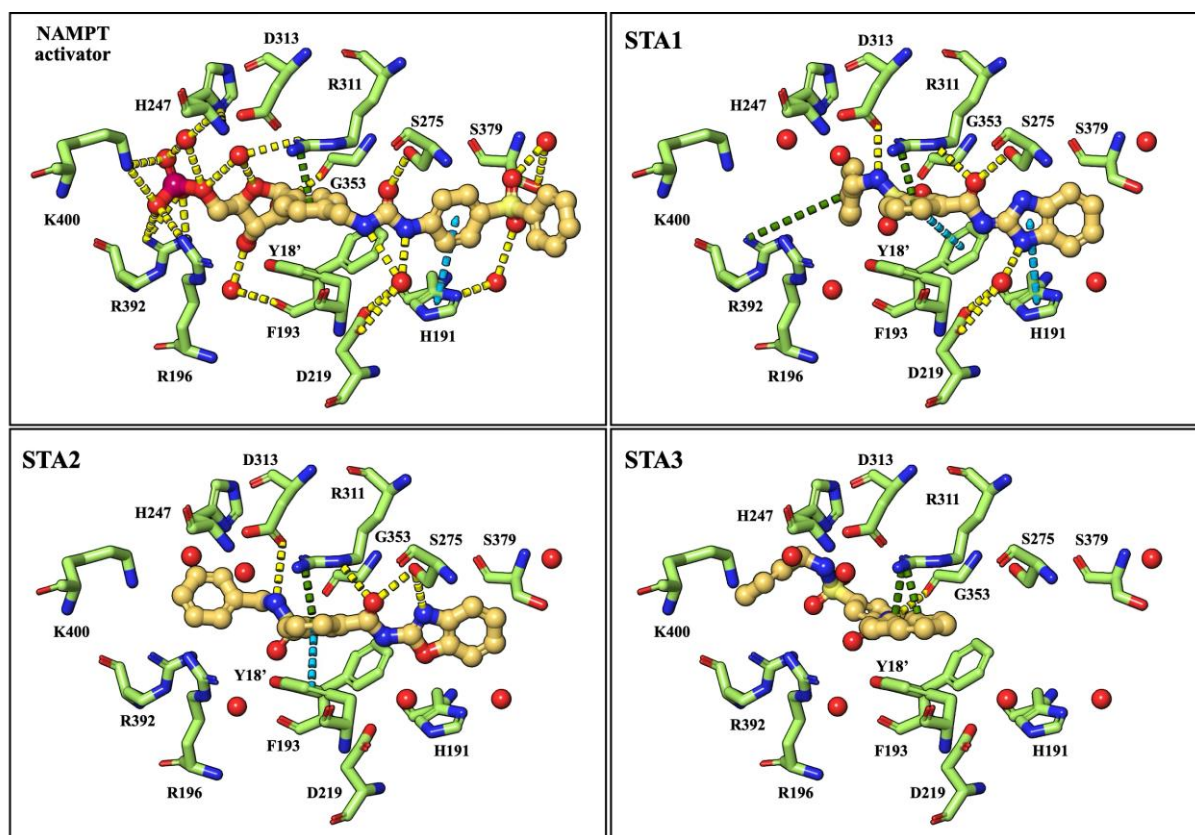


Figure 4: The bioactive conformation of the coligand (NAMPT activator) and predicted binding poses of the compounds within the catalytic binding site of NAMPT (PDB: 7Q8T) with binding energies of -7.61, -7.19, -6.50, and -5.28 kcal/mol, respectively. Water molecules are depicted as red spheres. H-bonds, π - π contacts, and π -cation interactions are represented by yellow, cyan, and green dashed lines, respectively.

To assess the potential allosteric binding of **STA1-STA3**, the compounds were docked into the allosteric site (PDB: 8TM7) located in the rear channel, which has been implicated in NAMPT activation (Gordon-Blake et. al., 2024). As shown in Figure 5, the allosteric NAMPT activator NP-A3-B2 occupied the rear channel primarily through hydrophobic interactions with residues P273, I351, Y188, V242, and R349 surrounding its aromatic rings and tetrahydropyran moieties, and additionally formed water-mediated hydrogen bonds with S275 and R349 *via* its hydroxyl and

carbonyl groups. Regarding **STA1-STA3**, all compounds adopted - remove 'it was shown that' adopted a bent conformation in contrast to the binding mode in the catalytic site and occupied the allosteric site *via* their aromatic rings interacting with hydrophobic residues similar to NP-A3-B2. The most active compound, STA1, formed an H-bond with H191 *via* sulfonamide NH, while its phenyl rings contributed hydrophobic interactions with Y188, P273, I351, V242, and R349. STA2 engaged in a water-mediated H-bond with S275 and hydrophobic interactions with Y188, V242,

R349, and I351. In contrast, the naphthalene derivative **STA3** exhibited a weaker profile, showing only π - π stacking with H191 and hydrophobic interactions through aromatic rings without forming H-bonds. Moreover, the docking scores of **STA1** (-7.51

kcal/mol), **STA2** (-7.19 kcal/mol), and **STA3** (-6.24 kcal/mol) the same ranking as their experimentally observed activity, while the coligand NP-A3-B2 showed a comparable score of -7.73 kcal/mol.

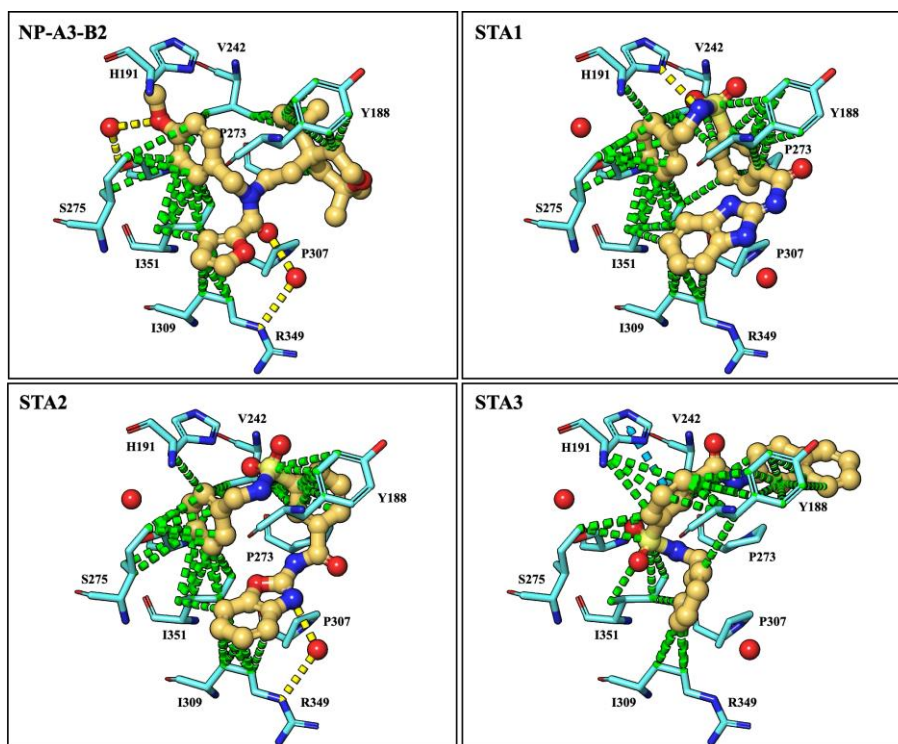


Figure 5. The bioactive conformation of the coligand (NAMPT activator, NP-A3-B2) and predicted binding poses of the compounds within the NAMPT allosteric binding site (PDB: 8TM7) with binding energies of -7.73, -7.51, -7.19, and -6.24 kcal/mol, respectively. Water molecules are depicted as red spheres. H-bonds, π - π , and hydrophobic interactions are represented by yellow, cyan, and green dashed lines, respectively.

CONCLUSIONS

Herein, we report the synthesis of **STA1**-**STA3**, and their in vitro effects on the enzymatic activity of NAMPT, exhibiting notable activation effects ranging from 2- to 38-fold. Moreover, molecular docking studies were performed at both catalytic and allosteric binding sites of NAMPT in order to support the biological data. Consistent with their biological efficacy, **STA1** and **STA2** formed molecular interactions

similar to those of the co-crystallized ligand, whereas the least active **STA3** exhibited fewer contacts in the catalytic site. Notably, the 1*H*-benzimidazole moiety of **STA1** participated in additional H-bonding, which may contribute to its greater activity superior activation potential compared to **STA2**. All compounds were predicted to occupy the allosteric binding site as well, mainly driven by hydrophobic

contacts and H-bonds. Overall, **STA1-STA3** were identified as NAMPT activators and may provide therapeutic potential in restoring cellular NAD⁺ levels and thereby NAD⁺-related diseases.

ACKNOWLEDGMENTS

The authors declare no conflict of interest.

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Hepatoprotective Influence Of Linalool Against Imatinib-Induced Hepatic Injury In Balb/C Mice

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Abstract

Imatinib (IMTB), a first-generation tyrosine kinase inhibitor, that is used to treat chronic myeloid leukemia has however been implicated in hepatic injury. Linalool is a natural monoterpene alcohol with strong biologic antioxidant property. This study investigated the hepatoprotective potential of linalool against imatinib-induced toxicity in male BALB/c mice. 30 male BALB/c mice were randomly divided into five groups: I, control; II, olive oil (0.1ml olive oil); III, IMTB (400 mg/kg b.w.); IV, IMTB (400 mg/kg B.W.) + LN (40 mg/kg B.W.) simultaneously administered and V, LN (40 mg/kg b.w.). The result showed that simultaneous treatment with linalool significantly reduces AST, ALT and ALP activities compared to the IMTB group. Moreover, linalool abrogated IMTB-induced increase in advanced oxidation protein products and abated IMTB-facilitated surge decrease in total thiol levels and arylesterase activity. Additionally, linalool prevented pathological lesions and imatinib-induced necrotic influence in the liver of co-treated mice. Overall, linalool abrogative influence improved liver damage occasioned by IMTB-mediated toxicities in mice by alleviating oxidative stress resulting from imatinib administration.

Keywords

Imatinib, linalool, hepatotoxicity, oxidative stress, antioxidant system.

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INTRODUCTION

Drug induced liver injury (DILI) is a major cause of acute liver failure, accounting for about half of all cases across developed nations and continues to be a major issue in clinical practice (Björnsson, 2016). The liver is one of the most important organs in the human body that carries out numerous critical tasks such as metabolic regulation, protein production and detoxification (Mitra and Metcalf, 2009). Due to its crucial role in drug metabolism, it is susceptible to harm caused by pharmaceuticals leading to drug induced liver injury. As a result, it is essential to investigate and comprehend the causes of liver damage and create preventive measures to enhance therapeutic results and patient safety. Comprehensive literature review revealed that many active chemotherapeutic agents either alkylating agent or antimetabolite therapy cause injury to vital organs most especially liver organ (Olayinka et al. 2016; Kara, 2023).

FDA approved imatinib mesylate (Gleevec®), a selective tyrosine kinase inhibitor, in 2001 that transformed the treatment of Philadelphia chromosome-positive acute lymphoblastic leukemia and chronic myeloid leukemia (CML) (Druker et al. 2001). Imatinib enhanced the management of CML by targeting the BCR-ABL fusion protein responsible for the

excessive proliferation of leukemic cells (Hochhaus et al. 2017). On the contrary, observation in clinical practice revealed that imatinib therapy exhibited a number of side effects notably liver damage. Earlier findings showed that 2–10% of individuals that are under imatinib treatment experience liver impairment (Trent et al. 2003; Rea et al. 2014). The induction of liver damage by imatinib has been attributed to oxidative stress, mitochondrial dysfunction and direct cell injury (Fang et al. 2010). Reactive oxygen species (ROS) are produced during drug metabolism, which can overpower the antioxidant defense systems in liver and cause DNA damage, protein oxidation and lipid peroxidation (Cichoż-Lach and Michalak, 2014). Numerous studies have shown that imatinib raises indicators of oxidative stress in both clinical and experimental research (Breccia et al. 2008).

The hepatotoxic potential of imatinib has limited its clinical significance, hence the search for a remediating agent remediating agent that can mitigate the side effect of imatinib without impairing its therapeutic efficacy. Natural products have been reported to have positive safety profiles and gained utility in a variety of medical systems especially in the treatment of liver (Stickel and Schuppan, 2007). Linalool

(3,7-dimethyl-1,6-octadien-3-ol) is a natural small molecule monoterpene that is found in abundance in the essential oils of lavender, coriander, bergamot, and many other aromatic plants (Peana et al. 2002). It gains significance in biological research for its various pharmacological effects, such as anxiolytic, sedative, anti-inflammatory, antimicrobial, and, most importantly, antioxidant properties (Aprotosoia et al. 2014). Linalool has earlier been reported to boost the activities of endogenous antioxidant enzymes, scavenge free radicals and decrease lipid peroxidation in several animal models (Huo et al. 2013). In addition, linalool has demonstrated protective benefits against chemical-induced organ toxicity in several studies (Celik and Ozkaya, 2002; Eftekhar et al. 2019). Linalool was reported to exhibit analgesic, sedative antimicrobial, anti-inflammatory, antitumor and antioxidant properties (De Cássia et al. 2013). The

antioxidative potential of linalool was utilized in preventing oxidative stress-induced neurodegeneration and preservation of redox status in diabetic condition (Migheli et al. 2019; Bar and Kara, 2024). Its anticancer activity especially anti-leukemic activity was also linked to its antioxidant potential and induction of cell cycle arrest and apoptosis in leukemia (Gu et al. 2010; Chang et al. 2015). Linalool displayed strong hepatoprotective potential against myriads of hepatotoxicants such as carbon tetrachloride, 2-acetylaminofluorine, diethylnitrosamine and benzene (Altınok-Yipel et al. 2019; Nagappana and Jayaram, 2019; Ola and Sofolahan, 2021). There is a paucity of information on the protective potential of linalool on imatinib induced toxicity. Therefore, the current study was conducted to examine the hepatoprotective effects of linalool against imatinib-induced toxicity in an experimental mouse model.

MATERIALS AND METHODS

Experimental animals

Male BALB/c mice weighing between 25 and 35 g were used in this study. The mice were bred and housed in the animal house of the Department of Chemical Sciences, Ajayi Crowther University, Oyo, Nigeria. They were kept in wire meshed cages at room temperature and under controlled light cycle of twelve-hour light and twelve-

hour dark. Both the commercial animal feed and water were supplied to them *ad libitum*. The mice were handled and used following the international guide for the care and use of laboratory animals (*National Research Council*). The use of anesthesia was not involved in this research and the study was approved by the Research Ethics Committee, Faculty of

Natural Sciences, Ajayi Crowther University, Oyo in 2024 with approval code: FNS/ERC/2024021.

Chemicals, reagents, and kits

Linalool was purchased from Santa Cruz Biotechnology (Germany). Imatinib mesylate drug was from GSMS Inc. (Camarillo, CA, USA) and donated by Mr. Asiyambi Ayomide. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and Alkaline phosphatase (ALP) assay kits were procured from Fortress Diagnostics Limited (UK). All other chemicals and reagents used were of analytical grade and obtained from reputable suppliers, including BDH Chemicals Limited (England) and Sigma-Aldrich.

Experimental design

Mice were randomized into five experimental groups (n=6/group). Group I mice served as control and received distilled water while Group II served as vehicle control and received 0.1ml olive oil orally. On daily basis, mice in Group III; received 400 mg/kg/body weight (bwt) IMTB orally for 14 days, group IV received 400 mg/kg/d IMTB plus 40 mg/kg/d LN, and group V received 40 mg/kg/d LN While 3 mg/kg/d IMTB represents the dosage for the treatment of Hodgkin's disease, 40 mg/kg/d LN p.o. was selected on the basis of its effectiveness in earlier studies (Ola and Sofolahan, 2021).

Blood and tissue sample collection and processing

Twenty-four (24) hours after the final administration and two (2) hours preceding the sacrifice, the animals were administered with 0.04% colchicine (1 mL/100 g body weight) by intraperitoneal route. The blood samples were collected into lithium heparinized tubes from each animal through retro-orbital plexus. Plasma was processed for subsequent liver function enzyme analyses and determination of oxidative stress markers by centrifuging the whole blood for five minutes at 4000 rpm in a conventional bench centrifuge. Animals were thereafter sacrificed. Livers were also removed and processed for histopathological assessment and percentage DNA fragmentation.

Assay of biomarkers of hepatotoxicity

Assessment of hepatotoxicity were carried out by determining the activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and Alkaline Phosphatase (ALP) using Randox diagnostic kits based on the procedure of manufacturer. Determination of plasma ALT and AST activities were executed based on the principle highlighted by Reitman and Frankel in 1957 (Reitman and Frankel, 1957) and the results were expressed in U/L. The ALP activity was determined by the principle described by Kind and King (1954). This method is

predicated on the idea that alkaline pH allows ALP to catalyze the hydrolysis of p-nitrophenyl phosphate, yielding p-nitrophenol and inorganic phosphate. At 405 nm, the released p-nitrophenol is spectrophotometrically quantified. The unit of enzyme activity was U/L.

Assay of oxidative stress markers in the plasma

Plasma AOPPs content was estimated by following the procedure described by Witko et al. (1996) and modified by Zhang et al. (2014). The spectrophotometric detection of oxidatively modified proteins that interact with potassium iodide in an acidic environment serves as the principle for this test. Chloramine-T was used as a reference and the absorbance was measured at 340 nm. The concentration was reported in micromoles per liter ($\mu\text{mol/L}$). In brief, 100 μL of plasma was added to 400 μL of PBS solution (phosphate buffer saline) and 25 μL of 1.16 M potassium iodide (KI) was then pipetted followed 2 min later by 50 μL of acetic acid. The absorbance of the mixture was then immediately read at 340 nm against a blank which contained 500 μL of PBS, 25 μL of 1.16 M potassium iodide and 50 μL of acetic acid. Plasma total thiol was measured spectrophotometrically using DTNB (2,2'-dinitro-5,5'-dithiodibenzoic acid) (Motchnik et al. 1994). This approach is based on the interaction between thiols and DTNB,

which results in the formation of 2-nitro-5-thiobenzoic acid, a yellow substance that has a strong absorbance at 412 nm. The total thiol concentration was determined in micromoles per liter ($\mu\text{mol/L}$) using the molar extinction coefficient of $13,600 \text{ M}^{-1}\text{cm}^{-1}$. Arylesterase activity was determined using phenylacetate as the substrate following the procedure described by Erdem et al. (2010). The concept is based on the enzymatic breakdown of phenyl acetate by arylesterase to produce phenol and acetic acid. A spectrophotometer operating at 270 nm is used to track the pace of phenol production.

Percentage DNA fragmentation assay

The assay assessed the endonuclease cleavage of the end product of apoptosis where the extracted DNA from the liver homogenate was treated with diphenylamine (DPA). The chromophore formed was measured on the spectrophotometer at 620 nm. Briefly, the excised liver was homogenized in x10 volumes of Tris-EDTA buffer (TE) of pH 8.0 containing Triton-X100. The homogenate was centrifuged at 27,000g for 20 minutes to separate the intact chromatin (pellet tagged as A) from fragmented ones (supernatant tagged as B). The pellet (A) was suspended in Tris-EDTA buffer (TE!) of pH 8.0 without Triton-X100. 0.5 mL aliquot of each sample (A and B) was placed in separate test tubes and 1.5 mL of

freshly prepared diphenylamine solution was added to each sample. Reaction mixture was incubated at 37 °C for 20 h. Absorbance of mixture was then measured at 620 nm.

Quantity of fragmented DNA was estimated by using the formula;

$$\% \text{ DNA fragmentation} = \frac{A}{(A + B)} \times \frac{100}{1}$$

Histopathological assessment of liver

The Baker and Silvertown method of 1985 was followed to process liver for histology (Baker and Silvertown, 1985). The procedure

started by fixation of tissue section, dehydrating tissue, clearing, infiltration, tissue casting, sectioning of tissue and mounting to stain it for view and scoring

Statistical analysis

For each group of n=6 animals, data were presented as mean $\pm$ standard deviation (SD). The statistical analysis made use of one-way analysis of variance (ANOVA), which was then followed by post-hoc tests for several comparisons. Statistical significance was defined as a difference at $p < 0.05$. Appropriate statistical software was used for all statistical analyses.

RESULTS

Influence of linalool on imatinib-Induced changes in hepatic function biomarkers in the plasma of mice

Imatinib significantly elevated the activities of ALT, AST and ALP when compared with

the control group ($p < 0.05$). However, treatment with linalool significantly ameliorated the imatinib-induced increase in plasma activities of ALT, AST and ALP in mice.

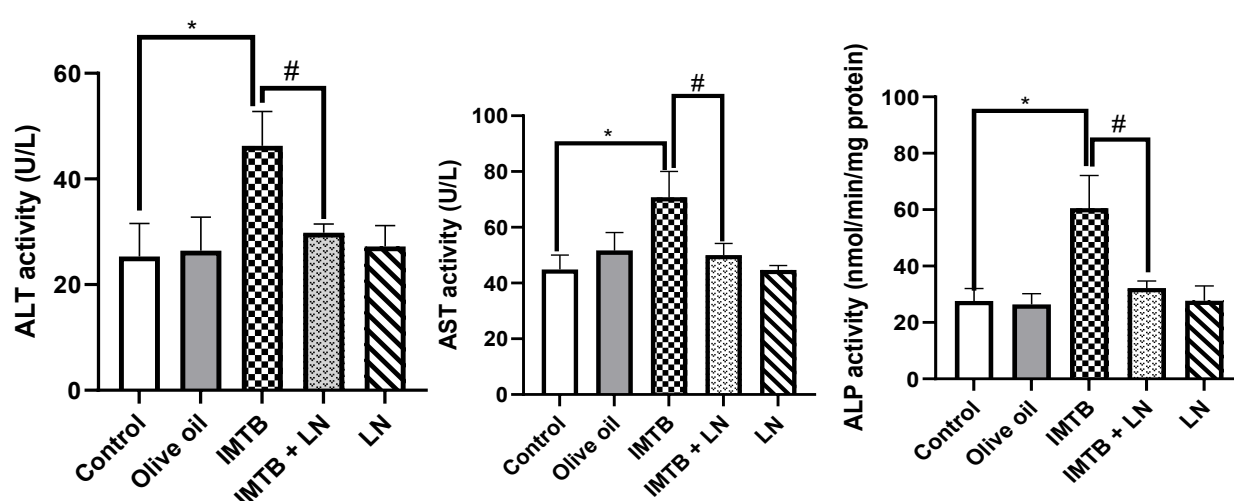


Figure 1: Protective effects of linalool on imatinib-induced changes in the plasma activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) in mice.

Protective effects of linalool on imatinib-induced changes in the levels of oxidative stress biomarkers

Figure 2a shows the effect of linalool on imatinib-induced toxicity on protein oxidation in mice. AOPPs levels used in the assessment of protein oxidation were significantly increased by 51.52% in the plasma in the imatinib-treated group compared with the control group ($p < 0.05$). However, when the animals were treated with imatinib, it attenuated this increase in AOPPs levels by 21.74% when compared to imatinib alone group. Moreover, in Figure 2b, imatinib-administered mice

showed a significant reduction in the Plasma levels of total thiol concentration when compared to the control group. Simultaneous treatment with linalool significantly increased the content of total thiol in the plasma of co-treated mice when compared with the mice that were solely intoxicated with imatinib. Also, administration of imatinib resulted in a significant decrease in the plasma activity of arylesterase enzyme compared with the control group in Fig. 2C. Treatment with linalool significantly increased arylesterase activity when compared with the mice exposed to imatinib alone.

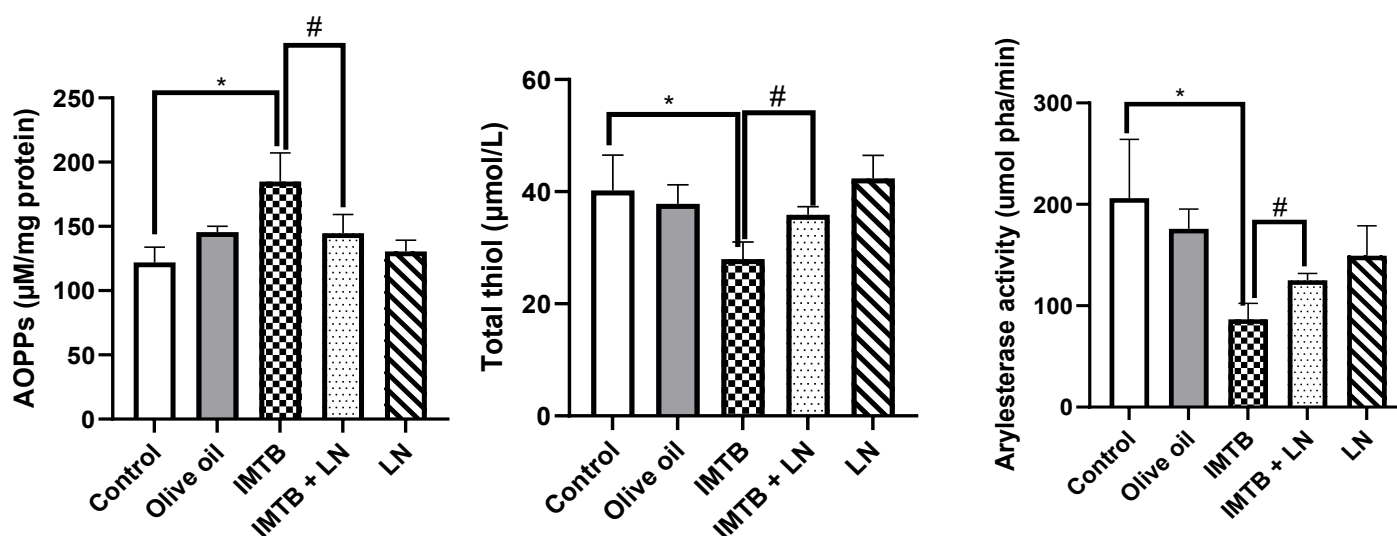


Figure 2: Protective effects of linalool on imatinib-induced changes in the levels of plasma advanced oxidation protein products (AOPPs), total thiol content and arylesterase activity in mice. Data are presented as mean SD for six mice per group. *Significantly different from control, # significantly different from IMTB at $p < 0.05$.

Protective effects of linalool on imatinib-induced changes in the percentage DNA fragmentation in liver of mice

The disruption of antioxidant molecules induced by imatinib administration was accompanied by a significant increase in the percentage of DNA fragmentation in the liver relative to control as shown in Figure

3. However, co-treatment with linalool significantly alleviated this increase

compared with the imatinib-treated group that did not receive linalool.

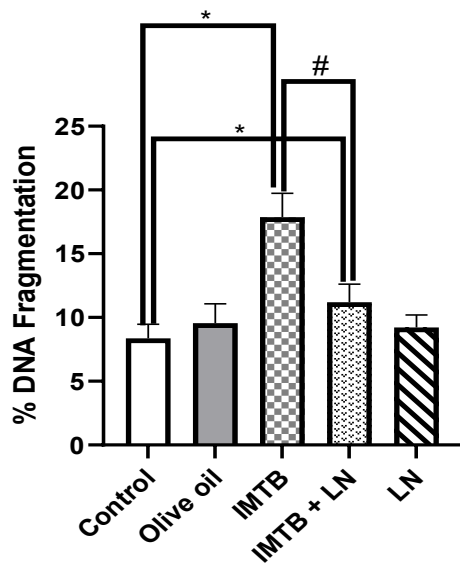


Figure 3: Effects of linalool on hepatic DNA fragmentation percentage in IMTB-induced liver damage in mice. Data are presented as mean SD for six mice per group. *Significantly different from control, # significantly different from IMTB at $p < 0.05$

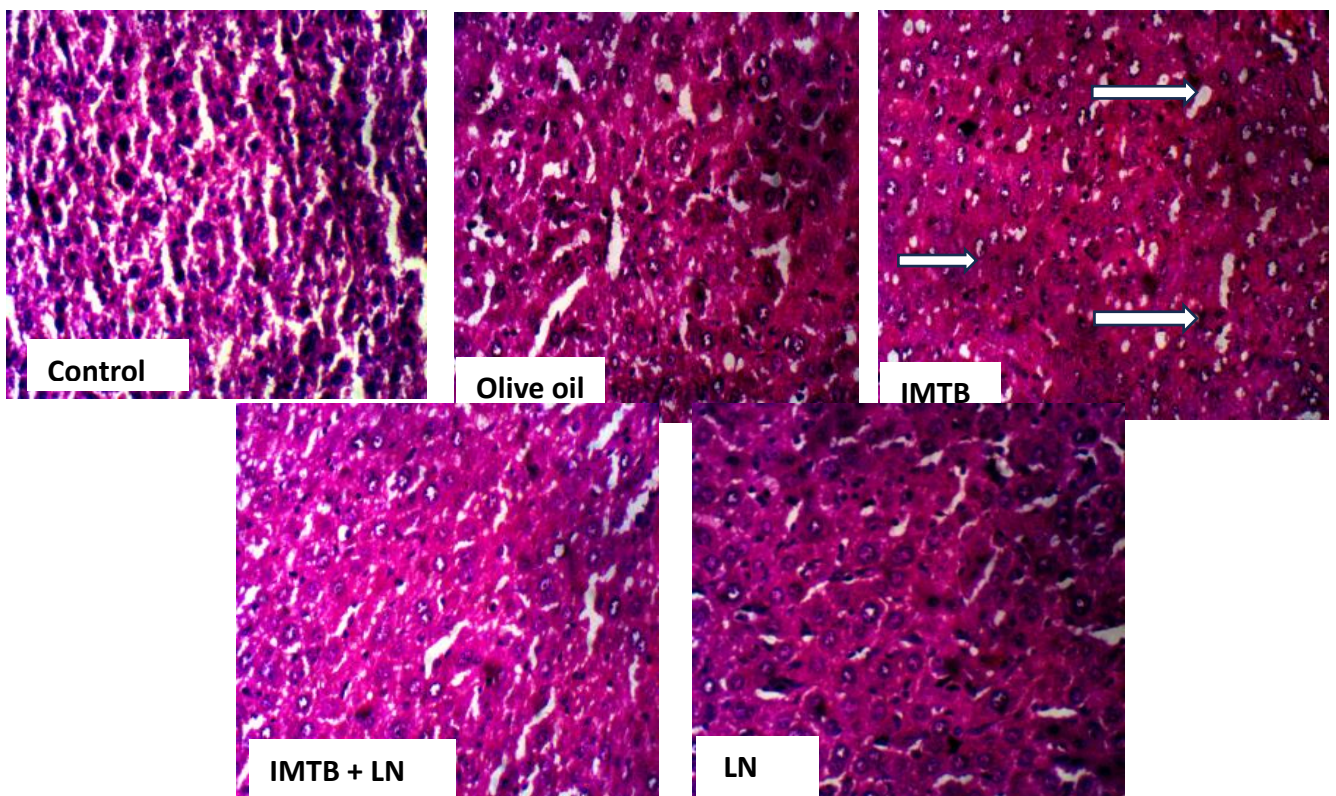


Figure 4: Photomicrograph ($\times 400$) shows Protective role of linalool extract on imatinib-induced Damages on Architecture of mice liver (Eosin & HX Stained).

Control: No visible lesion; Olive oil: No visible lesion; IMTB: Moderate infiltration and mild necrosis; IMTB + LN: No visible lesion observed; LN: No visible lesion is observed.

DISCUSSION

The treatment of Chronic myeloid leukemia (CML) with imatinib has limitations as 5–10% of patients receiving imatinib treatment are at risk of developing liver injury (Ferrero et al. 2005; Mindikoglu et al. 2004). The imatinib-induced hepatotoxicity mediated primarily through oxidative stress and mitochondrial dysfunction necessitates dose reduction or treatment discontinuation (Kim et al. 2020). However, linalool has shown strong antioxidant, anti-inflammatory, and hepatoprotective effects in several experimental models (Pereira et al. 2018; Altınok-Yipel et al. 2019). This research investigated the protective capacity of linalool against imatinib-induced liver toxicity in mice.

The present study reports the significant elevation in plasma ALT, AST, and ALP activities in imatinib-treated mice compared with the control group. This observation is consistent with previous reports of imatinib hepatotoxicity in both clinical and experimental studies (Trent et al. 2003, Fang et al. 2010). ALT is predominantly localized in hepatocytes and is considered the relatively specific marker for hepatocellular injury (Ramaiah, 2007). The 82.81% increase in ALT activity observed in our study compared with the control group indicates substantial

hepatocyte membrane damage and cytoplasmic enzyme leakage, consistent with the findings of Rea et al. (2014), who reported elevated transaminases in approximately 5-10% of patients receiving imatinib therapy. AST is a useful indicator when interpreted in conjunction with ALT (Giannini et al. 2005). The 57.73% increase in AST activity seen in our imatinib-treated mice relative to control is consistent with clinical findings of imatinib hepatotoxicity and indicates mitochondrial injury as AST exists in both cytoplasmic and mitochondrial isoforms (Nyblom et al. 2004). The AST/ALT ratio is used in assessment of pattern of liver damage; in our investigation, the ALT value increased proportionally more than the AST value (ratio<1), which is indicative of hepatocellular injury as opposed to alcoholic liver disease, which usually has an AST/ALT ratio greater than two (Botros and Sikaris, 2013). The elevation in the activity of ALP is frequently used to indicate cholestatic injury or hepatobiliary dysfunction. This is because an increase in ALP activity reflects biliary tree injury or decreased bile flow, and it is mostly connected with the biliary epithelium and canalicular membrane of hepatocytes (Sharma et al. 2014). The observed significant elevation in ALP observed in

our study supports case reports of imatinib-induced cholestatic hepatitis (Tonyali et al. 2008) and implies that imatinib toxicity may have an impact on biliary function in addition to causing direct hepatocellular damage. On the other hand, in this study, there is a noticeable decline in ALT (35.58%), AST (29.33%), and ALP (46.82%) activities after the simultaneous administration of linalool. According to these data, linalool protects the integrity of the hepatocyte membrane and reduces the leakage of these enzymes into the systemic circulation. This observation agrees with previous reports of protective capacity of linalool against chemical-induced hepatotoxicity (Celik and Ozkaya, 2002; Eftekhar et al. 2019)

The liver extensively metabolizes imatinib, primarily through cytochrome P450 enzymes, especially CYP3A4 and CYP2C9 (Peng et al. 2004). This metabolism may generate reactive metabolites that can form adducts with cellular macromolecules, resulting in oxidative stress and cellular damage (Kiang et al. 2010). Furthermore, imatinib has been shown to impair mitochondrial respiratory chain activity, resulting in ATP depletion and increased ROS production (Damaraju et al. 2015). Through necrosis or apoptosis, imatinib metabolism can cause oxidative stress that overpowers cellular antioxidant defenses, leading to lipid peroxidation, protein

oxidation, and eventually hepatocyte death (Breccia et al. 2008). Our result showed a significant rise in AOPPs in mice treated with imatinib compared with control. This reflected the evidence of oxidative protein injury, in addition to its consequences on liver function enzymes. The formation of dityrosine cross-links, characteristic of AOPPs, indicates severe oxidative damage and has been associated with various pathological conditions, including chronic kidney disease, diabetes, and cardiovascular disorders (Piwowar et al. 2007). Chlorinated oxidants can react with plasma proteins, leading to the formation of AOPPs, which serve as reliable markers of oxidative stress and inflammation (Witko-Sarsat et al. 1996; Kayali et al. 2006). The formation of oxidized proteins reveals oxidative damage and inflammation that may contribute to a vicious cycle of oxidative injury and inflammation (Kalousová et al. 2005). The accumulation of AOPPs in imatinib-treated animals is consistent with the known pro-oxidant effects of imatinib metabolism (Kiang et al. 2010). However, co-treatment with linalool significantly alleviated the increase in AOPPs in mice administered with imatinib. This protective effect may likely involve both direct free-radical scavenging and indirect enhancement of antioxidant enzyme systems as phenolic hydroxyl group in the structure of linalool can donate

hydrogen atoms to neutralize free radicals, converting them to less reactive species (Huo et al. 2013). Additionally, linalool has been shown to activate the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway, a major regulator of antioxidant response that upregulates the expression of numerous cytoprotective genes (Li et al. 2015).

The increase in plasma transaminases and oxidative stress in the mice exposed to imatinib corroborated the histopathological liver lesions that was observed in this study (Figure 3). The histopathological result of liver organ showed a moderate infiltration and mild necrosis in the livers of mice treated exclusively with imatinib mice compared with no visible lesions were observed in control group. However, co-treatment with linalool protected against alterations in hepatic architecture induced by imatinib. Previous report has shown that activities of serum aminotransferases return to normalcy when hepatic parenchyma heals up and hepatocytes regenerate which supported our finding on the healing effect of imatinib in histopathological result (Thabrew et al. 1987).

In this study, there was also, there is a significant reduction in total thiol concentration following imatinib administration compared with control. Thiols represent the important component of the non-enzymatic antioxidant defense

system in hepatocytes (Lu, 2013). The depletion of thiols during imatinib treatment can occur through direct conjugation with reactive metabolites generated during imatinib metabolism depleting cellular stores (Kiang et al. 2010). However, co-administration of linalool restored thiol levels in the co-treatment group. Linalool can decrease thiol consumption and preserve the cellular antioxidant reservoir through direct radical scavenging and enhancement of antioxidant enzyme activities (Aprotosoie et al. 2014). Furthermore, linalool may facilitate GSH regeneration by maintaining the activity of glutathione reductase, the enzyme responsible for converting GSSG back to GSH (Huo et al. 2013).

Moreover, the present study demonstrated a significant decrease in arylesterase activity following imatinib administration compared with the control group. Paraoxonase-1 (PON1), especially arylesterase is essential for detoxification and defense against oxidative stress (Mackness et al. 2010). Under normal physiological circumstances, arylesterase hydrolyzes a variety of harmful esters and lactones while protecting lipoproteins from oxidation (Furlong et al. 2016). The reduced arylesterase activity observed in mice treated with imatinib may be an indication of higher levels of oxidative stress and toxin exposure. Studies have

shown that under severe oxidative stress, PON1 itself may undergo oxidation, potentially altering changes in its catalytic activity or substrate specificity (Aviram et al. 1999). However, co-treatment with linalool restored the activity of arylesterase relative to imatinib-treated group. This normalization may indicate a reduction in the need for compensatory detoxification reactions and an improvement in hepatocellular function. The free radical-induced oxidation of macromolecules such as lipids has been reported to interact with DNA leading to oxidative DNA damage (Agarwal and Draper, 1992). This establishes the association between oxidative stress and genotoxicity (Mitchell et al. 2004). The capacity of linalool to prevent genotoxicity induced by imatinib in liver of mice was investigated. In this study, administration of imatinib increased the liver percentage of DNA fragmentation in the liver suggesting the induction of DNA strand breaks or genomic instability. This corroborates earlier evidence that imatinib induced chromosomal, mitotic and karyotype aberrations (Fabarius et al. 2007). More importantly, the finding of this study indicated that linalool was potent

against imatinib-induced genotoxicity as revealed by the significant reduction in percentage DNA fragmentation in the group simultaneously treated with linalool when compared to imatinib intoxicated mice. The potency of linalool in genoprotection may be associated with its antioxidant capability.

In conclusion, this study demonstrates that linalool possesses significant hepatoprotective and antigenotoxic properties against imatinib-induced liver toxicity in mice. Its protective effects were evidenced by substantial modulation of plasma transaminases and alkaline phosphatase. It also reduced increase in oxidative protein damage of oxidative protein damage (AOPPs) with concomitant preservation of total thiols and arylesterase activity. These findings suggest that linalool may exert hepatoprotective potential possibly through antioxidant activity. Therefore, linalool may have potential as linalool as a natural adjuvant treatment to lessen the hepatotoxic adverse effects in individuals receiving imatinib therapy for treatment of chronic myeloid leukemia.

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The author declares no conflict of interest.

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Phytochemical Studies of the Fruits of *Vitex agnus-castus* L.

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Abstract

The present study focused on the phytochemical constituents of *the fruits of Vitex agnus-castus* L. (Lamiaceae, subfamily Viticoideae), whose medicinal applications include traditional use in the management of premenstrual syndrome, menstrual irregularities, and hormonal imbalances. It is native to the Mediterranean region and has a long history of utilization in folk medicine, gaining scientific interest due to its rich phytoprofile. The study was performed on the methanolic extract. Using several chromatographic techniques, six compounds (**1–6**) were isolated. Three polar phytochemicals were successfully isolated and identified from the aqueous extract, namely, two iridoid glycosides, agnuside (**1**) and eurostoside (**2**), and a flavonoid C-glycoside, isoorientin (**3**). Three compounds were isolated from the dichloromethane (DCM) extract two of which were identified as methoxylated flavonoids, casticin (**4**), and artemetin (**5**) and the third as the sesquiterpene alcohol, spathulenol (**6**). The structures of all the isolated compounds were determined using 1D (¹H, ¹³C-NMR, DEPT-135, APT) and 2D NMR (COSY, HSQC, HMBC) spectroscopic techniques. Thus, this work contributes to elucidating the chemical composition of *V. agnus-castus* to support its ethnopharmacological use and provide further information on the isolated phytochemicals.

Keywords

Flavone C-glycosides, iridoids, *Vitex agnus-castus* L., methoxy flavonols, sesquiterpene.

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INTRODUCTION

Vitex agnus-castus L. (known as chaste tree or “hayıt” in Turkish) belongs to the *Vitex* genus and is widely distributed in the Mediterranean regions and Western Asia (Wichtl, 2004; Harley et al., 2004). It is a dicotyledonous shrub or small tree, approximately 2–6 m in height characterized by aromatic leaves and terminal inflorescences (Wichtl, 2004). The fruits are small drupes (approximately 3–5 mm in diameter), typically dark purple to black when mature, and each containing a single seed (Wichtl, 2004). *Vitex agnus-castus* is native to the Mediterranean region and parts of Central Asia and has been traditionally cultivated and utilized since antiquity because of medicinal properties, particularly in women’s reproductive health disorders (Van Die et al. 2013; Christie et al. 2008).

Vitex belongs to the Lamiaceae family, which comprises approximately 236 genera and over 7,000 species, many of which are known to produce a variety of monoterpenes, sesquiterpenes, flavonoids, and iridoid glycosides (Harley et al. 2004). The plant has traditionally been used to treat reproductive system disorders in women, especially menstrual problems, PMS, and menopause symptoms, according to Mediterranean and Anatolian folk medicine practices (Van Die et al. 2013;

Christie et al. 2008). Due to these effects, scientists have studied its bioactive compounds, such as iridoids, flavonoids, diterpenoids, and essential oils, making the plant the subject of extensive phytochemical and pharmacological investigation. (Hajdú et al. 2007; Webster et al. 2011).

Vitex agnus-castus is characterized by the presence of several iridoid glycosides such as agnuside, eurostoside, and mussaenosidic acid derivatives, which have been considered important chemotaxonomic markers of the genus (Ono et al. 2003; Webster et al. 2011). Additionally, flavonoids such as casticin, vitexin, artemetin, and orientin derivatives have been identified, and have been reported to exhibit antioxidant, anti-inflammatory, and hormonal regulatory activities (Hajdú et al. 2007; Kucukboyaci and Sener, 2010). The essential oil fraction of *Vitex agnus-castus* fruits and leaves is rich in monoterpenes and sesquiterpenes such as 1,8-cineole, sabinene, limonene, and β -caryophyllene, which contribute to the plant’s characteristic aroma and biological effects (Stojković et al. 2011).

Secondary metabolites, such as iridoid glycosides, terpenoids, and essential oils, are mainly found in high concentrations in the fruits and leaves. They protect the plant

from herbivores, pathogens, abiotic stressors, diseases, and environmental conditions (Hajdú et al. 2007; Webster et al. 2011). Phenolic compounds and flavonoids, on the other hand, occur in reproductive parts and have been associated with the plant's reported biological activities (Hajdú et al. 2007; Kucukboyaci and Sener, 2010). Recent studies have focused on the extraction of diterpenoids and flavonoids because of their anti-inflammatory and antioxidant effects and their ability to regulate neuroendocrine

functions (Hajdú et al. 2007; Kucukboyaci and Sener, 2010).

The present study aims to investigate the phytochemical composition of the fruit extracts of *Vitex agnus-castus* focusing on iridoid glycosides and flavonoid constituents. Various chromatographic techniques were employed to fractionate and isolate pure compounds from the crude extracts. Structural elucidation of the isolated secondary metabolites was performed using 1D-NMR (^1H and ^{13}C NMR, DEPT) and 2D-NMR techniques (COSY, HSQC, and HMBC).

MATERIALS AND METHODS

General experimental procedures

Isolation procedures for secondary metabolites were performed using column chromatography and gradient medium-pressure liquid chromatography (Büchi MPLC, equipped with C-601 and C-605 pump modules, a C-610 pump controller, and a C-605 pump manager) with a Büchi C-615 fraction collector. Silica gel (0.063–0.200 mm, Merck) and LiChroprep C-18 (0.063–0.200 mm, Merck) stationary phases were used in normal- and reverse-phase column chromatography studies, respectively. Sephadex LH-20 was also used in purification studies of the compounds when necessary. NMR measurements of the isolated secondary metabolites were performed on a Bruker

Neo Ascend 500 MHz spectrometer operating at 500 MHz for ^1H NMR and at 125 MHz for ^{13}C , using the XWIN NMR 3.5 software package for data acquisition and processing. CDCl_3 , MeOD and $\text{DMSO-}d_6$ were used as solvents. Chemical shifts (δ) are reported in ppm relative to residual solvent signals. One- and two-dimensional NMR experiments, including ^1H , ^{13}C , DEPT, COSY, HSQC, and HMBC, were performed for structural elucidation. Lyophilization was performed using the CHRIST Alpha 1–4 LD Plus.

Column chromatography was carried out using silica gel 60 (0.063–0.200 mm, Merck) and Sephadex LH-20. Reversed-phase vacuum liquid chromatography (RP-VLC) was performed using LiChroprep

RP-18. Thin-layer chromatography (TLC) analyses were conducted on silica gel 60 F254 aluminum plates and visualized under UV light (254 and 365 nm), followed by spraying with vanillin, anisaldehyde, and sulfuric acid reagents.

Plant material

The fruits of *Vitex agnus-castus* L. were collected from the Güzelyurt region in Northern Cyprus (KKTC). The plant material was air-dried in the shade and stored until use. The plant material was authenticated, and a voucher specimen (NEUN 6884) was deposited at the NEU Herbarium, Near East University, Cyprus.

Extraction and fractionation

The dried fruits (80 g) were powdered and extracted with methanol (500 mL) at room temperature for 48 h. The extract was concentrated under reduced pressure at 40 °C to yield a crude methanolic extract. The extract was diluted with distilled water (25 mL) and partitioned with dichloromethane (50 mL), yielding an aqueous fraction (4.73 g) and a dichloromethane (DCM) fraction (5.91 g).

Isolation of compounds

The isolated compounds were identified as agnuside (**1**), eurostoside (**2**), isoorientin (**3**), casticin (**4**), artemetin (**5**), and spathulenol (**6**).

I- Isolation studies on the aqueous extract of *vitex agnus-castus*:

A total of 4.7360 g of the aqueous extract of *V. agnus-castus* was subjected to reverse-phase vacuum column chromatography. Fractionation was carried out using a gradient elution system composed of methanol and water (10% MeOH in H₂O, 20% MeOH in H₂O, 30% MeOH in H₂O, 40% MeOH in H₂O, 50% MeOH in H₂O, 60% MeOH in H₂O, 70% MeOH in H₂O, 80% MeOH in H₂O, 90% MeOH in H₂O), yielding 13 major fractions, which were subsequently combined based on their thin-layer chromatography (TLC) profiles, as follows: Group A (307 mg), Group B (415 mg), Group C (415 mg), and Group D (140 mg).

Fraction Group B was further purified using column chromatography (CC) on 25 g of silica gel. Elution was performed with a solvent gradient of dichloromethane (DCM), methanol (MeOH), and water (H₂O) in the following ratios: 80:20:1, 80:20:2, 75:25:2.5, 70:30:3, and 60:40:4, with a total volume of approximately 700 mL. Pure compounds **1** (45 mg) and **3** (43 mg) were successfully isolated.

Fraction Group C was subjected to gel chromatography using a Sephadex LH-20 column, with a methanol-water (MeOH-H₂O) gradient solvent system ranging from 50:50 to 100:0, followed by further purification by silica gel column chromatography (10 g) using a solvent system of DCM-MeOH-H₂O in the ratios

86:12:1 and 80:20:1. This process led to the isolation of compound **2** (32 mg).

II- Isolation studies on DCM extract of *vitex agnus-castus*:

A total of 5.9178 g of the *V. agnus-castus* DCM extract was subjected to silica gel column chromatography. The solvent system was meticulously prepared, starting with a first solvent composed of Toluene-EtOAc in a 9:1 ratio, followed by a series of solvents with varying ratios: 8:2, 7:3, 6:4, 5:5, 4:6. Based on the thin-layer chromatography (TLC) observations, the fractions collected from the column chromatography were combined into eight distinct fraction groups (Fr. E– Fr. L)

Fraction Group L (636 mg) was subjected to gel chromatography using a Sephadex LH-20 column for purification, with a

Hexane-DCM-MeOH solvent system. Compound **4** (15 mg) was successfully isolated.

Fraction Group G (266 mg) underwent gel chromatography for purification using a Sephadex LH-20 column, using a solvent system composed of Hexane, DCM, and MeOH. From this process, compound **5** (23 mg) was successfully isolated.

Fraction group K (580 mg) was subjected to column chromatography on 25 g of silica gel, using a solvent system comprising several mixtures: Hexane-Acetone in a 9:1 ratio; Hexane-Acetone-EtOAc in an 8:1:1 and 7:1.5:1.5 ratio; and finally, Hexane-Acetone-EtOAc-MeOH in a 6:1.5:1.5:1 ratio. The pure compound **6** (44 mg) was successfully isolated.

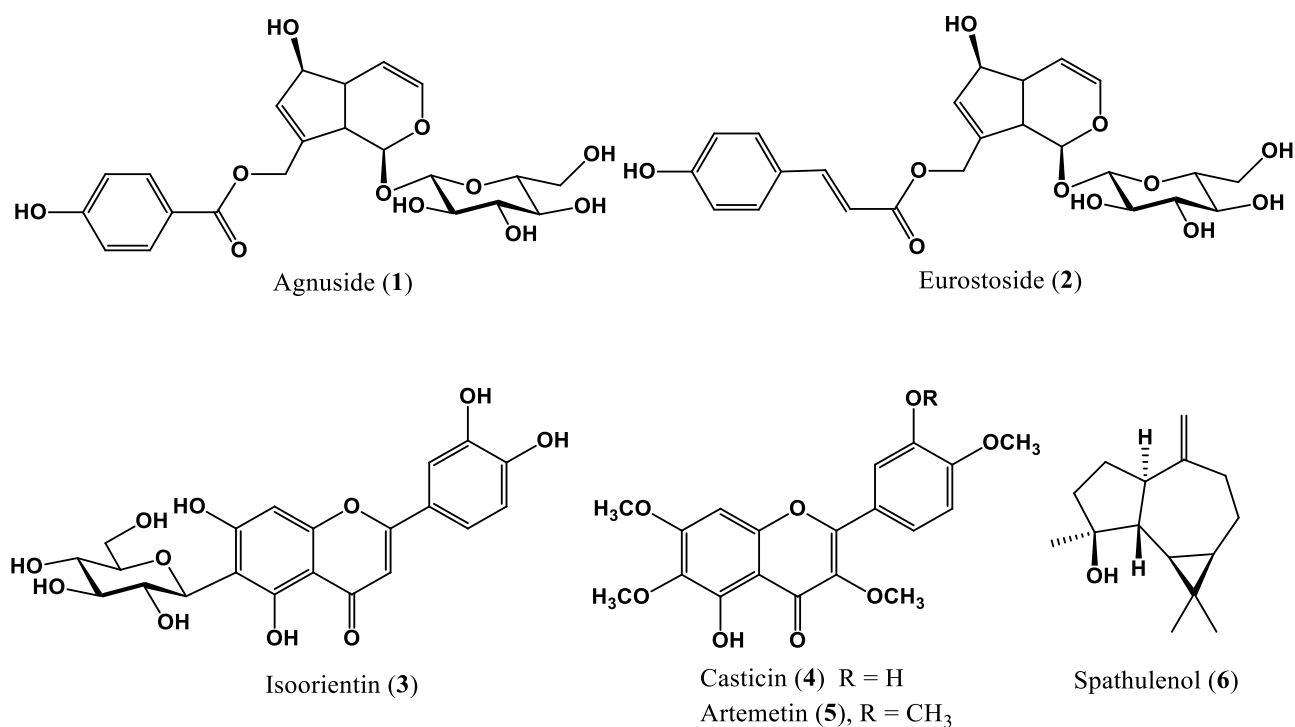


Figure 1: Compounds **1** – **6** isolated from the fruits of *V. agnus-castus* L.

RESULTS AND DISCUSSION

The structures of the isolated compounds (**1** – **6**; Figure 1) were elucidated by extensive spectroscopic analyses, including ^1H and ^{13}C NMR, as well as 2D NMR techniques (COSY, HSQC, HMBC), and by comparison with literature data.

Agnuside (**1**) and Eurostoside (**2**):

The structures of **1** and **2** were elucidated using ^1H -NMR, ^{13}C -NMR, and HMBC spectroscopic analyses, revealing that both compounds are cinnamoylated iridoid glucosides. In the ^{13}C -NMR spectra, both compounds exhibited characteristic signals of an iridoid glycoside skeleton, including an anomeric carbon signal at δ_{C} ~98.11 (C-1), confirming the presence of a β -glycosidic linkage to a glucose moiety. The signals at δ_{C} ~141–143 (C-3 and C-8) and ~132 (C-7), associated with the conjugated double bond of the iridoid core, are consistent with the oxygenated cyclopentanoid-pyran system. Additionally, the iridoid framework skeleton was further supported by the presence of oxygenated carbons such as C-6 (δ_{C} ~83) and C-10 (δ_{C} ~63–64). The sugar unit displayed typical resonances in the range of δ_{C} 62–100, consistent with a glucopyranosyl moiety. In both compounds, aromatic carbon signals at δ_{C} ~122–130 (C-1''), ~132–134 (C-2''/6''),

~116–117 (C-3''/5''), and ~161–164 (C-4''), together with ester carbonyl signals at δ_{C} ~168–169, confirmed the presence of a para-substituted cinnamoyl group esterified to the molecule. Additionally, in **2**, extra olefinic carbon signals at δ_{C} 115.02 and 147.91 indicated an extended conjugated side chain compared to **1**.

The ^1H -NMR spectra supported these findings, with both compounds exhibiting exhibited a β -glucosidic linkage through the presence of an anomeric proton signal at δ_{H} ~5.25 (d, $J \approx 7$ Hz), along with iridoid protons at δ_{H} ~6.36–6.38 (H-3) and ~5.85 (H-7). The oxygenated methylene protons assigned to H₂-10 as an AB system at δ_{H} 5.12 and 5.25 ($J_{\text{AB}} = 14.8$ Hz) were found to be shifted downfield in comparison to those reported for aucubin (δ_{H} 4.37 and 4.19) indicating the site of esterification (Ersoz et al. 2002).

Sugar protons resonated within δ_{H} 3.29–4.70, typical for glucose. Aromatic protons in both compounds appeared as doublets, confirming para-substitution of the phenyl ring, while **2** additionally showed olefinic proton signals at δ_{H} 7.74 (α) and 6.38 (β), supporting the presence of an extended conjugated system arising from two olefinic protons with a large coupling ($J_{\text{AX}} = 15.9$ Hz) of an AX system.

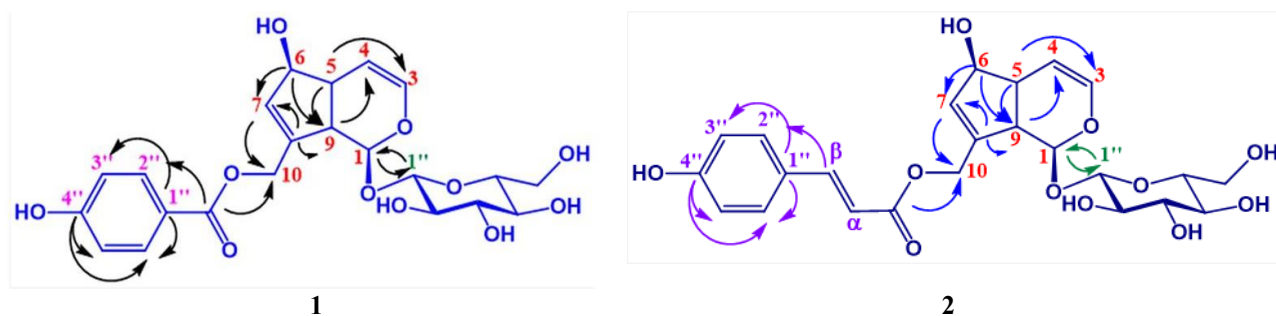


Figure 2: The significant ^{13}C , ^1H -long-range correlations in the HMBC of compounds **1** and **2**.

HMBC correlations further confirmed the structures by establishing key connectivities (Figure 2). In both compounds, the carbonyl carbon of the acyl units (δ_{C} 167.97 and 167.42, respectively) showed a long-range correlation to the oxymethylene protons of the iridoid skeletons (δ_{H} 5.09/4.93; and 5.12/5.25; H_2 -10, J_{AB} 14.8 Hz for **1** and **2**, respectively),

indicating the sites of esterification. Correlations from H-5 to C-3 and C-9, and from H-6 to C-7 and C-9, confirmed the iridoid ring system. Based on these spectroscopic data, **1** was identified as agnuside, while **2** was determined to be eurostoside, both classified as acylated iridoid glucosides previously reported from *Vitex agnus-castus* (Ono et al. 2003; Webster et al. 2011).

Table 1: The ^1H and ^{13}C -NMR Data of Agnuside (**1**) and Eurostoside (**2**) (δ_{H} 500 MHz; δ_{C} 125 MHz, MeOD).

Agnuside (1)				Eurostoside (2)	
C/H	DEPT-135	δ_{C} ppm	δ_{H} ppm, J (Hz)	δ_{C} ppm	δ_{H} ppm, J (Hz)
1	CH	98.11	5.00 d (7.4)	98.11	4.97 d (7.5)
3	CH	141.90	6.35 dd (6.1, 2.0)	141.91	6.34 dd (6.2, 2.0)
4	CH	105.67	5.12 (12.0, 6.1)	105.68	5.12 dd (6.2, 3.9)
5	CH	46.48	2.70 m	46.51	2.69 dddd (1.4, 2.0, 3.9, 7.6)
6	CH	83.03	4.47 ddd (	83.04	4.47 ddd (5.6, 3.5, 1.4)
7	CH	132.61	5.83 br. t (1.4)	132.65	5.81 br. t (1.4)
8	C	143.05	—	142.94	—
9	CH	48.72	2.99 br. t (7.4)	48.72	2.96 br. t (7.7)
10	CH_2	63.80	5.09, 4.92; J_{AB} = 14.8	63.58	5.12, 5.25; J_{AB} = 14.8
Glucose					
1'	CH	100.36	4.70 d (8.0)	100.33	4.70 d (8.0)
2'	CH	75.05	3.24 dd (8.0, 9.0)	75.06	3.25 dd (8.0, 9.0)
3'	CH	78.11	3.38 dd “t” (9.0)	78.11	3.38 dd “t” (9.0)
4'	CH	71.62	3.31†	71.64	3.30†
5'	CH	78.42	3.29 ddd (9.0, 6.0, 2.0)	78.43	3.28 ddd (9.0, 5.4, 1.8)
6'	CH_2	62.90	3.86 dd (11.9, 2.0) 3.66 dd (11.9, 6.0)	62.92	3.87 dd (11.9, 1.8) 3.85 dd (11.9, 5.4)
Acyl moiety					
1''	C	122.26	—	130.04	—
2''/6''	CH	133.86	7.92 d (8.8)	132.42	7.48 d (8.6)
3''/5''	CH	116.39	6.85 d (8.8)	117.00	6.81 d (8.6)
4''	C	163.84	—	161.54	—
α	CH	—	—	115.02	7.66 d (15.9)
β	CH	—	—	147.91	6.38 d (15.9)
C=O	C	167.97	—	169.42	—

† Signal pattern unclear because of overlap.

Isoorientin (3):

The ^{13}C -NMR spectrum of compound 3 displayed 21 carbon resonances, consistent with the presence of a flavone glucoside structure. Among these signals, 15 carbons were attributed to the flavone aglycone skeleton, while the remaining six signals corresponded to a sugar moiety. The characteristic carbonyl carbon of the flavone nucleus was observed at δC 182.82 (C-4), confirming the flavone framework. Oxygenated aromatic quaternary carbons resonated at δC 168.27 (C-2), 165.31 (C-7), 161.75 (C-9), 145.72 (C-3'), and 149.59 (C-4'), indicating extensive hydroxylation of the flavonoid skeleton.

In the ^1H -NMR spectrum, two singlet aromatic proton signals appeared at δH 6.64 and 6.28, assigned to H-3 and H-8, respectively. The presence of the singlet at δH 6.28 assigned to H-8, together with the absence of H-6, indicated substitution at C-6, which is characteristic of C-glycosyl flavones such as isoorientin. The B-ring proton system exhibited signals at δH 6.89 (d, $J \approx 2.0$ Hz, H-2'), 6.65 (d, $J \approx 8.0$ Hz, H-5'), and 7.42 (dd, $J \approx 8.0, 2.0$ Hz, H-6'), demonstrating an ABX coupling pattern typical of a 1,3,4-trisubstituted aromatic

ring. A strongly deshielded hydroxyl proton signal at δH 13.18 was assigned to the chelated 5-OH group, which is characteristic of flavones possessing a free hydroxyl group at C-5 involved in intramolecular hydrogen bonding with the C-4 carbonyl group.

The sugar moiety was identified based on the characteristic proton and carbon resonances between δC 61.59–81.95 and δH 3.16–4.85. The anomeric proton signal at δH 4.85 (d, $J \approx 9.8$ Hz) together with the anomeric carbon at δC 73.33 indicated the presence of a β -glucopyranosyl unit. The relatively upfield chemical shift of the anomeric carbon compared with that typically observed for O-glycosides supported C-glycosylation of the sugar moiety. The remaining sugar carbons resonated at δC 70.72, 78.71, 70.66, 81.95, and 61.59, consistent with a glucose unit directly attached to the flavone skeleton.

Based on these spectroscopic observations, this compound was identified as isoorientin, namely, luteolin-6-C- β -D-glucopyranoside, a well-known C-glycosyl flavone previously reported from *Vitex agnus-castus* (Kucukboyaci and Sener, 2010).

Table 2: Combined ^1H and ^{13}C -NMR Data of Isoorientin (**3**) (δ_{H} 500 MHz; δ_{C} 125 MHz, DMSO- d_6).

C No.	DEPT	δ_{C} ppm	δ_{H} ppm, J (Hz)
2	C	168.27	—
3	CH	104.50	6.64 s
4	C	182.82	—
5	C	128.52	—
6	C	113.00	—
7	C	165.31	—
8	CH	113.32	6.28 s
9	C	161.75	—
10	C	119.45	—
1'	C	122.25	—
2'	CH	119.23	6.89 (d, $J \approx 2.0$)
3'	C	145.72	—
4'	C	149.59	—
5'	CH	122.31	6.65 (d, $J \approx 8.0$)
6'	CH	128.25	7.42 (dd, $J \approx 8.0, 2.0$)
5-OH	-	-	13.18
Glucose			
1''	CH	73.33	4.85 (d, $J \approx 9.8$)
2''	CH	70.72	
3''	CH	78.71	
4''	CH	70.66	3.93–3.16 [†]
5''	CH	81.95	
6''	CH	61.59	

[†] Signal pattern unclear due to overlapping.

Casticin (**4**) and Artemetin (**5**):

The structures of **4** and **5** were elucidated using ^1H -NMR, ^{13}C -NMR, and HMBC analyses, confirming that both compounds are polymethoxylated flavonoids (Table 3). In the ^{13}C -NMR spectra, both compounds exhibited 15 carbon signals consistent with a flavonoid skeleton. Signals between δ 55–61 ppm confirmed the presence of methoxy ($-\text{OCH}_3$) groups, with four methoxy groups in compound **4** and five methoxy groups in compound **5**. The ^1H -

NMR spectra of both compounds showed aromatic proton resonances in the range of δ 6.47–7.71 ppm. The B-ring proton system displayed a characteristic pattern at δ ~7.6–7.7 (d, $J \approx 2.0$ Hz), δ ~7.6–7.7 (dd, $J \approx 8.8, 2.0$ Hz), and δ ~6.9–7.0 (d, $J \approx 8.8$ Hz), corresponding to a 1,3,4-trisubstituted aromatic system. In both compounds, the A-ring proton H-8 appeared as a singlet at δ ~6.47–6.48, and the absence of the H-3 proton signal confirmed substitution at C-3, supporting the flavonol-type structure.

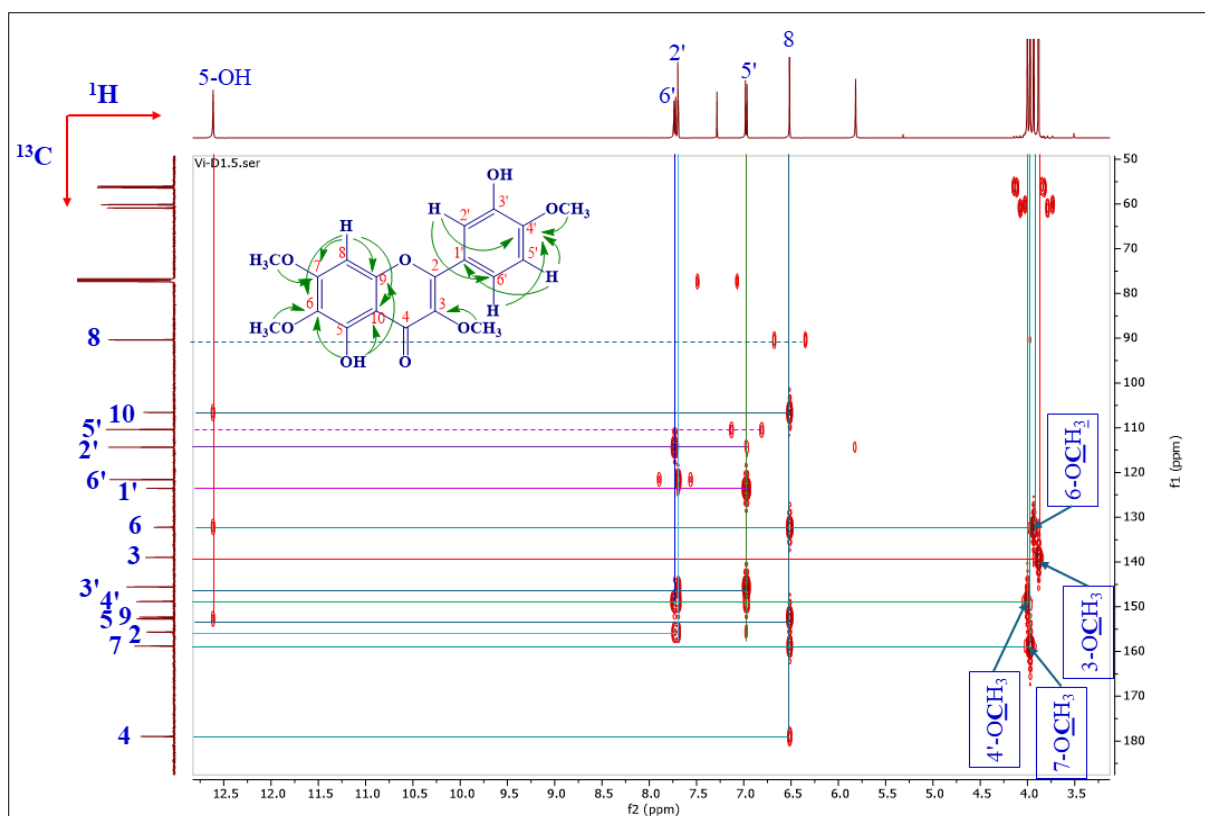


Figure 3. The HMBC of Casticin (**4**) and the significant ^{13}C , ^1H -long-range correlations.

HMBC correlations further established the substitution pattern, where H-8 showed correlations with C-6, C-7, C-9, and C-10, confirming A-ring substitution, while methoxy proton signals ($\delta \sim 3.90$) exhibited correlations with key quaternary carbons. Accordingly, the methoxy groups were assigned at C-3, C-6, C-7, and C-4' in **4**

(Figure 3), and at C-3, C-6, C-7, C-3', and C-4' in **5**. Based on these spectroscopic data, **4** was identified as 5,3'-dihydroxy-3,6,7,4'-tetramethoxyflavone (casticin), while **5** was determined to be 5-hydroxy-3,6,7,3',4'-pentamethoxyflavone (artemetin). The NMR data for these compounds were in good agreement with those previously reported data [6,8].

Table 3: ^1H and ^{13}C -NMR Data of Casticin (**4**) and Artemetin (**5**) with HMBC Correlations (δ_{H} 500 MHz; δ_{C} 125 MHz, CDCl_3).

Casticin (4)				Artemetin (5)		
C/H	δ_{C} , ppm	δ_{H} , ppm), J (Hz)	HMBC	δ_{C} , ppm	δ_{H} , ppm), J (Hz)	HMBC
2	152.88	—	—	151.4	—	—
3	139.16	—	—	138.0	—	—
4	179.15	—	—	180.0	—	—
5	155.85	—	—	151.9	—	—
5-OH	—	12.57 s	—	—	12.58 s	C-6, C-9, C-10
6	132.41	—	—	131.5	—	—
7	158.97	—	—	158.0	—	—
8	90.54	6.47 s	C-6, C-7, C-9	89.8	6.48 s	C-6, C-7, C-9, C-10
9	152.51	—	—	105.7	—	—
10	106.76	—	—	164.0	—	—
1'	123.72	—	—	110.7	—	—
2'	114.52	6.92 d (2.2)	C-4', C-6'	116.8	7.67 d (2.2)	C-4', C-6'
3'	145.73	—	—	121.5	—	—
4'	149.01	—	—	150.0	—	—
5'	110.57	6.93 d (8.6)	C-3'	122.1	6.98 d (8.6)	—
6'	121.76	7.69 dd (8.6, 2.2)	C-4'	135.0	7.71 dd (8.6, 2.2)	C-4'
3-OMe	60.33	3.84 s	C-3	59.7	3.85 s	C-3
6-OMe	61.08	3.89 s	C-6	60.1	3.87 s	C-6
7-OMe	56.51	3.93 s	C-7	55.4	3.93 s	C-7
3'-OMe	—	—	—	55.7	3.85 s	C-3'
4'-OMe	56.24	3.96 s	C-4'	55.5	3.91 s	C-4'
3'-OH	—	5.77 s	C-2'	—	—	—

Spathulenol (6):

The ^{13}C -NMR spectrum of compound **6** revealed 15 carbon signals, consistent with a sesquiterpene skeleton (Table 4). DEPT-135 analysis revealed the presence of three methyl groups, five methylene carbons, four methine carbons, and three quaternary carbons. Among these, signals at δ_{C} 153.66 (C-10) and 106.49 (C-15) indicated the presence of an exocyclic double bond, characteristic of spathulenol-type sesquiterpenes, which was further supported by the ^1H -NMR spectrum, which showed two broad doublets at δ_{H} 4.67 and 4.64 assigned to the olefinic methylene protons H-15a and H-15b, supporting the presence of the exocyclic double bond

attached to C-10. The oxygenated quaternary carbon observed at δ_{C} 81.21 was assigned to C-4, confirming the presence of an oxygenated quaternary carbon at this position.

The ^1H -NMR spectrum shows three methyl singlets at δ_{H} 1.23 (H-12), 1.02 (H-13), and 1.25 (H-14), indicating three tertiary methyl groups attached to quaternary carbons. The methine proton H-1 resonated at δ_{H} 2.18 as a ddd ($J = 6.1, 10.8, 16.5$ Hz), while H-5 appeared at δ_{H} 1.28 as a doublet ($J = 10.8$ Hz). The highly shielded methine proton at δ_{H} 0.44 (dd, $J = 9.5, 11.2$ Hz) was assigned to H-6, which is characteristic of the compact tricyclic skeleton of spathulenol.

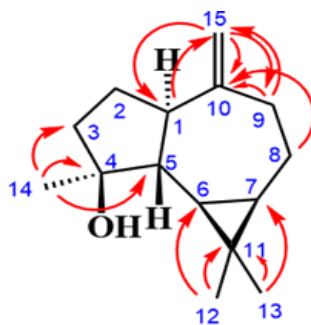


Figure 4: Significant HMBC correlations observed for Spathulenol (**6**).

The HMBC experiment further supported the proposed structure. The olefinic methylene protons H-15 showed correlations with C-1 and C-10, confirming the attachment of the exocyclic double bond at C-10. H-6 exhibited a correlation with the oxygenated quaternary carbon C-4, supporting the hydroxyl substitution at this position. The methyl protons H-12 correlated with C-6 and C-11, whereas H-13 showed correlations with C-7 and C-11,

confirming their relative positions within the bicyclic framework. Additionally, H-14 displayed correlations with C-3, C-4, and C-5, supporting its attachment adjacent to the oxygenated carbon C-4.

Based on these spectroscopic findings and comparison with reported literature data, compound **6** was identified as spathulenol, a tricyclic sesquiterpene alcohol commonly reported in essential oils and medicinal plants (De Albuquerque et al. 2007).

Table 4: The ^1H - and ^{13}C -NMR data of Spathulenol (**6**) and significant HMBC correlations (^1H 500 MHz; ^{13}C 125 MHz, CDCl_3).

C/H Atom	DEPT 135	^{13}C ppm	Spathulenol (6)	
			^1H ppm, J (Hz)	HMBC from H to C
1	CH	53.61	2.18 ddd (6.1/10.8/16.5)	C-15
2	CH ₂	26.93	1.87 m / 1.60 m	-
3	CH ₂	41.96	1.76 m / 1.55	-
4	C	81.21	-	-
5	CH	54.55	1.28 d (10.8)	-
6	CH	30.12	0.44 dd (9.5/11.2)	C-4
7	CH	27.70	0.69 ddd (6.1/9.5/11.4)	-
8	CH ₂	24.99	1.95 m / 0.98 m	-
9	CH ₂	39.08	2.40 ddd (13.2/6.2/1.2) 2.01 br. d (13.2)	-
10	C	153.66	-	C-15
11	CH ₂	20.49	-	C-15
12	CH ₃	28.87	1.23 s	C-6, C-11
13	CH ₃	16.56	1.02 s	C-7, C-11
14	CH ₃	26.30	1.25 s	C-3, C-4, C-5
15	CH ₂	106.49	4.67 br. d / 4.64 br. d	C-1, C-10

CONCLUSION

This study reports on the phytochemical investigation of the fruits of *Vitex agnus-castus*, which led to the isolation of six compounds, including flavonoids, iridoid glycosides, and a sesquiterpene. Their structures were elucidated using comprehensive 1D and 2D NMR spectroscopic techniques.

The identified compounds, namely agnuside (1), eurostoside (2), isoorientin

(3), casticin (4), artemetin (5), and spathulenol (6), contribute to the chemical characterization of this medicinal plant.

The results support previous reports on the richness of *V. agnus-castus* in bioactive secondary metabolites and highlight the importance of NMR spectroscopy in the structural elucidation of natural products.

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The author declares no conflict of interest.

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Coumarin-Piperazine Hybrids: An Investigation of Molecular Structure and Biological Activity (A Mini Review)

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Abstract

In this review, the effects of coumarin-piperazine hybrids on various biological targets are evaluated in light of current literature data (2013-2026). Coumarin-piperazine hybrids have been demonstrated to possess a wide range of pharmacological potential, including antibacterial, antimicrobial, antimalarial, antiviral, anticancer, antidiabetic, anti-Alzheimer's, and antitumor activities. In the context of the global health challenges posed by microbial resistance and neurodegenerative diseases, the high efficacy demonstrated by these hybrid molecules in preclinical studies is noteworthy. Furthermore, structure-activity relationship (SAR) studies indicate that the linker structure connecting the coumarin ring to the piperazine unit plays a decisive role in determining biological activity.

Overall, coumarin-piperazine hybrids have emerged as one of the most notable hybrid systems in modern drug design due to their structural diversity and ability to interact with multiple biological targets. While studies reported in the literature demonstrate significant progress, particularly in applications targeting anticancer and neurodegenerative diseases, it is evident that areas such as tyrosinase inhibition, multitarget antibacterial approaches, and translational pharmacology still hold considerable potential for further development.

Keywords

Coumarin, piperazine, hybrid molecules, pharmacological activity.

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INTRODUCTION

The primary objectives of contemporary pharmaceutical research are the development of molecules that exhibit high efficacy, low toxicity, and suitable pharmacokinetic properties. Such challenges may often stem from an inability to achieve the desired efficacy, or alternatively, a necessity to minimise adverse effects. Consequently, researchers have adopted multitarget drug design approaches, with the objective of overcoming these limitations. The hybrid molecule strategy developed in this context aims to simultaneously modulate multiple biological mechanisms within a single molecular structure by covalently linking different pharmacophores (Shaveta et al. 2016). The combination and derivatization of different pharmacophore groups at appropriate positions on a single molecule have been shown to result in significant changes in biological activity profiles, thereby contributing to the development of new drug candidates. This approach offers several advantages, including increased therapeutic efficacy, reduced drug resistance, and minimized adverse effects (Feng et al. 2020; Xiao et al. 2020).

The coumarin ring is a heterocyclic structure that is notable for its significant biological activity (Phutdhawong et al. 2021; Annunziata et al. 2020). It is a natural

constituent of many sources and can be synthesized through a variety of procedures (Pandhurnekar et al. 2026). The broad range of properties exhibited by these molecules, including anticancer (Koley et al. 2024), anti-inflammatory (Han et al. 2025), antioxidant (Santu and Manoj, 2025), antiviral (Auwal et al. 2026), antimicrobial (Wilinski et al. 2025), and neuroprotective effects (Carneiro et al. 2026), is well established, contributing to their extensive utilization in medicinal chemistry research over recent decades. The development of selective compounds targeting different biological targets is facilitated by various structural modifications of coumarin (Stefanachi et al. 2018).

Moreover, the piperazine ring is one of the most widely used nitrogen-containing heterocyclic structures in pharmaceutical chemistry (Faizan et al. 2024). Molecules containing piperazine offer several advantages, including high bioavailability, favorable pharmacokinetic properties, and water solubility (Zhang et al. 2021). Furthermore, due to their capacity to interact with a wide range of biological targets, particularly serotonin, dopamine, and histamine receptors, these compounds are incorporated into the structures of numerous pharmaceuticals employed in the treatment of neurological diseases,

infections, and cancer. The fact that a significant number of FDA-approved drugs contain a piperazine ring, such as olaparib, palbociclib, imatinib, abemaciclib, and rociletinib, clearly demonstrates the importance of this structure in drug design. Hybrid systems, which are obtained by combining coumarin and piperazine structures within a single molecule, combine the advantages of both pharmacophores to offer a broad spectrum of biological activities. The combination of the coumarin scaffold's capacity for strong interactions with target proteins and the

piperazine ring's contributions to solubility and pharmacokinetic properties facilitates the development of a new generation of multitarget drug candidates. Consequently, research on coumarin-piperazine hybrids has increased significantly in recent years, and the anticancer, antibacterial, anti-inflammatory, antituberculosis, neuroprotective, antidepressant, and acetylcholinesterase inhibitory activities of these molecules have been investigated. The studies and molecules discussed in this article are summarized in Table 1.

Table 1: Overview of therapeutic targets, linker types, structure-activity relationship (SAR) findings, and biological activity outcomes of coumarin-piperazine hybrids.

Therapeutic Area / Target	Structure	Key SAR Findings	Key Results	Ref
Tyrosinase	<i>N</i> -phenylacetamide	2,4-dimethyl substituents enhance activity.	Best activity in 2,4-dimethyl derivative.	(Mahdi et al. 2026)
Antibacterial	Alkyl chain / indole and cyanoenone /cinnamic acid	Electron- donating and withdrawing groups affects activity.	Successful against <i>E. Coli</i> , <i>P. aeruginosa</i> , <i>S. pyogenes</i> , and <i>S. aureus</i>	(Patel et al. 2023) (Zeng et al. 2023) (Zala and Kumari 2023)
Anti-inflammatory	C8-Mannich base	C8-aryl-piperazine substitution is critical.	p-nitro and naphthylmethyl derivatives stronger than IND and L-NAME	(Buran et al. 2021)
Antitubercular (MTb)	acetamide / Triazole	-	Active for Mycobacterium tuberculosis	(Kuldeep et al. 2025) (Jaggal et al. 2025)
Anti-tumor	thiazole	Functional groups on the piperazine ring is crucial.	Over 50% inhibition of Gal-1	(Sethi et al. 2024)
Anticancer	Carboxamide	-	Inhibite eEF-2K, selective anticancer agents	(Onder et al. 2020)
Anticancer (CA IX/XII)	Derivatives as Sulfonamide	Thiophene-2-yl enhances activity	hCA IX inhibition	(Ghouse Mahammad et al. 2023)
Anticancer (EGFR)	1,2,3-triazole	Phenyl ring substituents are decisive.	4-chloro-3,5-dimethoxy more potent than erlotinib	(Gangadhar et al. 2023)
Neurological (5-HT)	-	-	Antidepressant/Antipsychotic potential comparable with commercial drugs	(Wang et al. 2021) (Chen et al. 2013) (Ostrowska et al. 2021)
Anti-Alzheimer (AChE)	-	Electron-donating groups and saturation affects the activity	Multi-targetting	(Sanad and Mekky 2022) (Shukur et al. 2021)

Dermatological application and tyrosinase inhibition

Scientific studies have demonstrated the effectiveness of coumarin derivatives as tyrosinase inhibitors. Tyrosinase is an enzyme that plays a critical role in melanin biosynthesis, and functional abnormalities in this enzyme have been linked to various dermatological disorders, such as hyperpigmentation. The inhibitory effects of the newly synthesized coumarin-piperazine-*N*-phenylacetamide derivatives (Figure 1) on tyrosinase enzyme were evaluated under *in vitro* conditions, and all derivatives were found to exhibit significant

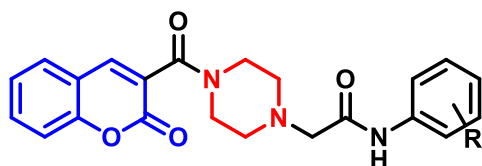


Figure 1: Coumarin-piperazine-*N*-phenylacetamide derivatives acting as tyrosinase inhibitors.

Antibacterial and anti-inflammatory activities

The combination of the coumarin core's antimicrobial properties and the piperazine ring's capacity to exhibit high affinity for biological targets has facilitated the development of new molecules particularly for the treatment of infectious diseases. The growing need for new antimicrobial agents due to increasing antibiotic resistance has led to intensive research into the potential of coumarin-piperazine hybrids in this field. It has been elucidated that coumarin-piperazine hybrids (Figure 2) derived from

inhibitory activity. The 2,4-dimethyl-substituted derivative exhibited the highest activity, with the lowest IC₅₀ value. These findings demonstrate that these hybrid molecules have the potential to serve as effective molecular scaffolds for the development of new tyrosinase inhibitors (Mahdi et al. 2026).

Although the potential of coumarin-piperazine hybrids for dermatological applications has been demonstrated through tyrosinase inhibition, the scope of these hybrid systems is not limited to pigmentation disorders.

coumarin, synthesized from malonic acid and *m*-cresol, have been reported to exhibit antibacterial activity against strains of *E. coli*, *P. aeruginosa*, *S. pyogenes*, and *S. aureus*. In addition, it was found that the compound 4-(3-(4-benzhydrylpiperazin-1-yl)propoxy)-7-methyl-2*H*-chromen-2-one was found to exhibit both antibacterial and anticancer activities. The IC₅₀ values of this compound were found to be better than those of doxorubicin. Based on this finding, molecular modelling studies proposed the compound as a potential drug candidate (Patel et al. 2023).

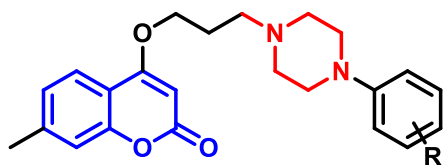


Figure 2: Representative coumarin-piperazine hybrids synthesized from malonic acid and m-cresol showing antibacterial activity.

A study was designed to develop strategies to overcome antibacterial resistance. Zeng et al. integrated indole and cyanoenone units into the coumarin-piperazine structure (Figure 3). These compounds were found to exhibit antibacterial activity by disrupting bacterial cell integrity and interacting with

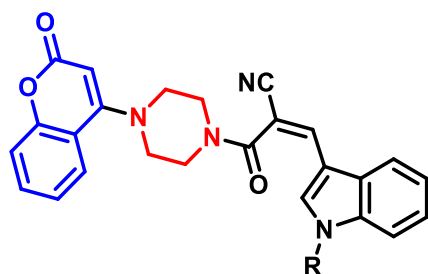


Figure 3: Indole and cyanoenone-integrated coumarin-piperazine hybrids with antibacterial potential.

Another study with a similar objective involved the synthesis of 13 different cinnamic acid derivatives of coumarin-piperazine hybrids (Figure 4). These compounds were found to exhibit antimicrobial activity by forming hydrogen bonds with the Arg136 amino acid residue of the DNA gyrase enzyme. The 4-fluorophenyl piperazine derivative in particular, exhibited the strongest activity against an *E. coli* strain,

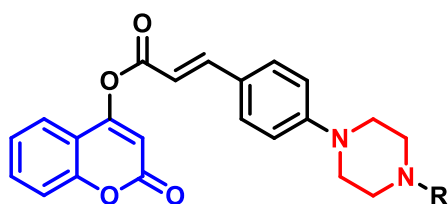


Figure 4: Cinnamic acid derivatives of coumarin-piperazine hybrids as potential DNA gyrase inhibitors.

DNA gyrase, which plays a pivotal role in DNA replication. Specifically, the 4-chlorobenzyl derivative was found to be effective against *P. aeruginosa* at a lower minimum inhibitory concentration (MIC) than norfloxacin, with negligible hemolytic activity (Zeng et al. 2023).

with a minimum inhibitory concentration (MIC) of 12.5 $\mu\text{g/mL}$. Molecular docking analyses revealed that combining the piperazine core with the coumarin structure and adding an electron-withdrawing substituent could make the compound effective against bacteria and fungi. ADME confirmed that the compounds possessed suitable pharmacokinetic properties (Zala and Kumari, 2023).

While derivatization at the C3, C4, and C7 positions of the coumarin ring is a common strategy, coumarin-piperazine compounds derived from the C8 position through the Mannich reaction have also been investigated for their potential anti-inflammatory activity (Figure 5). These compounds have been shown to reduce prostaglandin E2 (PGE2) levels and inhibit nitrite production.

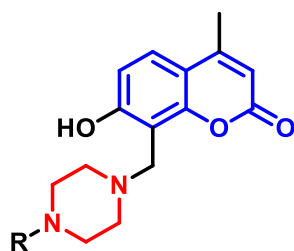


Figure 5: C8-substituted coumarin-piperazine derivatives investigated for anti-inflammatory potential.

Studies on antimicrobial activity have shown that coumarin-piperazine hybrids can inhibit bacterial growth and modulate various biological processes. This has provided a new focus for research into the impact of these hybrid systems on infection-related inflammatory processes and the mechanisms underlying chronic diseases. Subsequent studies have also demonstrated the anti-inflammatory antitubercular potential of these compounds, further expanding their pharmacological applications.

Antitubercular potential

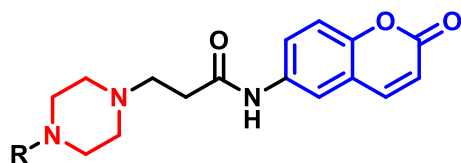


Figure 6: Lipophilic coumarin-piperazine-acetamide derivatives targeting Mycobacterium tuberculosis thymidylate monophosphate kinase.

Notably, p-nitro and naphthylmethyl piperazine derivatives have been reported to exhibit greater activity than indomethacin (IND) and N(G)-nitro-L-arginine methyl ester hydrochloride (L-NAME). These results suggest that aryl-piperazine groups linked to the coumarin structure at the C8 position may play an important role in anti-inflammatory activity (Buran et al. 2021).

Subsequent studies have also demonstrated the anti-inflammatory and antituberculosis potential of these compounds, further expanding their pharmacological applications. Kuldeep et al. Synthesized 14 different lipophilic coumarin-piperazine-acetamide derivatives (Figure 6) with the aim of achieving antitubercular activity and inhibiting the Mycobacterium tuberculosis thymidylate monophosphate kinase (MTb TMPK) enzyme. The results suggest that these compounds could be evaluated in preclinical studies (Kuldeep et al. 2025).

Furthermore, studies investigating the antituberculosis, anticancer, anti-inflammatory, and antioxidant activities of coumarin and piperazine units fused to a triazole ring (Figure 7) were conducted. It was found that the 7-methyl, 7,8-benzo, 7-

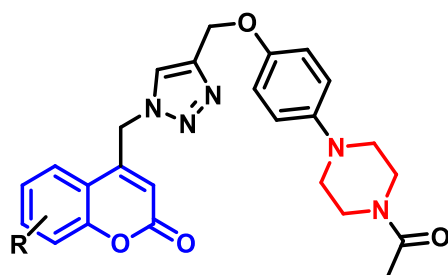


Figure 7: Triazole-fused coumarin-piperazine hybrids with diverse pharmacological activities.

Anti-tumor and anti-cancer properties

One of the most extensively researched areas for coumarin-piperazine hybrids is cancer treatment. As cancer is a multifactorial disease, treatment approaches targeting a single biological target often fail to produce satisfactory results. This has led to a growing focus on hybrid designs that combine different pharmacophores within the same molecule. These hybrid designs represent a key strategy in the development of new anticancer agents capable of targeting multiple signalling pathways at once. Coumarin-piperazine hybrids have also produced effective results against various tumour-associated targets, including

chloro, and 7,8-dimethyl derivatives exhibited high activity against the *Mycobacterium tuberculosis* H37Rv strain, and the 7,8-benzo derivative also exhibited anti-inflammatory, antioxidant, and anticancer properties (Jaggal et al. 2025).

carbonic anhydrases, tyrosine kinases, and translation-regulating proteins.

Studies aimed at improving the poor pharmacological properties of carbohydrate-based inhibitors resulted in the synthesis of new hybrid molecules by adding a thiazole ring to the coumarin-piperazine structure (Figure 8). The resulting compounds exhibited over 50% inhibition of Galectin-1 (Gal-1), a protein associated with tumour survival and immune suppression. The coumarin core has been shown to enhance binding affinity, while the functional groups on the piperazine ring can positively or negatively affect activity through hydrogen-bond interactions (Sethi et al. 2024).

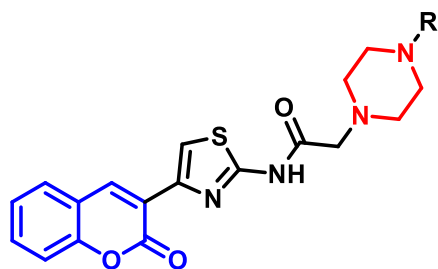


Figure 8: Thiazole-linked coumarin-piperazine hybrids evaluated as Galectin-1 inhibitors.

In anticancer research, the effects of coumarin-piperazine hybrids on carbonic anhydrase (CA) enzymes have been extensively investigated. Chandra et al. found that the synthesized hybrid molecules (Figure 9) exhibited selective inhibition

against the hCA IX and hCA XII isoforms. The compound with a K_i value of $0.58 \mu\text{M}$ effectively inhibited the hCA IX enzyme, an enzyme associated with malignant proliferation and metastasis (Chandra et al. 2021).

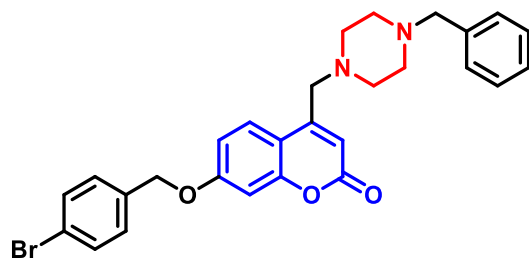


Figure 9: Coumarin-piperazine hybrid molecules evaluated for selective inhibition of hCA IX and hCA XII isoforms.

Synthesized coumarin-piperazine-sulfonamide hybrids (Figure 10) have also been reported to exhibit antitumour activity and inhibit CA IX/XII enzymes. Structure-activity relationship studies have shown that hydroxyl groups on the coumarin ring

reduce activity, whereas phenyl and thiophene-2-yl groups in the piperazine ring increase activity. Notably, the thiophene-2-yl derivative exhibited significant selectivity for CA IX enzyme (Ghouse Mahammad et al. 2023)..

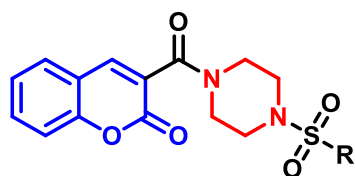


Figure 10: Coumarin-piperazine-sulfonamide hybrids with antitumor activity.

In another study conducted as part of cancer research, the eEF-2K enzyme, which plays a role in the development of malignant tumors, was targeted. It was demonstrated that the synthesized coumarin-piperazine

hybrids (Figure 11) effectively inhibited the eEF-2K protein in breast cancer cells at concentrations ranging from 1.0 to $2.5 \mu\text{M}$ and could be considered as selective anticancer agents (Onder et al. 2020).

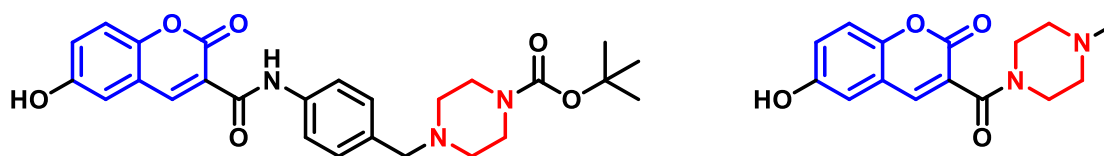


Figure 11: Coumarin-piperazine hybrids targeting eEF-2K protein for anticancer activity.

The importance of the piperazine ring in anticancer drug design is further supported by its presence in FDA-approved drugs such as olaparib, palbociclib, imatinib, abemaciclib, and rociletinib. In this context, the newly synthesized coumarin-piperazine-1,2,3-triazole hybrids (Figure 12) target the EGFR tyrosine kinase enzyme, a key target in breast cancer. The

4-chloro-3,5-dimethoxy derivative exhibited higher activity than the standard drug 5-fluorouracil and stronger EGFR inhibition than erlotinib. Structure-activity relationship studies have shown that substituents on the phenyl ring directly affect activity (Gangadhar et al. 2023).

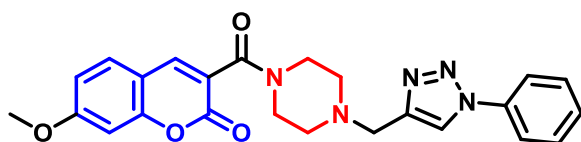


Figure 12: Coumarin-piperazine-1,2,3-triazole hybrids designed to target EGFR tyrosine kinase.

Examining coumarin-piperazine hybrids exhibiting anticancer activity reveals that these molecules can affect different biological targets. The inhibition of proteins that play critical roles in tumor development, such as carbonic anhydrase IX/XII, EGFR, and eEF-2K, demonstrates that the hybrid molecule approach is suitable for multitarget therapeutic strategies. Notably, aromatic groups attached to the piperazine ring and appropriate electronic arrangements in the coumarin core may enhance cytotoxic activity.

Neurological effects and neurodegenerative diseases

The ability of coumarin and piperazine pharmacophores to interact with numerous biological targets affecting the central nervous system has led to the evaluation of these hybrid structures for the treatment of neuropsychiatric and neurodegenerative diseases. Specifically, studies targeting proteins associated with the serotonin, dopamine, and cholinergic systems demonstrate that coumarin-piperazine hybrids represent an important area of research in central nervous system drug design.

In investigations conducted on the central nervous system, the results demonstrate potent antidepressant properties for some coumarin-piperazine derivatives (Figure

13), with -CH₂Ph para-fluoro substituted variants exhibiting activity comparable to that of existing drugs. Molecular docking studies have shown that this activity may

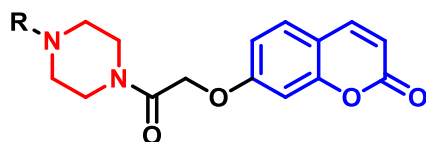


Figure 13: Coumarin-piperazine derivatives with potential antidepressant effects targeting the 5-HT receptor.

In the context of psychiatric disorders, the effects of coumarin-piperazine derivatives (Figure 14) on dopamine D₂, D₃, and serotonin 5-HT_{1A}, 5-HT_{2A} receptors have been examined, and some derivatives were

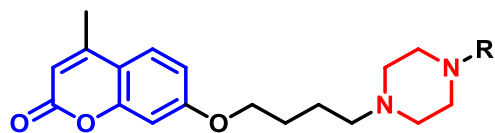


Figure 14: Coumarin-piperazine derivatives evaluated for antipsychotic activity.

In studies targeting the central nervous system, the effects of coumarin-piperazine hybrids (Figure 15) on the 5-HT_{1A} and 5-HT_{2A} receptors have also been investigated. The effects of substituents on the piperazine

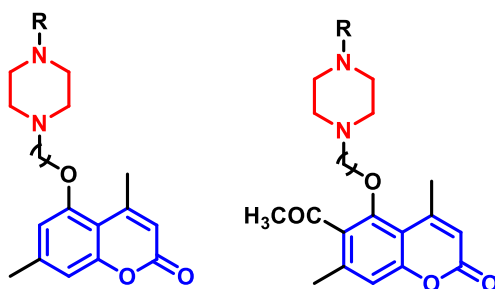


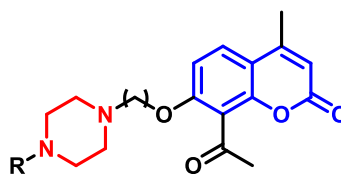
Figure 15: Coumarin-piperazine hybrids investigated for 5-HT_{1A} and 5-HT_{2A} receptor antagonism.

Another area of research in which coumarin-piperazine hybrids have attracted attention is neurodegenerative diseases. Alzheimer's disease (AD) is the most common cause of dementia worldwide,

involve interactions with 5-hydroxytryptamine (5-HT) receptors which are involved in anxiolytic and sedative effects (Wang et al. 2021).

found to exhibit antipsychotic activity with a lower side-effect profile than risperidone. These results suggest these hybrids may be potential candidates for the treatment of schizophrenia (Chen et al. 2013).

ring and the length of the alkyl bridge on receptor binding were evaluated, and it was demonstrated that derivatives bearing a methoxyphenyl group were found to exhibit the highest antagonist activity (Ostrowska et al. 2021).



significantly significantly reduces quality of life. The acetylcholinesterase (AChE) enzyme plays a key role in the pathogenesis of the disease and is responsible for the hydrolysis of acetylcholine in the synaptic

cleft. AChE inhibitors are among the primary drug classes currently used for the symptomatic treatment of Alzheimer's disease treatment.

Sanad and Mekky showed coumarin-piperazine hybrids (Figure 16), synthesized in high yields using ultrasound-assisted methods, exhibit both acetylcholinesterase inhibitory and antioxidant activities. The

attachment of electron-donating groups to the 6th position of the coumarin ring enhances inhibitory activity. Specifically, the 6-methoxy-2H-chromen-2-one derivative exhibits AChE inhibitory and antioxidant activities at a concentration of 25 μ M, comparable to that of donepezil (Sanad and Mekky, 2022).

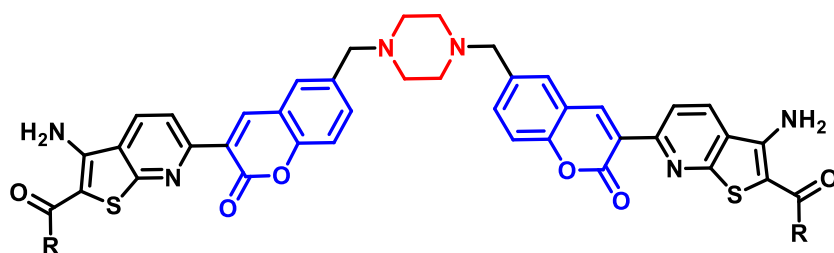


Figure 16: Coumarin-piperazine hybrids with acetylcholinesterase inhibitory and antioxidant properties.

Finally, in studies where urolithins, which are coumarin derivatives, were fused to piperazine structures via an amide bridge (Figure 17), it was determined that the synthesized compounds were found to act as both cholinesterase inhibitors and monoamine oxidase B (MAO-B) inhibitors. MAO-B enzyme is responsible for the metabolic breakdown of dopamine in the synaptic cleft. Among the compounds synthesized in

two different series, the THU series, which has a saturated ring structure, was reported to exhibit stronger activity, while the THU derivative bearing a propargyl group was found to be the most effective MAO-B inhibitor. These results indicate coumarin-piperazine hybrids may contribute to multitarget drug design for the treatment of neurodegenerative diseases (Shukur et al. 2021).

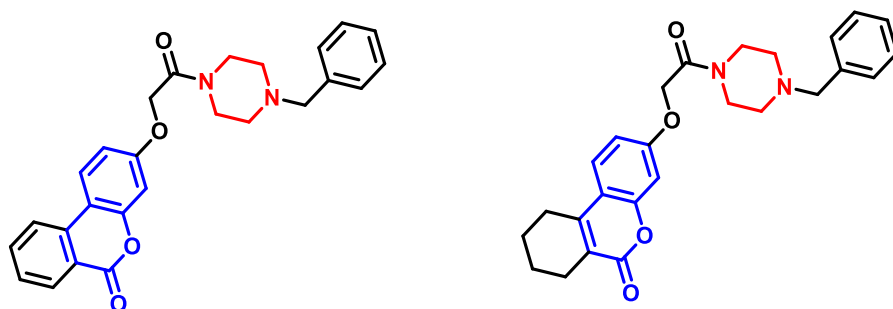


Figure 17: Urolithin-piperazine amide hybrids as multitarget agents for Alzheimer's disease.

Studies on neurodegenerative diseases have revealed that coumarin-piperazine hybrids exhibit acetylcholinesterase, antioxidant, and MAO-B inhibitory properties,

potentially targeting the complex pathology of Alzheimer's disease through multiple mechanisms.

CONCLUSION

Investigations of coumarin-piperazine hybrids clearly demonstrate that these molecular systems possess a broad spectrum of pharmacological activity. These hybrid structures can exhibit antibacterial, antitubercular, anti-inflammatory, antidepressant, antipsychotic, and anti-Alzheimer's, and anticancer effects, and they can also provide inhibitory effects against various biological targets such as tyrosinase, acetylcholinesterase, carbonic anhydrase, DNA gyrase, EGFR, and eEF-2K.

When the studies reported in the literature are considered collectively, it is evident that the biological activities of coumarin-piperazine hybrids appear to depend not only on the presence of the coumarin or piperazine core but also, to a significant extent, on the properties of the substituents on the molecule and the structure of the linker connecting the two pharmacophores. In particular, aromatic and heteroaromatic groups attached to the piperazine ring may enhance biological activity by promoting hydrophobic and π - π interactions with target proteins. Similarly, modifications at

different positions of the coumarin ring affect target selectivity and binding affinity. Various linkers, including amide, ether, and triazole groups, have been incorporated and these structures can influence their conformation, flexibility, and interactions with biological targets. Specifically, the reported activities of triazole- and amide-containing hybrids against various biological targets highlight the potential importance of the linking group in hybrid molecule design. In addition, electron-withdrawing and electron-donating substituents have been associated with changes in antimicrobial and anticancer activity, while in some enzyme inhibitor derivatives, electron-donating groups can enable stronger interactions.

The results obtained in these studies demonstrate that coumarin-piperazine hybrids offer versatile molecular platforms that can be investigated not only for a single therapeutic area but also against different disease groups within the framework of a multitarget drug design approach. When considering current health challenges such as antibiotic resistance, neurodegenerative

diseases, and cancer, these hybrid systems hold significant potential for the development of new drug candidates that may complement or provide alternatives to existing treatment options.

Overall, the combination of the coumarin core's ability to interact with biological targets and the pharmacokinetic advantages provided by the piperazine ring has enabled these hybrid systems to have contributed to promising findings in a wide variety of therapeutic areas. However, while current research highlights the importance of certain structural features in determining biological activity, there is a need for more comprehensive structure-activity relationship studies, and new molecular modification strategies are needed. As the

available evidence is largely based on in vitro and in silico studies, it is clear that comprehensive in vivo pharmacokinetic and toxicological studies are required to clarify the clinical potential of these molecules. Accordingly, it is critically important for future research to focus on the in vivo efficacy, pharmacokinetic profiles, and safety analyses of the most active compounds.

Consequently, interdisciplinary research integrating the processes of synthesis, biological evaluation, and pharmacokinetic optimization will play a key role in transforming coumarin-piperazine hybrids into drug candidates suitable for clinical use.

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