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A comprehensive assessment of phytochemicals from Phyla nodiflora (L.) Greene as a potential enzyme inhibitor, and their biological potential: An in-silico, in-vivo and in-vitro approach

--Manuscript Draft--

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Abstract:	This work explored Phyla nodiflora (L.) Greene as a potential source of the bioactive medicinal agent. In this aspect, methanol (PN-M) and dichloromethane (PN-D) extracts were prepared from the whole plant and evaluated for phytochemical composition (total bioactive contents, UHPLC-MS analysis, and HPLC-PDA polyphenolic quantification), biological (antioxidant and enzyme inhibition) potential and in-vivo toxicity. The PN-M was found to contain higher phenolic (26.08 mg GAE/g extract) and flavonoid (50.25 mg QE/g extract) contents which might correlate to the higher radical scavenging (DPPH: 52.94 mg TE/g extract; ABTS: 72.11 mg TE/g extract) and reducing power (FRAP: 71.96 mg TE/g extract; CUPRAC: 142.65 mg TE/g extract) antioxidant potential, as well as AChE (4.33 mg GALAE/g extract), tyrosinase (125.36 mg KAE/g extract), and amylase (1.86 mmol ACAE/g extract) inhibition activity of this extract. In contrast, the PN-D extract was found to be most active for phosphomolybdenum (1.30 mg TE/ g extract) and metal chelation (54.84 mg EDTAE/g extract) assays in addition to BChE (4.70 mg GALAE/g extract) and glucosidase (0.62 mmol ACAE/g extract) enzyme inhibition activity. The PN-M extract on UHPLC-MS analysis revealed the tentative identification of 24 different secondary metabolites, most of which belonged to the flavonoid, glycoside, and terpenoid classes of phytochemicals. The polyphenolic composition of the extracts was appraised by HPLC-PDA. Seven phenolic compounds were identified in the extracts. PN-M was found to be rich in catechin (0.25 µg/extract) and 3-OH benzoic acid (0.64 µg/extract), while PN-D contained epicatechin (0.30 µg/extract), 3-OH-4-MeO benzaldehyde (0.21 µg/extract), and 2,3-Di-Meo benzoic acid (0.97 µg/extract) in higher amounts. The methanol extract was found to be non-toxic even at higher doses. Furthermore, the relationship between the phytochemicals and the tested enzymes was highlighted by molecular docking studies. In sum, this research showed that the studied extracts were effective as enzyme inhibitors and antioxidants, suggesting it would be worth investigating in more depth for further advanced studies to explore its pharmacological properties.
Suggested Reviewers:	Adriano Mollica adriano.mollica@unich.it
	Fawzi Mahomoodally f.mahomoodally@uom.ac.mu
	Muhammad saleem m.saleem@iub.edu.pk

To Whom It May Concern

I **Hammad Saleem** is submitting a revised manuscript entitled **"A comprehensive assessment of phytochemicals from *Phyla nodiflora* (L.) Greene as a potential enzyme inhibitor, and their biological potential: An in-silico, in-vivo and in-vitro approach"** for possible publication in **Arabian Journal of Chemistry**. All authors agreed to submit the paper.

Sincerely Yours,

Dr. Hammad Saleem

Arabian Journal of Chemistry
To: Editor in Chief **Dated: 28:08:2023**
Greetings

Dear Professor,

We are pleased to submit our revised manuscript (**ARABJC-D-23-00554)** entitled **"A comprehensive assessment of phytochemicals from *Phyla nodiflora* (L.) Greene as a potential enzyme inhibitor, and their biological potential: An in-silico, in-vivo and in-vitro approach"** for possible publication in **Arabian Journal of Chemistry.**

The genus Phyla belonging to the family Verbenaceae, have been traditionally used for treating various common ailments. Nonetheless, one of the important species of this genus i.e., Phyla nodiflora is yet to be further exploited in terms of its chemical, biological, and toxicological effects. We have investigated the methanol and DCM extracts of P. nodiflora for computational studies, chemical composition (total bioactive contents UHPLC-MS secondary metabolites, and HPLC-polyphenolic quantification) and biological activities. Antioxidant potential was appraised using a panoply of assays including DPPH, ABTS, FRAP, CUPRAC, phoshomolybdenum, and metal chelating. Whereas, the enzyme inhibition activities of all the extracts were tested against cholinesterases, α-amylase, α-glucosidase, urease, lipoxygenase, and tyrosinase. The in-vivo toxicity was studied was also performed. Moreover, in-silico studies were also performed to highlight possible interactions between the bioactive contents and tested biological assays.

We believe that our findings could be of interest to the readers of **Arabian Journal of Chemistry** because this plant species can be further considered as a source of bioactive-functional agents for the food industry and pharmaceutical applications.

We hope that the editorial boards and reviewers will agree on the interest of this study.

Sincerely yours,

Dr. Hammad Saleem

Response to Comments:

To:

Editor in Chief **28:08:23**

Greetings

Revision of Manuscript ARABJC-D-23-00554

First of all, I would like to pay thanks to you for granting me this opportunity to improve our manuscript, which we opine will benefit readers of your high-ranked journal.

Please find below our point-by-point response to the addressed comments. We have accepted all the suggestions made by the reviewers, and the manuscript has been revised accordingly.

We look forward to your positive response concerning the revision undertaken.
Thank you for your quick response.

Sincerely Yours

Dr. Hammad

Reviewers' comments:

Reviewer #2:

Authors studied in this paper about phytochemicals from Phyla nodiflora (L.) Greene as a potential enzyme inhibitor and choose methanol and dichloromethane extracts for this study through UHPLC-MS analysis, and HPLC-PDA polyphenolic quantification. There are so many papers published on phytochemicals and itself cited by the author in this manuscript. Still many new concepts and detailed analysis of many phytochemicals have been done in this study which makes it appealing for readers. Thus I recommend the editor to accept this paper in the revised form with following points which should be addressed before consideration for publication.

Dear Reviewer, we thanks to you for your comments. All the revisions as suggested by you are marked as YELLOW in the revised manuscript.

(1) Target analysis

Authors described in abstract "methanol (PN-M) and dichloromethane (PN-D) extracts were prepared from the whole plant and evaluated for phytochemical composition (total bioactive contents, UHPLC-MS analysis, and HPLC-PDA polyphenolic quantification)". However, in experimental part, 2.2.2. UHPLC-MS analysis it is stated that "the methanol extract was subjected to analysis". There is no information about dichloromethane extract. Author should clarify it.

Response: Dear reviewer, we have provided the explanation about the reason for which only the methanol extract was used for the UHPLC-MS analysis.

(2) In section 2.2.3. HPLC-PDA polyphenolic quantification; author presented analysis for 22 different polyphenols and particularly mentioned names like benzoic acid. Is benzoic acid a polyphenol? Again the HPLC-PDA polyphenolic quantification is not showing information about methanol extract and dichloromethane extract.

Response: Dear Reviewer, we have changed/corrected the term polyphenolic as phytochemicals and also provided the information about the methanol and dichloromethane extracts.

(3) In section 2.5. In-vivo toxicological studies; the author demonstrated the board of studies approval of title of MS Thesis. I suggest such king of information should be presented in acknowledgment not in the experimental part.

Response: Dear Reviewer, we have made the changes as suggested.

(4) In results and discussion section 3.1. Antioxidant potential; "The higher antioxidant activity of methanol extract might be linked to the higher amount of phenolic and flavonoid contents for this extract". Is there any experimental proof of this statement? The author further stated that these findings are agreed with previous data in literature but he did not cite any reference.

Similarly some activities are linked with non-phenolic components. Can author give biological logic of this behavior?

Response: Dear reviewer, we have provided the references.

(5) Several places author used complete word methanol. But instead of dichloromethane only DCM is mentioned which makes the statements ambiguous. Therefore it is suggested to use full name of DCM wherever applicable.

Response: Dear Reviewer, we have made these changed throughout the manuscript.

(6) Figure 2 is not clear. The axes are not visible; the author should replace it with high resolution Figure.

Response: Dear Reviewer, we have improved the overall resolution of Figure 2.

(7) In results and discussion section 3.3.Phytochemical composition; the author stated that "The tested extracts contained higher total flavonoid contents than phenolic ones. However, the methanol extract was higher in polyphenolic contents (TPC: 26.08 mg GAE/g extract; TFC: 50.25 mg 281 QE/g extract) than DCM extracts." These statements are not clear. In one statement it seems flavonoid contents are higher than phenolic while in another statement the methanol extract was higher in polyphenolic contents. Author should make it clear.

Response: Dear Reviewer, we have corrected the statement as suggested.

(8) The author should add UHLPC-MS diagrams if any in the revised manuscript.

Response: Dear Reviewer, Figure 1 is the UHPLC diagram of the tested extract.

(9) In many places the references are missing. Author should check it carefully.

Response: Dear Reviewer, we have checked the whole manuscript for references and all of the missing references have been cited in the revised manuscript.

(10) The results of photochemical screening should be compared with data in literature of relevant plants.

Response: Dear reviewer, previously this plant has not been analyzed for the total bioactive contents, however, We have provided the references of the isolation of different phytocompounds from this plant in the phytochemical section.

Reviewer #3:

As a result of the conducted study the authors have carried out a large amount of experimental work, however, there are the following recommendations, namely:

The results of GC-MS quantitative analysis of the chemical composition of the studied extracts obtained might be considered uncorrected.

Response: Dear Reviewer, we thanks to you for your comments. All the revisions as suggested by you are marked in the revised manuscript.

In addition, it is necessary to indicate how the studied ecstarcts were standardized and explain why there is no comparison drug. It should also be indicated whether a statistically significant difference between the studied extracts has been found.

If possible, reduce the abstract of the article and skip the mention of the results and discussions, and have the breives of the work and an outline of the work method and finally the conclusion.

Response: Dear Reviewer, we have made all the corrections as per your kind suggestion and the manuscript has been modified accordingly.

Reviewer #4:

This work explored Phyla nodiflora (L.) Greene as a potential source of the bioactive 42 medicinal agent. In this aspect, methanol (PN-M) and dichloromethane (PN-D) extracts were 43 prepared from the whole plant and evaluated for phytochemical composition (total bioactive 44 contents, UHPLC-MS analysis, and HPLC-PDA polyphenolic quantification), biological 45 (antioxidant and enzyme inhibition) potential and in-vivo toxicity. I suggest the following revisions:

Response: Dear Reviewer, we thanks to you for your comments. All the revisions as suggested by you are marked as GREEN in the revised manuscript

- Format of manuscript should be significantly revised in terms of some things such as abbreviation, typos, word spacing and relative position.

Response: ***Response: Dear Reviewer, we have thoroughly revised the manuscript for the English grammar and typos.***

- Abbreviations should be defined at first mention and used consistently thereafter.

Response: Dear reviewer, all the abbreviations are defined at first mention, as suggested.

- It is recommended to enrich the present work by referring following works: ChemCatChem, 15(4). doi: https://doi.org/10.1002/cctc.202201351

ACS Catalysis, 11(9), 5100-5107. doi: 10.1021/acscatal.1c00913

Nature Communications, 13(1), 4672. doi: 10.1038/s41467-022-32364-3

ACS sensors, 7(3), 775-783. doi: 10.1021/acssensors.1c02305

Sensors and Actuators B: Chemical, 369, 132315.
doi: https://doi.org/10.1016/j.snb.2022.132315

Chemosphere, 281, 130718. doi: https://doi.org/10.1016/j.chemosphere.2021.130718

Green chemistry, 24(19), 7500-7518. doi: 10.1039/d2gc02467e

Science, 349(6251), 936. doi: 10.1126/science.aab0095

Frontiers in Sustainable Food Systems, 7. doi: 10.3389/fsufs.2023.1172522

Response: Dear reviewer, we have cited the references as suggested.

- What about the effect of different solvents on the results?

Response: Dear reviewer, the extracts were properly dried using a rotary evaporator, therefore there will be no effect of the solvents on the activities of the extract.

- Language of manuscript should be also significantly revised. I recommend that English of the manuscript needs to be improved by native speaker.

Response: Dear Reviewer, we have thoroughly revised the manuscript for the English grammar and typos.

- The necessity of doing this work should be stated in the first sentence of the abstract section.

Response: Dear Reviewer, we have added a statement as suggested.

- The future outline should be added to the last sentence of the abstract section.

Response: Dear Reviewer, we have added the sentence as required.

- Cite these works:

Chemical Engineering Journal, 407, 127212.
doi: https://doi.org/10.1016/j.cej.2020.127212

ACS Nano, 17(5), 4601-4618. doi: 10.1021/acsnano.2c10694

Response: Dear reviewer, we have cited the references as suggested.

- In the last paragraph of the introduction section, the authors should briefly state what they want to do in their project. Please follow the standard format of writing the introduction.

Response: We have added the required details in the introduction section.

- Please add the limitation and possible recommendations for future works.

Response: Dear Reviewer, we have added the limitation and future recommendations in the abstract as well as conclusion section.

- The novelty of the work should be established in the introduction. Overall, I think that the work presented in the current manuscript merit publication in the journal after above me

Response: Dear Reviewer, we have provided a sentence regarding the novelty of the study in the introduction section.

Reviewer #5:

Dear Author,

The results of this manuscript explored Phyla nodiflora (L.) Greene as a potential source of the bioactive medicinal agent. The results and discussion are well written. However, major corrections are needed on introduction section.

Response: Dear Reviewer, we thanks to you for your comments. All the revisions as suggested by you are marked as GREY in the revised manuscript

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Title: in-silico, in-vivo and in-vitro must be write in italic.

Response: Dear reviewer, we have made these changes as suggested.

Introduction: Paragraph 1: all references are old. Please cite the latest 2019++

Response: Dear reviewer, we have cited the latest references.

Line 82-84: Is this refer to Sharma and Singh 2013? It was 10 years ago.

Response: Dear reviewer, we have cited the latest references.

Introduction: Paragraph 2: 80% of references are old. Please cite the latest 2019++

Response: Dear reviewer, we have cited the latest references.

Please refer to Arabian Journal of Chemistry guidelines, How to cite the references in text.

Example: Line 111: Thirupathy, Tulshkar et al. 2011 to Tulshkar et al. 2011

Response: Dear reviewer all the references have been checked accordingly journal style and guidelines.

Line 139: Please explain in detail the shade drying methodology. Thank you.

Response: Dear reviewer, we have provided the details of drying methodology.

Line 301-304: All references for support result in Table 3 are old. Please rephrase and add on more information and cited the latest references 2019++.

Response: Dear reviewer, we have cited the latest references.

A comprehensive assessment of phytochemicals from *Phyla nodiflora* (L.) Greene as a potential enzyme inhibitor, and their biological potential: An *in-silico*, *in-vivo*, and *in-vitro* approach

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Abstract:

This work explored *Phyla nodiflora* (L.) Greene as a potential source of the bioactive medicinal agent. In this aspect, methanol (PN-M) and dichloromethane (PN-D) extracts were prepared from the whole plant and evaluated for phytochemical composition (total bioactive contents, UHPLC-MS analysis, and HPLC-PDA polyphenolic quantification), biological (antioxidant and enzyme inhibition) potential and *in-vivo* toxicity. The PN-M was found to contain higher phenolic (26.08 mg GAE/g extract) and flavonoid (50.25 mg QE/g extract) contents which might correlate to the higher radical scavenging (DPPH: 52.94 mg TE/g extract; ABTS: 72.11 mg TE/g extract) and reducing power (FRAP: 71.96 mg TE/g extract; CUPRAC: 142.65 mg TE/g extract) antioxidant potential, as well as AChE (4.33 mg GALAE/g extract), tyrosinase (125.36 mg KAE/g extract), and amylase (1.86 mmol ACAE/g extract) inhibition activity of this extract. In contrast, the PN-D extract was found to be most active for phosphomolybdenum (1.30 mg TE/ g extract) and metal chelation (54.84 mg EDTAE/g extract) assays in addition to BChE (4.70 mg GALAE/g extract) and glucosidase (0.62 mmol ACAE/g extract) enzyme inhibition activity. The PN-M extract on UHPLC-MS analysis revealed the tentative identification of 24 different secondary metabolites, most of which belonged to the flavonoid, glycoside, and terpenoid classes of phytochemicals. The polyphenolic composition of the extracts was appraised by HPLC-PDA. Seven phenolic compounds were identified in the extracts. PN-M was found to be rich in catechin (0.25 µg/extract) and 3-OH benzoic acid (0.64 µg/extract), while PN-D contained epicatechin (0.30 µg/extract), 3-OH-4-MeO benzaldehyde (0.21 µg/extract), and 2,3-Di-Meo benzoic acid (0.97 µg/extract) in higher amounts. The methanol extract was found to be non-toxic even at higher doses. Furthermore, the relationship between the phytochemicals and the tested enzymes was highlighted by molecular docking studies. In sum, this research showed that the studied extracts were effective as enzyme inhibitors and antioxidants, suggesting it would be worth investigating in more depth for further advanced studies to explore its pharmacological properties.

Keywords: *Phyla nodiflora*; phytochemicals; UHPLC-MS; HPLC-PDA; antioxidant; enzyme inhibition; toxicity

1. Introduction

Plant species have been used for medicine for as long as humans have existed. Different civilizations have provided documentary evidence of the medicinal use of plants (Phillipson 2001, Suroowan, Llorent-Martínez et al. 2023). The therapeutic effects of plants are due to the presence of different organic and inorganic salts (Kurmukov 2013, Acquaviva, Di Simone et al. 2023). After years of struggle, the use of plants was established as medicine against various ailments (Petrovska 2012). Primary and secondary metabolites produced by the plants are the sources for different drugs to be used medicinally (Balick and Cox 1996). Humans have been using folk medicines for thousands of years extracted from plants, which participated in the conception of modern medicines (Hassan 2015, Acquaviva, Di Simone et al. 2023). Drugs are obtained from plants by employing different botanical, biological, phytochemical, and molecular techniques (Balunas and Kinghorn 2005).

The medicinal plant "*P. nodiflora"* is a predominant member of the Verbenaceae family. Its common habitat is in Central and South America, Pakistan, Sri Lanka, and India. *P. nodiflora* grows best on moist soil. It is present in soil rich in moisture (Sharma and Singh 2013, Al-Snai 2019, Rahman, Javaid et al. 2021). Two flavones, luteolin-7-*O*-glucoside and 6-hydroxyluteolin-7-apioside, are mainly present in the flowers of *P. nodiflora* and the leaves of *P. nodiflora* were also found to be contain different flavone glycosides (Barnabas, Gunasingh et al. 1980, Jabeen, Jillani et al. 2016). Acetoside and demethoxycentaureidin are the two compounds yielded from the alcoholic extract of *P. nodiflora* (Khalil, Lahloub et al. 1995). The isolation of stigmasterol, beta-sitosterol, and triterpenoid lippacin was reported from the methanolic extract of *P. nodiflora* (Sharma and Singh 2013). The aerial parts also reported numerous steroidal constituents (Siddiqui, Ahmed et al. 2009). Methanol and dichloromethane extract of *P. nodiflora* leaves are reported to

contain the compounds halleridone and halleron (Ravikanth, Ramesh et al. 2000). A skin whitening agent, eupafolin, was yielded from dried aerial parts of the *P. nodiflora* (Yen, Wang et al. 2012). The methanol extract of *P. nodiflora* yields hispidulin, nodifloretin, and eupafolin (Ko, Chiang et al. 2014). The ethanolic extract of *P. nodiflora* contains a diverse group of chemical compounds, including betatifolin, nodifloridin, jaceosidin, lippiflorin, and nepetin (Regupathi and Chitra 2015). The ethanolic extract of *P. nodiflora* has also been reported as the main source of the ecteoside (Khalil, Lahloub et al. 1995). Likewise, stigmasterol, eugenol, α-copaene, *β*-bisabolene, and *γ*-sitosterol are among the various constituents isolated from the plant's methanolic extract (Ko, Chiang et al. 2014). Similarly, steam distillation of *P. nodiflora* has reported the presence of linalool, methyl salicylate, cymen-8-ol, and *β* carboline (Elakovich and Stevens 1985). *P. nodiflora* have been previously reported for a number of biological effects (Al-Snai 2019, Paua 2022), including antimicrobial (Gopal, Balkrishna et al. 1996, Patel Janki, Shah Kinjal et al. 2005, Pirzada, Iqbal et al. 2005, Durairaj, Vaiyapuri et al. 2007, Malathi, Cholarajan et al. 2011, Sharma and Singh 2013), antitumor (Vanajothi, Sudha et al. 2012, Cheong and Teoh 2014), hepatoprotective (Durairaj, Vaiyapuri et al. 2008, Arumanayagam and Arunmani 2015), antioxidant (Ashokkumar, Thamilselvan et al. 2008, Durairaj, Vaiyapuri et al. 2008), diuretic (Shukla, Patel et al. 2009, Balamurugan and Ignacimuthu 2011), antidiabetic, hypolipidemic (Balamurugan, Duraipandiyan et al. 2011, Balamurugan and Ignacimuthu 2011), neuropharmacological (Thirupathy, Tulshkar et al. 2011, AO and SL 2012), anti-urolithiasis (Dodoala, Diviti et al. 2010), anti-inflammatory (Ahmed, Selim et al. 2004, Balakrishnan, Janakarajan et al. 2010, Al-Snafi and Faris 2013), melanogenesis inhibition (Yen, Wang et al. 2012, Ko, Chiang et al. 2014), antihypertensive (Gadhvi, Mishra et al. 2012), skin whitening effect

(Yen, Wang et al. 2012), central inhibitory (AO and SL 2012), apoptotic (Cheong and Teoh 2014), and antidandruff (Regupathi and Chitra 2015) activities.

Hence, the estimation of phytochemical contents both quantitatively and qualitatively, along with the assessment of antioxidant and enzyme inhibition potential, as well as the *in-vivo* toxicological studies of methanol and dichloromethane extracts of *P. nodiflora*, are the main objectives of this paper. The phytochemical profile was evaluated by ascertaining preliminary phytochemical testing, total phenolic and flavonoid contents, UHPLC-MS analysis, and HPLC-PDA polyphenolic quantification. The antioxidant potential was assessed via different assays, including radical scavenging (DPPH and ABTS), reducing power (FRAP and CUPRAC), total antioxidant activity (phosphomolybdenum), and metal chelation assays. Likewise, the inhibitory potential of both extracts was tested against the five therapeutically relevant enzymes, including AChE and BChE (involved in neurological problems), amylase, glucosidase (involved in diabetes), and tyrosinase (skin problems). Furthermore, in-silico studies against the tested enzymes were also conducted. The *in-vivo* toxicity was tested on the chicks for 21 days. This work will provide evidence for the pharmacological effectiveness of these species by identifying the best methods for isolating their bioactive components.

2. Material and methods

2.1. Plant collection and extraction

The whole plant material was collected from the peripheries of Bahawalpur City, Pakistan (GPS co-ordinates: 29°23'19.7"N 71°42'07.0"E) . The plant was identified and authenticated as *P. nodiflora* by Mr. Hafiz Waris, Taxonomist at Cholistan Institute of Desert Studies (CIDS), The Islamia University of Bahawalpur. For future reference, a voucher specimen of the plant material (PN-WP-01-14-131) was also deposited at the herbarium of the Islamia University of

Bahawalpur's Faculty of Pharmacy and Alternative Medicines. The plant material was subjected to shade drying for 15 days. The dried plant material was grounded into a fine powder with the help of a grinding mill. The powdered plant material was extracted with dichloromethane and methanol successively. The filtrate collected was concentrated on a Rotary evaporator at 35 ºC under reduced pressure.

2.2. Phytochemical composition

2.2.1. Total phenolic and total flavonoid contents

The total bioactive contents, including total phenolic and flavonoid contents, were estimated using well-established Folin-Ciocalteu and aluminium chloride assays, respectively. (Saleem, Zengin et al. 2019). The standard for total phenolic content was gallic acid, with results expressed as mg GAE/g (gallic acid equivalents), while quercetin was used as a standard for total flavonoid content, and the results were expressed as mg QE/g (quercetin equivalent). The detailed protocols for these assays are presented in the supplementary material section.

2.2.2. UHPLC-MS analysis

UHPLC-MS analysis is a technique that is mostly used for the tentative identification of polar compounds in plant extracts. Therefore, the methanol extract (being polar one) was subjected to the UHPLC-MS analysis (negative ionization mode) for the tentative identification of possible secondary metabolites. The identification of the phytochemicals was made by using METLIN database library (Saleem, Zengin et al. 2019). The detailed instrumentation for this analysis is presented in the supplementary material section.

2.2.3. HPLC-PDA quantification of different phytochemicals

HPLC-PDA analysis (of both the methanol and dichloromethane extracts of *P. nodiflora*) for 22 different phytochemicals (including 4-hydroxybenzoic acid, 3-dimethoxybenzoic acid, quercetin, gallic acid, benzoic acid, catechin, epicatechin, harpagoside, naringin, vanillic acid, carvacrol, 3-hydroxybenzoic acid, naringenin, 3-hydroxy-4-methoxy benzaldehyde, *t*-cinnamic acid, *p*-coumaric acid, chlorogenic acid, rutin, syringic acid, sinapinic acid, *t*-ferulic acid, and *o*-coumaric acid) was done and quantified using previously reported method (Locatelli, Zengin et al. 2017). The gradient elution program used for HPLC analyses is given in Table S1 of the supplementary material section. Likewise, the standards used, retention times, and maximum wavelengths used for quantitative analyses are shown in Table S2 of the supplementary material section. The chemical standards chromatogram for the 22 standards used is depicted in Figure S1 of the supplementary material section. The detailed instrumentation for this analysis is presented in the supplementary material section.

2.3. Biological assays

2.3.1. Antioxidant assays

The metal chelating, phosphomolybdenum, FRAP, CUPRAC, ABTS, and DPPH activities were made as per previously described methods (Saleem, Zengin et al. 2019). The antioxidant activities were reported as Trolox equivalents, whereas EDTA was used for the metal-chelating assay. The detailed protocols for these assays are presented in the supplementary material section.

2.3.2. Enzyme inhibition activities

The possible inhibitory effects of the extracts against cholinesterases, tyrosinase, α-amylase, and α-glucosidase were assessed using standard bio-assays (Saleem, Zengin et al. 2019). Galantamine was used as a standard for AChE, and BChE inhibition activity was expressed as (mg

GALAE/g extract). In contrast, the inhibition activities for α-glucosidase and α-amylase were reported as millimoles of acarbose equivalent (mmol ACAE/g extract), whereases tyrosinase inhibition was noted as milligrams of kojic acid equivalent (mg KAE/g extract). The detailed protocols for these assays are presented in the supplementary material section.

2.4. Computation Methods:

2.4.1. Structures preparation and docking studies:

Sybyl-X1.3/SKETCH (Jain 2003) was used to create a three-dimensional conformation of inhibitors 6-hydroxyluteolin 5-rhamnoside (HLR), luteolin 7-rhamnosyl (1->6) galactoside (LRG), and torosaflavone D (TFD) (**Figure 3**). To get biologically active conformation of compounds under study, all three inhibitors were subjected to energy optimization using the Tripos force field with Gasteiger Hückel atomic charges (Powell 1978). The co-crystal structures of acetylcholinesterase (ACHE), butyrylcholinesterase (BCHE), and tyrosinase (TYR) were obtained from the RCSB Protein Data Bank under the following PDB entries; (PDB ID's: 4EY7 (Cheung, Rudolph et al. 2012), 6QAE (Meden, Knez et al. 2019) and 2Y9X (Ismaya, Rozeboom et al. 2011), respectively). Before docking investigations, all protein structures were further processed using the structure preparation tools contained in the SYBYL-X 1.3 biopolymer module (Ghersi and Sanchez 2011). The energy was minimized using the Powell algorithm with a convergence gradient of 0.5 kcal (mol)-1 for 1000 cycles, with missing hydrogens added, charges applied, and atom types assigned according to the AMBER 7 FF99 force field (Onufriev, Bashford et al. 2004). Finally, the energy-optimized bioactive conformation of selected compounds was docked into the active site of ACHE, BCHE, and TYR enzymes using the Surflex-Dock module of the SYBYL-X 1.3 software package, following the same protocol and parameters as those published in our prior work.(Chohan, Chen et al. 2016, Chohan, Qian et al. 2016). The experimentally determined active

conformation of donepezil in 4EY7, N1, N2-dimethylpropane-1,2-diamine in 6QAE, and tropolone in 2Y9X was used as initial conformation to define the potential binding pocket for protomol (an idealized active site) generation (Jain 2007). The top twenty docked conformations were saved and investigated for their binding modes into the active site of their respective target. The Hammerhead scoring method was used to rate these potential ligand poses (Jain 1996, Jain 2003).

2.5. In-vivo toxicological studies

The *in-vivo* toxicological parameters were studied on the broiler chicks. The animal's selection, grouping, toxicity parameters, hematological and biochemical analysis, and relative and absolute weight of body organs were calculated as reported in our previously published data (Saleem, Zengin et al. 2019).

2.6. Statistical analysis

All the biological experiments were done in triplet, and the estimation of results was made as mean value ± standard deviation (SD). One-way ANOVA test was used to find the mean difference, followed by data analysis using SPSS v.17.0 software.

3. Results and discussion

3.1. Antioxidant potential

The involvement of oxidative stress in disease development and progression is becoming more evident. Increasing endogenous antioxidant defenses or supplementing the body with exogenous antioxidants is a viable approach to combating ROS-induced oxidative damage (Pizzino, Irrera et al. 2017, Lan, Liu et al. 2021). The plant kingdom provides a rich supply of

beneficial substances to one's health, particularly natural antioxidants. (Zengin, Mahomoodally et al. 2022). In the current study, PN-M and PN-D extracts of the *P. nodiflora* plant were tested for a panoply of antioxidant assays, including radical scavenging (DPPH and ABTS), reducing power (FRAP and CUPRAC), total antioxidant activity (phosphomolybdenum) and metal chelation activities, and the results are presented in **Table 1**. A similar pattern to total bioactive contents was noted, and the methanol extract was found to have a higher antioxidant potential for radical scavenging (DPPH: 52.94 mg TE/g extract; ABTS: 72.11 mg TE/g extract) and reducing power (FRAP: 71.96 mg TE/g extract; CUPRAC: 142.65 mg TE/g extract) assays. This higher antioxidant activity of methanol extract might be linked to the higher amount of phenolic and flavonoid contents for this extract. These findings agree with some of the previous research data, which have provided a positive relationship between bioactive contents and antioxidant potential (Chang, Ye et al. 2022, Chen, Ran et al. 2022, Li, Shi et al. 2022, Zengin, Fernández-Ochoa et al. 2023). However, in the case of phosphomolybdenum and MCA assay, different results were obtained, and the dichloromethane extract was found to contain higher activity. This higher activity could be linked to the non-phenolic compounds present in this extract. These findings correlate with the previous studies, which also presented the same trend, and the methanolic extract of *P. nodiflora* has higher antioxidant potential for tested reducing power, scavenging free radical, and scavenging superoxide anion radical activities and other antioxidant assays (Ashokkumar, Thamilselvan et al. 2008, Durairaj, Vaiyapuri et al. 2008). Meticulous correlation values between these assays in plant extracts have been published by various researchers, consistent with our findings. However, we noticed a low correlation value for the PBD assay, which could be explained by additional reducing chemicals in the extracts (peptides, sugars, etc.). The poor correlation value obtained between total phenolics and the metal chelating assay can be explained

by the activity of non-phenolic chelators present in the examined extracts, as demonstrated in the previous study (References) (Zengin, Ak et al. 2022).

3.2. Enzyme inhibition

Drug discovery relies heavily on the use of enzyme inhibitors. The malfunction, overexpression, or hyperactivation of enzymes is the root cause of many diseases, thanks to advances in molecular biology. Enzyme inhibitors can be used to treat such hyperactivation or overexpression of enzymes. These efforts have resulted in the clinical use of various enzyme inhibitors, some of which are of natural origin. (Zengin, Mahomoodally et al. 2022).

Several clinically relevant enzymes involved in major pathologies, including neurodegenerative disorders (AChE and BChE), skin diseases (tyrosinase), and diabetes (amylase and glucosidase), were tested for enzyme inhibition potential using *P. nodiflora* methanol and dichloromethane extracts. The outcomes of these tests are summarised in **Table 2**. The PN-M extract was most active against AChE and tyrosinase enzymes with an inhibition potential of 4.33 mg GALAE/g extract and 125.36 mg KAE/g extract, respectively. Likewise, the dichloromethane extract was noted to show higher inhibition values against BChE (4.70 mg GALAE/g extract) and amylase (1.97 mmol ACAE/g extracts) enzymes. However, both extracts presented weak inhibition potential against the glucosidase enzyme.

Mice have been shown to benefit from extracts of P. nodiflora in chloroform and ethanol as anxiolytics and anticonvulsants (Thirupathy, Tulshkar et al. 2011). *P. nodiflora* methanol extract has previously shown anti-diabetic action in rats induced with Streptozotocin. A significant increase in insulin levels and body weight resulted in decreased blood glucose and glycosylated hemoglobin levels (Balamurugan and Ignacimuthu 2011). Eupafolin, an active flavonoid derived from *P. nodiflora*, exhibited skin-whitening effects in B16F10 mice via a melanin suppression

(Ko, Chiang et al. 2014). Likewise, another study has reported the tyrosinase inhibitory potential of *P. nodiflora* in the dose-dependent matter (Yen, Wang et al. 2012).

3.3.Phytochemical composition

Natural products play a key role in drug development; therefore, screening plants to identify and isolate active ingredients is a necessary step to developing effective drugs against a wide range of diseases (Bibi Sadeer, Sinan et al. 2022). Therefore, total flavonoid and phenolic contents were estimated, with results depicted in **Table 1**. The tested extracts contained considerable quantities of total phenolic and flavonoid contents. However, the methanol extract was higher in polyphenolic contents (TPC: 26.08 mg GAE/g extract; TFC: 50.25 mg QE/g extract) than dichloromethane extracts. The higher amount of phenolic and flavonoids in methanol extract might be attributed to the polar nature of the methanol solvent. Our findings are in line with the previous studies that had reported higher bioactive contents for polar extracts (Zekri, Zerkani et al. 2021, Wairata, Fadlan et al. 2022). Current studies made use of colorimetric methods to quantify phenolic compounds. Although, the effectiveness of these methods is quite controversial in producing accurate results (Zengin, Ak et al. 2022). Thus, a punctual UHPLC-MS and HPLC-PDA analysis was used to reveal the quantitative and qualitative composition of the phenolic compounds.

As mentioned above, to have further insight into the composition of the secondary metabolites, the methanol extract was subjected to UHPLC-MS analysis in negative ionization mode which resulted in the tentative identification of 24 different phytochemicals. The list of these identified compounds is presented in **Table 3**, while the total ion chromatogram (TIC) is shown in **Figure 1**. It can be noted from Table 2 that the maximum number of phytochemicals identified belong to the flavonoid group of secondary metabolites. The identified flavonoids were 6-

methoxytaxifolin, luteolin 7-rhamnosyl (1->6) galactoside, 6-hydroxyluteolin 5-rhamnoside, luteolin 7-rhamnosyl (1->6) galactoside, maysin, acacetin 7-(6"-methylglucuronide), demethyltorosaflavone D, grossamide, and torosaflavone D. Likewise, periandrin V, betavulgaroside X, spinacoside D, spinacoside C, betavulgaroside VI, and medicoside H were belonging to terpene derivatives. Similarly, three glycosides, including sarmentosin epoxide, citrusin F, and calendulaglycoside E, were also identified. One phenolic (formononetin 7-*O*-glucoside-6"-*O*-malonate), and saponin (durupcoside B) derivative was also identified. The presence of these phytochemical classes in the PN-M extract agrees with some previous studies that reported the isolation/identification of different flavonoids, glucosides, and triterpenoid derivatives (Barnabas, Gunasingh et al. 1980, Khalil, Lahloub et al. 1995, Ko, Chiang et al. 2014, Al-Snai 2019).

Likewise, the polyphenolic composition of the extracts was appraised by HPLC-PDA quantification for the 22 different phenolic standards, and the results of the quantified compounds are presented in **Table 4**, while their HPLC chromatograms are depicted in **Figure 2**. It was noted that seven phenolic compounds were quantified in both extracts. PN-M was found to be rich in catechin (0.25 µg/extract) and 3-OH benzoic acid (0.64 µg/extract). In comparison, PN-D contained epicatechin (0.30 µg/extract), 3-OH-4-MeO benzaldehyde (0.21 µg/extract), and 2,3-Di-Meo benzoic acid (0.97 µg/extract) in higher amounts.

3.4. Molecular docking studies

Molecular docking studies in the ligand-binding cavity of enzymes AChE, BChE, and TYR were done to explain the binding behavior of three phytoconstituents, HLR, LRG, and TFD (**Figure 3**). A greater understanding of the structures of AChE, BChE, and TYR may aid in identifying important molecular interactions involved in forming ligand-protein complexes. In

order to investigate the changes in interaction patterns that contribute to different docking scores, optimal docking conformations for each compound are saved and displayed graphically. As shown in Figure 2, the examined ligands occupy the same binding site of AChE, BChE, and TYR as observed in their co-crystallized complexes (4EY7, 6QAE, and 2Y9X) with their respective inhibitors.

A series of molecular docking simulations were performed to study the pattern of molecular interactions contributing to the variance in the conformations of the analyzed ligands to the target protein. Based on the cumulative score (cScore) (**Table 5**), the best probable poses of ligands in the active site of proteins were chosen. The docking scores (cScore) of selected inhibitors bonded to the AChE complex are 8.78, 7.94, and 7.20 for ligands HLR, LRG, and TFD, respectively. These results indicate that the compound HLR forms the strongest complex with the AChE enzyme among the studied ligands. Other compounds, such as LRG and TFD have also shown a comparable binding affinity towards the AChE. In the case of BChE-ligand bonded systems, compounds HLR and TFD demonstrated almost similar docking scores (cScore: 5.01 and 5.12, respectively), while the compound HLR demonstrated the highest binding affinity towards BChE (cScore: 6.52). Among TYR-ligand complexes, TFD showed substantial binding potential towards TYR (cScore: 7.80, whereas compounds HLR and LRG demonstrated weak to moderate binding affinity towards the TYR enzyme. To get detailed insight into the docking results, the docking scores and the list of residues involved in H-bond interactions are summarized in **Table 5**. Furthermore, the simplified 2D ligand interaction diagrams for all docking poses are depicted in **Figure 4** to get better insight into the interaction pattern of ligand-receptor complexes.

The best docking conformations were saved for each ligand-receptor complex and graphically inspected to determine the ligand-protein interactions responsible for the variation in

binding affinity. Moreover, only the top-ranking docking complexes for each ligand-protein system are presented in **Figure 5 (A-I)** to reveal the important molecular interactions responsible for the superior binding affinities of top-scored ligands for their corresponding molecular target. Graphical analysis of all docking complexes (Figures 3 and 4) demonstrates that all ligands occupy the same binding cleft to adopt identical binding orientations in their respective molecular target. However, the differences in binding affinities may originate from variations in the interaction pattern of the ligand-bound system. As presented in figure 5A-C, all three ligands (HLR, LRG, and TFD) penetrate deeply into the two main binding subsites (CAS and PAS) of AChE. In the active site of AChE, both ligands HLR, LRG, and TFD acquire such a binding conformation to establish several interactions with surrounding residues Y70, D72, Y121, W279, S286, I287, R289, F290, F330, F331, G335, Y334, and H440 (Figure 5A and 2B). In the HLR-bonded system, HLR covers a major area of AChE's active site to establish at least six H-bond interactions with D72, Y121, S286, Y334, and H440. In addition to the conserved H-bond contacts with Y121 and Y334, compound LRG also establishes four H-bond interactions with S81, G118, E199, and S200. Compound TFD was found to establish only three H-bond contacts with residues S286 and I287. Despite fewer H-bonds, considering the binding affinity of TFD towards AChE might be attributed to van der Waals's contact of TFD with nearby residues D72, W84, L282, F331, Y334. In addition, the benzyl moiety faces the aromatic indole moiety of W279 to establish pi-pi contact. Hence, these nonbonding interactions may play a crucial role in improved binding affinities of HLR, LRG, and TFD towards AChE. In BChE-ligand bonded systems, compound LRG demonstrated the highest binding affinities among studied ligands, which a network of nine H-bonds may explain between LRG and residues W82, N83, T120, G116, G117, S287, W231, P285of BChE. Compound LFD displays the least binding affinity towards BChE by establishing fewer H-bond contacts.

Interestingly, LFD demonstrated the highest binding affinity in TYR-ligand complexes by acquiring six H-bonds with residues H85, H244, H259, H263, and R268 of the TYR enzyme. Moreover, the aromatic moiety in LFD is sandwiched between V283 and H263 to contribute to sigma-pi and pi-pi contacts, respectively. Compounds of HLR and LRG with a moderate affinity towards AChE develop three to four H-bonds with the nearby residues in the active site of the TYR enzyme (Figure 5G and H). Overall, the docking analysis is consistent with the results and effectively explains the influence of interaction changes on the binding affinities of chosen inhibitors.

3.5. Toxicological studies

The PN-M extract was subjected to toxicological studies to assess the plant's toxic effects, including different hematological, biochemical, and physical parameters. The effect of oral administration of methanol extract of *P. nodiflora* on the haematological parameters of chicks is shown in **Table 6**, while the effect of oral administration of methanol extract of *P. nodiflora* on the biochemical parameters of chicks is shown in **Table 7; likewise**, the effect of oral administration of methanol extract of *P. nodiflora* on body organs of chicks is shown in **Table 8**. Broiler chicks were selected and distributed in four experimental groups. They were orally fed with the extract for 21 days. The chicks were physically evaluated, and the number of chicks remained the same with no abnormality. After comparing the weights of different groups, no significant variation was found. On day 21, absolute and relative weights of other body organs, including kidney, heart, spleen, trachea, liver, intestine, thymus, and lungs, were also done with no significant variation. Hematological and biochemical parameters were evaluated after collecting blood samples on days 7, 14, and 21. On account of statistical analysis, *P. nodiflora* was non-toxic, as there was no significant difference in experimental values to that of the control group.

Overall, we have used the methanolic extract (at different concentrations of 150 mg/kg, 200 mg/kg, 250 mg/kg, and 300 mg/kg) of *P. nodiflora* plant in order to check the in-vivo toxicity. The results have indicated that the tested extract was found safer even at the higher doses of 300 mg/kg.

4. Conclusion

Based on the results of current study, methanolic and dichloromethane extracts of *P. nodiflora* exhibit varying inhibitory effects on enzymes and antioxidant properties. More precisely, the methanol extract showed better antioxidant activity than the dichloromethane extract. In addition, weak inhibiting propensities were exhibited by the studied extracts against the screened enzymes. Significant quantities of TPC and TFC were yielded from the extracts. UHPLC-MS and HPLC-PDA analysis conform to the presence of several important phenolics, flavonoids, terpenes, and glycosides. The molecular docking highlighted the interaction between the identified phytochemicals and tested enzymes. In acute toxicity studies, oral doses of methanolic extract did not result in death or adverse effects on general behavior. Almost no significant changes were observed in any of the hematological and biochemical parameters of the animals. This medicinal plant was found to be effective as an enzyme inhibitor and antioxidant, making it a possible option for further advanced research into its pharmacological capabilities.

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Pakistan) for the thesis of M. Phil student topic entitled" Studies on Phytochemical, Biological and Toxicological impact of *Phyla nodiflora* (Verbenaceae)".

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Tables and Figures:

Table 1: Total bioactive contents and antioxidant properties of *P. nodiflora* methanol and dichloromethane extracts.

Extracts	Total bioactive contents		Antioxidant activities					
			Radical Scavenging activity		Reducing power		Total antioxidant capacity (TAC)	Ferrous chelating
	TPC (mg GAE/g)	TFC (mg QE/g)	DPPH (mgTE/g extract)	ABTS (mgTE/g extract)	FRAP (mgTE/g extract))	CUPRAC (mgTE/g extract)	PBD (mgTE/g extract)	Metal Chelating (mgEDTA/g)
PN-M	26.08±1.32	50.25±0.15	52.94±0.47	72.11±2.49	71.96±1.67	142.65±1.41	1.17±0.03	32.18±0.53
PN-D	18.85±0.38	31.80±0.84	3.44±0.58	12.86±0.86	35.66±0.67	86.61±1.01	1.30±0.09	54.84±0.43

PN-M: *P. nodiflora* methanol extract; PN-D: *P. nodiflora* dichloromethane extract.

GAE: gallic acid equivalent; QE: quercetin equivalent. TE: trolox equivalent; EDTAE: EDTA equivalent. Values expressed are means ± S.D. of three parallel measurements.

Table 2: Enzyme inhibition results of *P. nodiflora* methanol and dichloromethane extracts.

Extracts	Neurodegenerative diseases		Skin problems	Diabetes	
	AChE inhibition (mg GALAE/g extract)	**BChE inhibition (mg GALAE/g extract)**	**Tyrosinase (mg KAE/g extract)**	**Glucosidase (mmol ACAE/g extract)**	**Amylase (mmol ACAE/g extract)**
PN-M	4.33±0.05	2.11±0.06	125.36±1.65	0.60±0.02	1.86±0.03
PN-D	3.48±0.53	4.70±0.14	116.04±1.12	0.62±0.01	1.97±.01

PN-M: *P. nodiflora* methanol extract; PN-D: *P. nodiflora* dichloromethane extract.
GALAE: galantamine equivalent; KAE: kojic acid equivalent; ACAE: acarbose equivalent. All values expressed are means ± S.D. of three parallel measurements.

Table 3: UHLPC-MS tentative secondary metabolites identification of methanol extract of *P. nodiflora* (negative ionization mode).

S.no	RT (min)	BP (*m/z*)	Compounds tentatively identified	Mol. Formula	Comp class	Mol. mass	AUC
1	0.63	333.06	6-Methoxytaxifolin	$C_{16}H_{14}O_8$	Flavonoid	334.06	180964
2	0.762	290.09	Sarmentosin epoxide	$C_{11}H_{17}NO_8$	C. glycoside	291.09	1010800
3	8.462	593.15	Luteolin 7-rhamnosyl(1->6)galactoside	$C_{27}H_{30}O_{15}$	Flavonoid	594.15	665330
4	8.504	225.12	12-hydroxyjasmonic acid	$C_{12}H_{18}O_4$	Acid	226.12	559385
5	8.512	447.10	6-Hydroxyluteolin 5-rhamnoside	$C_{21}H_{20}O_{11}$	Flavonoid	448.10	1264764
6	8.66	519.18	Citrusin F	$C_{22}H_{32}O_{14}$	Phenolic Glycoside	520.18	257059
7	8.979	577.16	Isovitexin 7-O-rhamnoside	$C_{27}H_{30}O_{14}$	Flavonoid	578.16	1665617
8	8.987	593.15	Luteolin 7-rhamnosyl(1->6)galactoside	$C_{27}H_{30}O_{15}$	Flavonoid	594.15	665330
10	9.504	575.14	Maysin	$C_{27}H_{28}O_{14}$	Flavonoid	576.14	379636
11	9.505	577.16	Isovitexin 7-*O*-rhamnoside	$C_{27}H_{30}O_{14}$	Flavonoid	578.16	561198
12	9.604	515.12	Formononetin 7-*O*-glucoside-6"-*O*-malonate	$C_{25}H_{24}O_{12}$	Phenol	516.12	225692
13	9.939	473.11	Acacetin 7-(6"-methylglucuronide)	$C_{23}H_{22}O_{11}$	Flavonoid	474.11	536694
14	10.77	355.05	Demethyltorosaflavone D	$C_{18}H_{12}O_8$	Flavonoid	356.05	536952
15	11.37	777.41	Periandrin V	$C_{41}H_{62}O_{14}$	Triterpene	778.41	335538
16	11.39	909.45	Betavulgaroside X	$C_{46}H_{70}O_{18}$	Diterpenoid	910.45	296898
17	11.85	793.44	Calendulaglycoside E	$C_{42}H_{66}O_{14}$	Glycoside	794.44	599057
18	11.99	623.24	Grossamide	$C_{36}H_{36}N_2O_8$	Flavonoid	624.24	793947
19	12.03	763.39	Spinacoside D	$C_{40}H_{60}O_{14}$	Oleane triterpenoid	764.39	321379
20	12.08	925.45	Spinacoside C	$C_{46}H_{70}O_{19}$	Oleane triterpenoid	926.45	773840
21	12.081	971.45	Betavulgaroside VI	$C_{47}H_{72}O_{21}$	Diterpenoid	972.45	421626
22	12.36	369.06	Torosaflavone D	$C_{19}H_{14}O_8$	Flavonoid	370.06	311323
23	12.43	941.48	Medicoside H	$C_{47}H_{74}O_{19}$	Triterpene glycoside	942.48	356757
24	13.37	925.48	Durupcoside B	$C_{47}H_{74}O_{18}$	Saponin	926.48	3474724

RT: retention time; B.P: base peak; Mol. Formula; AUC: area under curve

Table 4: HPLC-PDA polyphenolic quantification of *P. nodiflora* methanol and dichloromethane extracts.

Tested extracts	Polyphenolics quantified						
	Catechin	***p*-OH benzoic acid**	**Vanillic acid**	**Epicatechin**	**3-OH benzoic acid**	**3-OH-4-MeO benzaldehyde**	**2,3-di-MeO benzoic acid**
PN-M	0.25±0.02	BLD	BLD	nd	0.64±0.05	nd	nd
PN-D	nd	nd	nd	0.30±0.02	nd	0.21±0.01	0.97±0.08

PN-M: *P. nodiflora* methanol extract; PN-D: *P. nodiflora* dichloromethane extract.
nd: not detected; BLD: below limit of detection (<0.1 µg/mL); Chlorogenic acid, *p*-coumaric acid, rutin, sinapinic acid, *t*-ferullic acid, *o*-coumaric acid, quercetin, harpagoside, *t*-cinnamic, carvacrol, benzoic acid, naringin, syringic acid, vanillic acid, and epicatechin were not detected in any of the tested extracts.

Table 5: Surflex scores of docked ligands HLR, LRG, and TFD in the binding site of acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and tyrosinase (TYR) enzymes.

Protein	Ligand	CScore[a]	Crash score[b]	Polar score[c]	D score[d]	PMF score[e]	G score[f]	Chem score[g]	Amino acid interaction
AChE	HLR	8.78	-3.20	3.71	-133.143	-86.23	-187.93	-35.13	D72, Y121, S286, Y334, H440
	LRG	7.94	-2.17	5.37	-170.15	-97.31	-206.44	-25.41	S81, G118, Y121, E199, S200, Y334,
	TFD	7.20	-3.70	1.74	-165.67	-83.16	-241.91	-18.35	S286, F288
BChE	HLR	5.09	-2.38	4.83	-92.17	-24.4	-151.4	-24.7	D70, W82, E197, S198, P285, L286, Y332, W430,
	LRG	6.52	-2.11	4.91	-102.12	-28.1	-161.6	-19.6	W82, N83, T120, G116, G117, S287, W231, P285
	TFD	4.12	-2.81	5.12	-166.7	-44.4	-212.1	-14.0	D70, S72, G116, S198, A199, P285
TYR	HLR	4.41	-1.49	1.23	-98.21	33.14	-121.10	-34.45	N260, R268
	LRG	5.98	-4.73	7.10	-170.3	62.053	-319.8	-6.22	H85, N260, E322
	TFD	7.80	-2.62	5.56	-148.0	32.933	-191.8	-4.97	H85, H244, H259, H263, R268

[a]**CScore** is a consensus scoring which uses multiple types of scoring functions to rank the affinity of ligands, [b]**Crash**-score revealing the inappropriate penetration into the binding site, [c] **Polar** region of the ligand, [d] **D-score** showing hydrogen bonding, complex (ligand-protein), and internal (ligand-ligand) energies, [e] **PMF-score** indicating the Helmholtz free energies of interactions for protein-ligand atom pairs (Potential of Mean Force, PMF), [f] **G-score** for charge and van der Waals interactions between the protein and the ligand, [g] **Chem-score** points for hydrogen bonding, lipophilic contact, and rotational entropy, along with an intercept term.

Table 6: Effect of oral administration of methanol extract of *P. nodiflora* on haematological parameters of chicks

Parameters	Group (ZP)				
	Control	**150 mg/kg**	**200 mg/kg**	**250 mg/kg**	**300 mg/kg**
Erythrocyte counts					
Day 7	2.5 ± .12	2.63 ± .08	2.43 ± .08	2.48 ± .08	2.74 ± .07
Day 14	2.60 ± 0.13	2.46 ± 0.07	2.70 ± 0.02	2.43 ± 0.04	2.48 ± 0.05
Day 21	2.40 ± 0.07	2.54 ± 0.07	2.74 ± 0.07	2.73 ± 0.07	2.56 ± 0.05
Leucocyte count (103/ul)					
Day 7	197.38 ± 3.62	193.9 ± 2.45	190.8 ± 5.05	195.3 ± 4.31	222.2 ± 5.30
Day 14	202.8 ± 6.11	196.1 ± 3.73	193.5 ± 4.25	213.0 ± 10.9	233.5 ± 3.75
Day 21	213.3 ± 16.3	221.3 ± 3.82	198.1 ± 4.90	224.7 ± 2.81	229.4 ± 6.31
Lymphocyte count (10^3 / µ L)					
Day 7	168.98 ± 2.85	168.90 ± 2.60	214.10 ± 3.64	174.90 ± 11.89	167.50 ± 3.48
Day 14	167.6 ± 3.28	170.4 ± 1.67	211.0± 2.23	215.0 ± 0.89	216.2 ± 0.71
Day 21	213.3 ± 16.3	209.8 ± 3.79	210.8 ± 2.67	162.1 ± 5.18	159.7 ± 2.58
Haemoglobin (gm/dl)					
Day 7	10.14 ± 0.37	10.50 ± 0.28	11.36 ± 0.49	11.40 ± 0.18	10.14 ± 0.47
Day 14	10.28 ± 0.63	10.34 ± 0.37	9.46 ± 0.26	11.28 ± 0.28	10.8 ± 0.29
Day 21	10.34 ± 0.37	10.54 ± 0.34	9.58 ± 0.22	11.43 ± 0.43	11.10 ± 0.27
MCV (FL)					
Day 7	125.10 ± 0.96	123.90 ± 1.33	137.24 ± 1.07	129.20 ± 2.90	124.80 ± 1.89
Day 14	124.10 ± 1.18	116.80 ± 0.77	134.20 ± 1.08	136.70 ± 1.78	136.90 ± 1.78
Day 21	136.30 ± 1.91	137.3 ± 2.73	132.7 ± 1.57	135.0 ± 1.49	137.0 ± 1.15
MCHC					
Day 7	31.60 ± 0.29	31.20 ± 0.14	38.20 ± 2.85	40.80 ± 0.57	42.20 ± 1.06
Day 14	29.86 ± 1.90	31.50 ± 0.40	37.40 ± 2.72	38.30 ± 1.90	41.10 ± 0.73
Day 21	30.10 ± 0.28	31.70 ± 0.18	40.40 ± 0.62	41.20 ± 0.53	41.60 ± 0.57
HCT (%)					
Day 7	31.48 ± 0.23	31.66 ± 0.65	30.98 ± 1.10	33.58 ± 2.41	36.74 ± 1.23
Day 14	31.90 ± 1.49	30.74 ± 0.87	29.92 ± 0.33	29.64 ± 1.53	37.60 ± 1.12
Day 21	32.10 ± 0.91	32.12 ± 0.68	30.0 ± 0.49	31.90 ± 0.77	32.80 ± 2.03

Table 7: Effect of oral administration of methanol extract of *P. nodiflora* on biochemical parameters of chicks.

Biochemical parameters	Control	Plant extract			
		150 mg/kg	200 mg/kg	250 mg/kg	300 mg/kg
Alkaline Phosphatase	2650.0 ± 111.10	2637.2 ± 144.50	3066.5 ± 153.60	2345.0 ± 141.90	2012.5 ± 141.90
Aspartate Aminotransferase	6.0 ± 0.47	8.50 ± 1.44	10.50 ± 0.64	8.75 ± 0.85	7.0 ± 0.85
Alanine Aminotransferase	149.4 ± 13.44	174.50 ± 7.90	174.25 ± 7.43	183.75 ± 6.20	160.0 ± 7.43
Urea level	14.0 ± 1.08	21.0 ± 2.08	17.0 ± 1.88	18.0 ± 0.85	14.0 ± 0.85
Creatinine	0.25 ± 0.01	0.18 ± 0.03	0.34 ± 0.03	0.39 ± 0.02	0.40 ± 0.04

Table 8: Effect of oral administration of methanol extract of *P. nodiflora* on body organs of chicks.

Parameters/Day	Group (PN)				
	Control	**150 mg/kg**	**200 mg/kg**	**250 mg/kg**	**300 mg/kg**
Liver weight					
Absolute	53.11 ± 1.70	52.62 ± 1.83	44.73 ± .85	37.09 ± 1.32	35.30 ± 1.37
Relative	2.10 ± 0.08	2.33 ± 0.13	1.88 ± 0.02	1.67 ± 0.04	1.68 ± 0.05
Kidney weight					
Absolute	4.03 ± 0.01	12.4 ±0.94	3.73 ± 0.43	4.16 ± 0.26	3.5 ± 0.20
Relative	0.48 ± 0.03	0.55 ± 0.05	0.15 ± 0.01	0.18 ± 0.009	0.16 ± 0.003
Lung's weight					
Absolute	4.03 ± 0.01	11.36 ± 0.82	4.43 ± 0.08	3.73 ± 0.26	3.73 ± 0.31
Relative	0.41 ± 0.03	0.17 ± 0.008	0.16 ± 0.01	0.18 ± 0.002	0.50 ± 0.02
Thymus weight					
Absolute	4.59 ± 0.54	5.36 ± 0.31	3.66 ± 0.17	4.4 ± 0.36	4.1 ± 0.11
Relative	0.18 ± 0.02	0.19 ± 0.005	0.19 ± 0.01	0.15 ± 0.006	0.23 ± 0.007
Intestine weight					
Absolute	143.47 ± 10.7	141.7 ± 18.50	146.6 ± 5.89	149.0 ± 13.01	132.2 ± 13.11
Relative	5.62 ± 0.28	6.85 ± 1.51	6.64 ± 0.29	6.30 ± 0.58	5.89 ± 0.82
Heart weight					
Absolute	10.44 ± 0.76	10.60 ± 0.85	8.53 ± 0.54	10.96 ± 0.74	8.56 ± 0.17
Relative	0.41 ± .03	0.40 ± .01	0.49 ± .03	0.36 ± .02	0.47 ± .05
Spleen weight					
Absolute	3.27 ± 0.03	1.97 ± 0.16	2.35 ± 0.02	1.64 ± 0.14	1.36 ± 0.15
Relative	0.13 ± .002	0.08 ± .005	0.09 ± .005	0.07 ± .007	0.06 ± .004
Trachea weight					
Absolute	3.43 ± 0.31	3.65 ± 0.43	2.51 ± 0.08	2.59 ± 0.17	2.46 ± 0.26
Relative	0.13 ± .010	0.16 ± .020	0.10 ± .004	0.11 ± .008	0.11 ± .006
Bursa weight					
Absolute	1.17 ± 0.01	1.19 ± 0.005	1.77 ± 0.18	1.07 ± 0.08	1.04 ± 0.07
Relative	0.04 ± 0.002	0.05 ± 0.001	0.07 ± 0.007	0.04 ± 0.004	0.05 ± 0.005

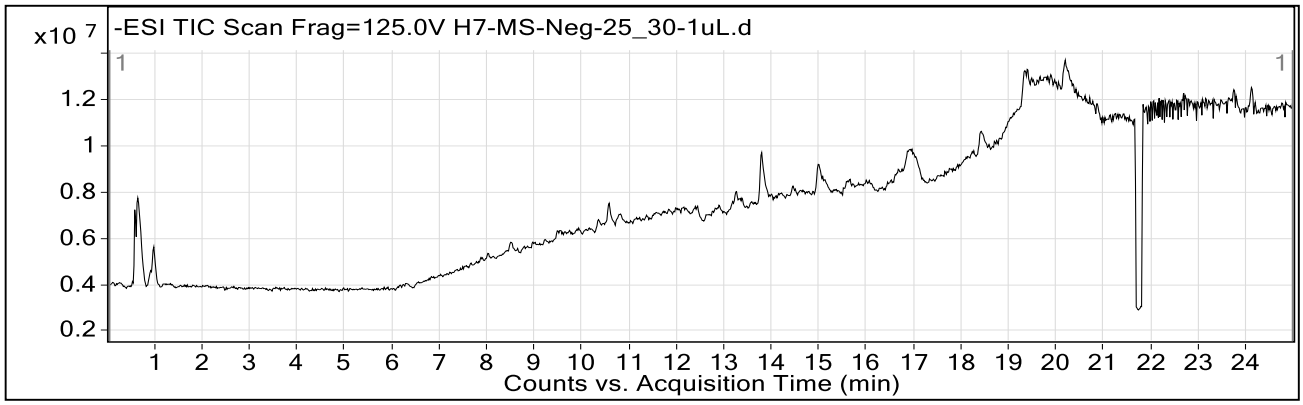

Figure 1: Total ion chromatogram (TIC) of UHPLC-MS analysis *P. nodiflora* methanol extract.

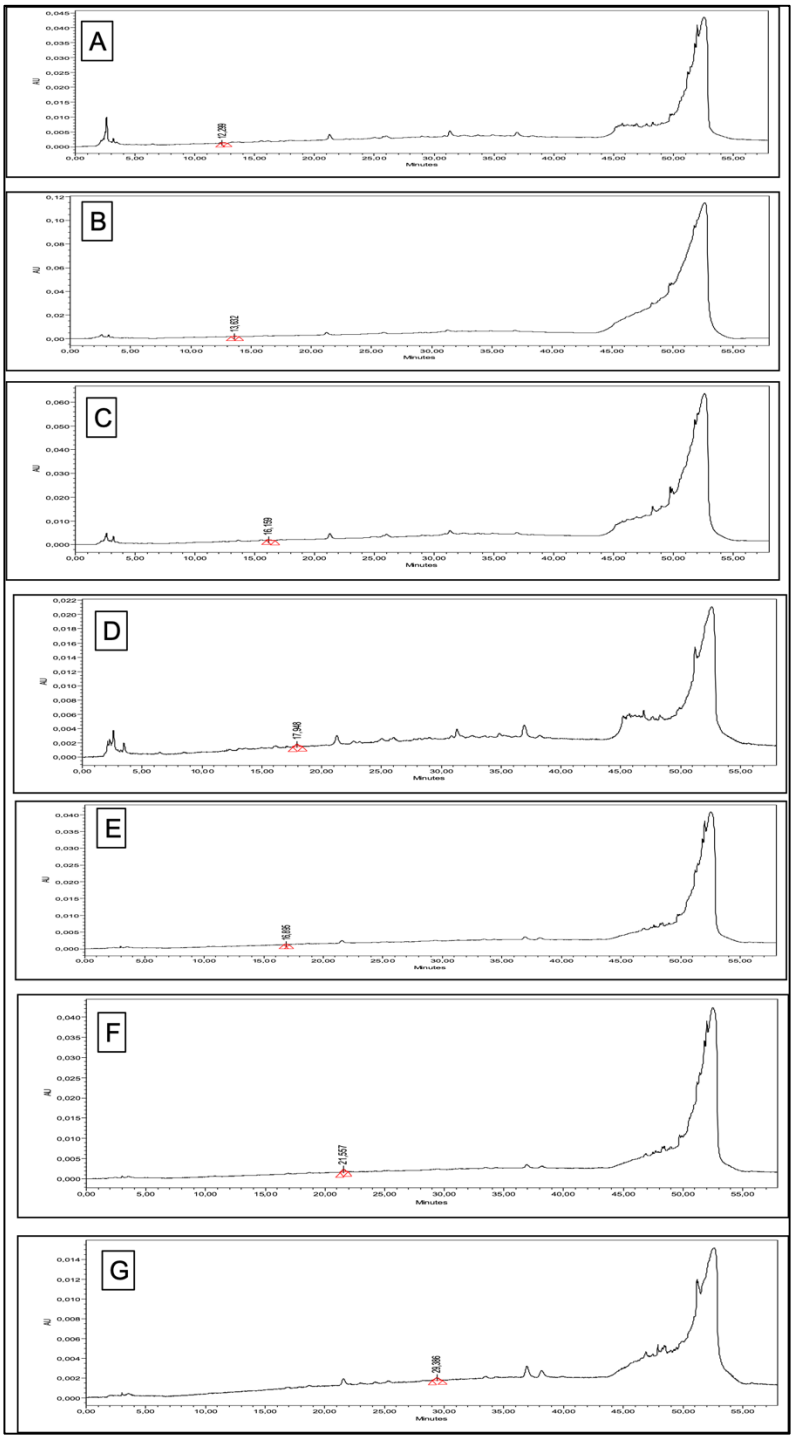

Figure 2: HPLC chromatograms of the quantified compounds from *P. nodiflora* extracts. (A: catechin 278 nm; B: *p*-OH benzoic acid 256 nm; C: vanillic acid 260 nm; D: 3-OH benzoic acid 295 nm; E: epicatechin 278 nm; F: 3-OH 4-MeO benzaldheyde 275 nm; G: 2,3-di-MeO benzoic acid 299 nm)

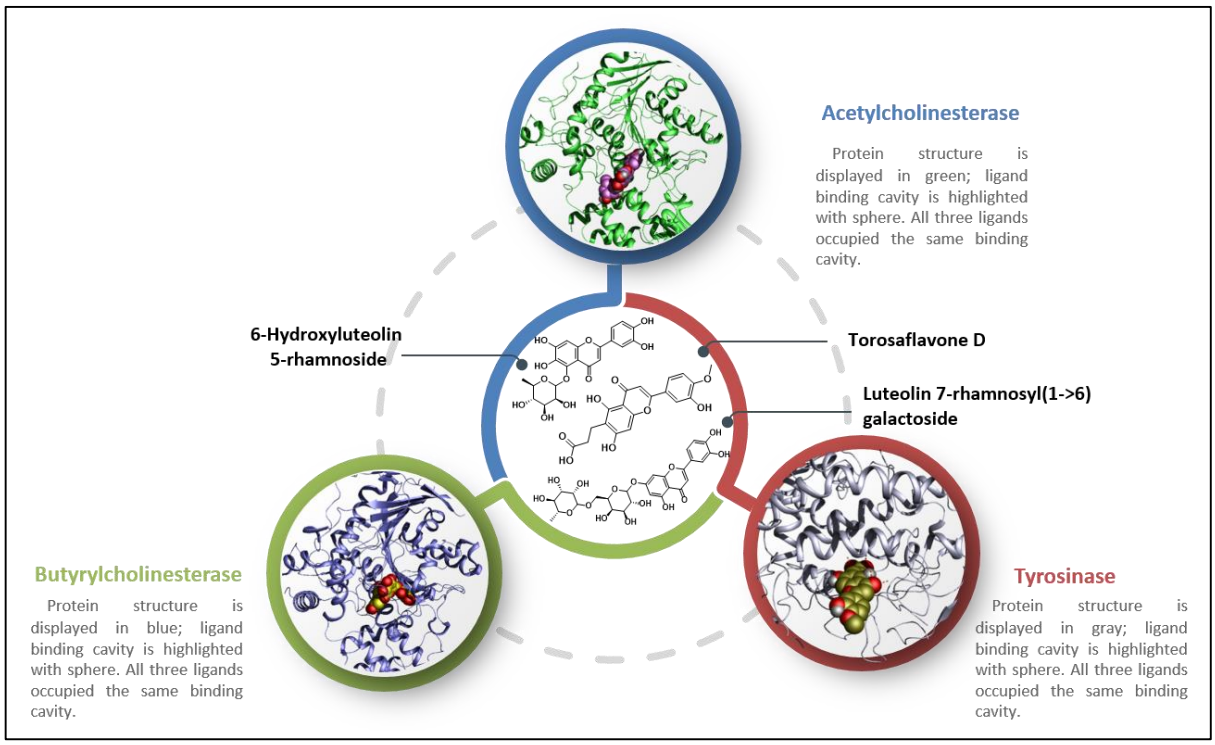

Figure 3: Chemical structures of selected ligands HLR, LRG, and TFD for molecular docking analysis. 3D-conformation of selected hits in their corresponding molecular targets.

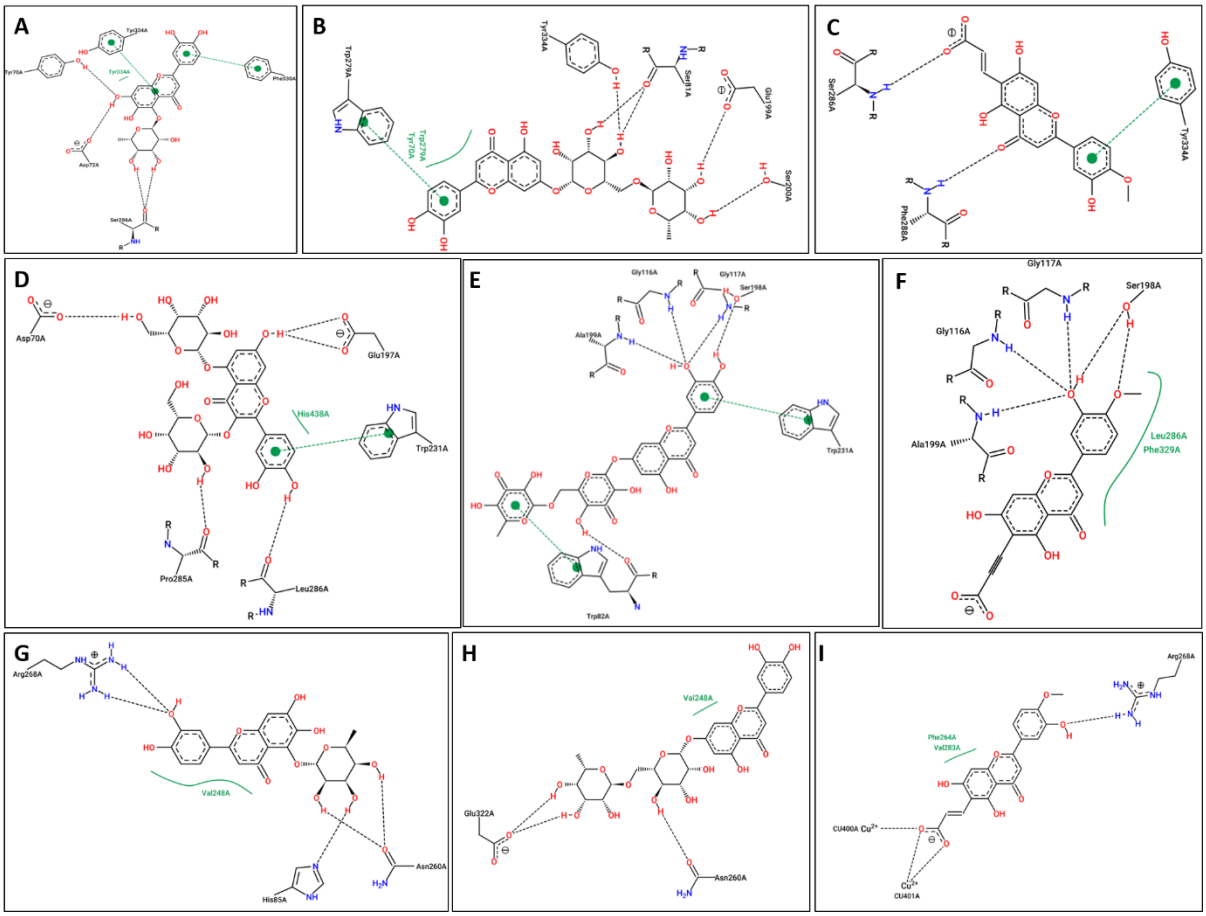

Figure 4: 2D-interaction diagram of studied ligands in complex with AChE, BChE and TYR enzymes. (A) AChE-HLR, (B) AChE-LRG, (C) AChE-TFD, (D) BChE-HLR, (E) BChE-LRG, (F) BChE-TFD, (G) TYR-HLR, (H) TYR-LRG, (I) TYR-TFD.

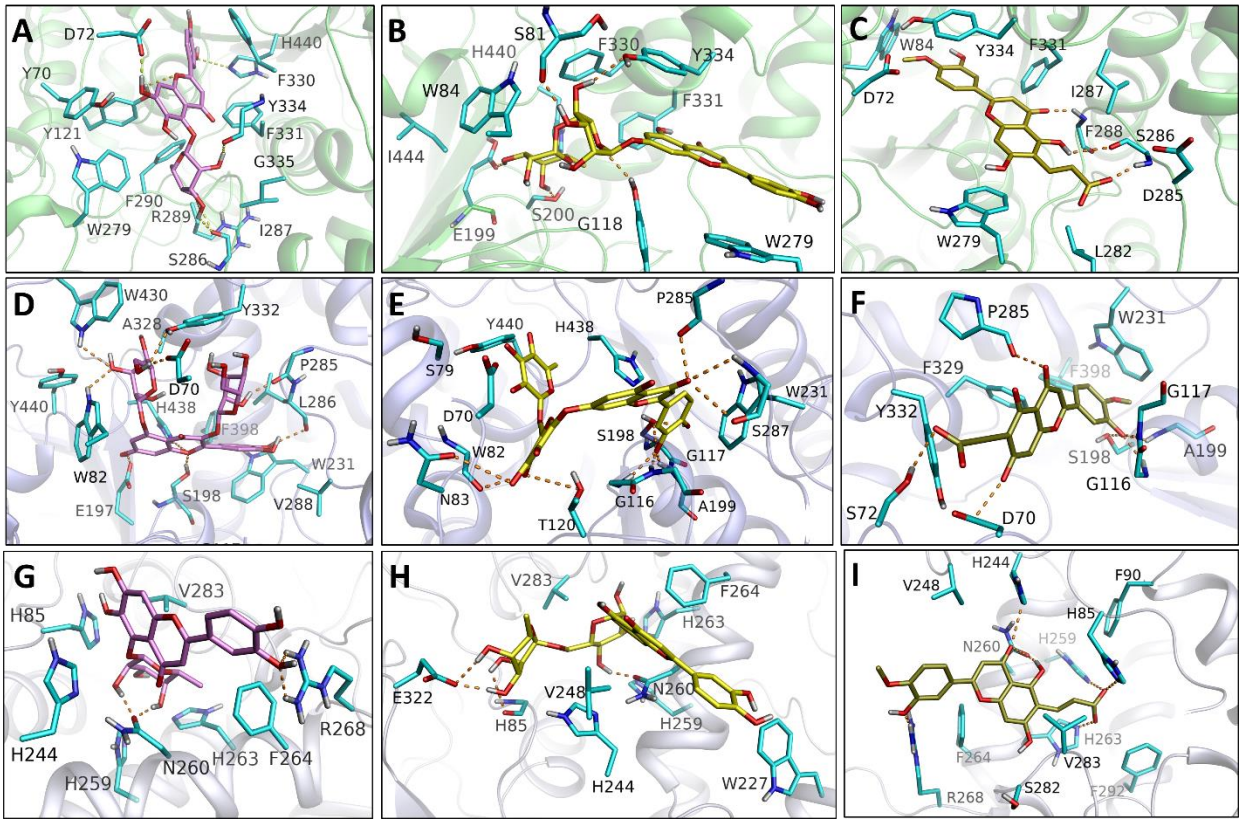

Figure 5: Docking generated complexes of proteins bonded to their tested compounds: (A) AChE-HLR, (B) AChE-LRG, (C) AChE-TFD, (D) BChE-HLR, (E) BChE-LRG, (F) BChE-TFD, (G) TYR-HLR, (H) TYR-LRG, (I) TYR-TFD.

Supplementary

To Whom It May Concern

I **Hammad Saleem** is submitting a manuscript entitled **"A comprehensive assessment of phytochemicals from *Phyla nodiflora* (L.) Greene as a potential enzyme inhibitor, and their biological potential: An in-silico, in-vivo and in-vitro approach"** for possible publication in **Arabian Journal of Chemistry**. It is stated that there is no Conflict of Interest for the submitted paper.

Sincerely Yours,

Dr. Hammad Saleem