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Insilico And Invitro Study Of The Anti-Inflammatory Activity Of The Chloroform And Methanolic Extracts Of Hibiscus Sabdariffa

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Abstract
Background*: Hibiscus Sabdariffa is traditionally a well-known medicinal plant, widely used for the treatment of hypertension, hyperglycemia, hepatotoxicity, inflammation, microbial infections and many more. The different extracts were analyzed, both in vitro and in vivo, thoroughly for the biological activities. The anti-inflammatory activity of the plant extract was investigated independently in different solvents of varying polarity. Here the methanolic amd chloroform extracts of the plant are compared for the anti-inflammatory activity.*
Materials and Methods*: The methanol and chloroform extract was collected by soxhlet extraction. The invitro anti-inflammatory study is carried out using protein denaturation assay. Diclofenac is taken as the standard. Also, the docking study of the phyto constituents, which are believed to be responsible for the anti-inflammatory activity are subjected to docking study.*
Results*: It is now proved that the methanolic extract supersede the other one in the activity. The docking study of the compounds responsible for anti-inflammatory activity has been conducted and concluded that they have comparable anti-inflammatory activity with the standard drug, diclofenac.*
Keywords: *Chloroform, Methanol**,** Diclofenac, BSA solution, protein denaturation, docking*

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I. Introduction

Hibiscus sabdariffa (family: Malvaceae), commonly referred to as Roselle, is a plant native to tropical and subtropical regions and widely consumed as a herbal tea, especially in Africa, Asia, and the Caribbean. Traditionally, various parts of the plant—particularly the calyces—have been used in folk medicine for treating a range of conditions. Modern scientific research has increasingly validated its pharmacological potential. The extracts of Hibiscus Sabdariffa is intensely red in colour due to high content of anthocyanins1. The plant is popularly used for making herbal tea due to the presence of anthocyanins, phenolic compounds, flavonoids and hibiscus acids, which are water soluble2.

Biological activity**,**

Numerous studies have confirmed the strong antioxidant activity of *H. sabdariffa*, primarily attributed to its rich polyphenolic and anthocyanin content. Delphinidin-3-sambubioside and cyanidin-3-sambubioside are major anthocyanins found in the calyces. These compounds scavenge reactive oxygen species (ROS), inhibit lipid peroxidation, and enhance endogenous antioxidant enzymes such as superoxide dismutase (SOD) and

catalase 1. It was demonstrated that hibiscus extract significantly reduced oxidative stress markers in animal models. Another most well-documented biological effect of *Sabdariffa* is its blood pressure-lowering ability. A thorough analysis confirmed that hibiscus tea moderately but significantly reduces blood pressure in hypertensive and mildly hypertensive adults 3. Extracts of *H. sabdariffa* have demonstrated hypolipidemic effects in both animals and humans. Reduction in LDL, total cholesterol, triglycerides, and increase in HDL levels was observed4. Reports are there that hibiscus extract reduced body weight and improved lipid profiles in obese mice on a high-fat diet. It has shown potential in lowering blood glucose and improving insulin sensitivity. Mechanism involves the Inhibition of α-glucosidase and α-amylase, enhancement of pancreatic β-cell function, and protection against oxidative damage5. It is shown to be active against *Staphylococcus aureus*, *E. coli*, *Salmonella typhi*, and *Helicobacter pylori*. It also demonstrates antimalarial effects in vitro against *Plasmodium falciparum***.** Mechanism involves membrane disruption and inhibition of microbial enzymes6. Preclinical studies suggest that *H. sabdariffa* protects liver and kidney tissues against chemically induced damage7.

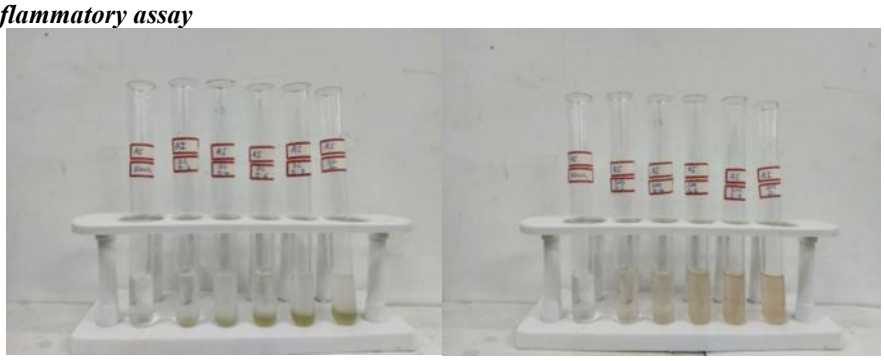
Hibiscus sabdariffa is a plant of significant medicinal interest with proven antioxidant, antihypertensive, lipid-lowering, antidiabetic, and antimicrobial properties8. The invivo anti-inflammatory studies were done and found that there is no significant effect on the rat paw edema, but effective against yeast induced pyrexia9. The anti-inflammatory activity of Roselle is due to the presence of large amount of polyphenols. The presence of ten phenolic acids, ten flavonoids, and two anthocyanins was determined by 1H NMR analysis10. Flavonoids in Hibiscus sabdariffa can inhibit NF-κB, a protein complex that regulates the expression of genes involved in inflammation. The plant has been shown to reduce the production of pro-inflammatory cytokines like nitric oxide (NO), IL-6, and TNF-α, which are crucial mediators of inflammation. The potential anti-inflammatory activities of the *sabdariffa* leaf extracts were screened against RAW 264.7 murine macrophage cells in vitro. Inhibition of nitric oxide synthetase (NOS) was determined by treating the cells with lipopolysaccharide induced inflammatory response. Cells treated with the extracts exhibited a dose-dependent inhibition of NOS11. The in-vitro anti oxidant study of the extract was previously studied by protein denaturation method12-14. The aqueous, chloroform and methanolic extracts of the plant were employed for different bioactivity study. In this work, we compare the chloroform and methanolic extracts of Roselle for the anti inflammatory activity. Dried Calyces of *Hibiscus Sabdariffa* collected from the Kollam region of Kerala during January. The plant material was authenticated by the Department of Botany, University College, Thiruvananthapuram.

The *insilico* analysis was carried out with different phytochemical constituents of the plant extract for its diverse biological activities like antidiabetic activity, antihypertensive activity etc15-16. Cyclooxygenase (COX) is a key enzyme involved in the inflammatory process and serves as a common target for anti-inflammatory drugs. In silico studies investigating the anti-inflammatory potential of Hibiscitrin, Delphinidin, Hibiscetin, Gossypetin, and Diclofenac were conducted by targeting COX. Molecular docking analyses were carried out to assess their interactions with the enzyme's active sites. Suppressing COX enzymatic activity is crucial for reducing inflammation, making it a significant focus for therapeutic intervention.

II. Materials And Methods

A 2% BSA solution, Diclofenac Sodium, Phosphate buffered Saline, Methanol, Chloroform were employed. All chemicals were purchased from Merck and are of AR grade. Dried, powdered sample was soxhlet extracted with chloroform and methanol for about four hours. The extracts were cooled, filtered and concentrated. The anti-inflammatory assay was done by protein denaturation method**.**

Invitro anti-inflammatory assay



The standard and the extracts were prepared at five different concentrations ranging from 0.2 µg/ml to 1 µg/ml . A total volume of 5 mL of the control was created by combining 2 mL of the standard, 0.2 mL of 1-

2% BSA solution, and 2.8 mL of phosphate-buffered saline. The test solutions were also prepared by taking the different extracts. The reaction mixtures were then incubated at 37±2°C for 30 min and heated in a water bath at 70±2°C for 15 min. After cooling, the absorbance was measured at 280 nm by UV/Vis spectrophotometer using triple distilled water as the blank. The following equation was used to determine the % inhibition of protein denaturation.

Percentage inhibition = [Absorbance of control - Absorbance of sample]100/ Absorbance of control

Then concentration for 50% inhibition (IC50) was determined by plotting percentage inhibition concerning control against concentration. Absorbance of control = 0.994nm

Docking studies.

Recently, docking and scoring softwares are widely used to hasten the drug design and product development in pharmaceutical industry17,18.

Protein Data Bank: Binding energies of protein-ligand interactions play a crucial role in evaluating the potential of a molecule as a drug candidate19. For molecular docking studies, the three-dimensional structure of the target protein was selected and retrieved from the Protein Data Bank (PDB). The protein with PDB ID: 3LN1 was obtained and used for subsequent analyses. Docking simulations were carried out to identify the optimal binding pose and interactions between the protein and ligand. The docking procedure was performed using iGEMDOCK v2.1 (Generic Evolutionary Method for Molecular Docking), a graphical and automated drug design platform employed for molecular docking, virtual screening, and post-docking analysis.

Structure Drawing Software: The two-dimensional (2D) structures of the compounds were drawn using MarvinSketch v5.3. The geometries of the structures were optimized using the cleaning function, and the finalized structures were saved in PDB format for further studies.

Visualization Software: Discovery Studio Visualizer 2024, a freely available molecular visualization tool, was used for the visualization and analysis of biological and chemical data. Following post-docking analysis, two-dimensional (2D) interaction diagrams of the protein–ligand complexes were generated and visualized using this software.

Docking Procedure: All docking processes were carried out in Windows 10 software The crystal structure of protein was downloaded from protein data bank with protein id: 3LN1 with resolution 2.40 Aº. The water molecules were deleted, and the binding sites at 8A0 were identified. The docking was conducted using Hibiscitrin, Delphinidin, Hibiscetin, and Gossypetin as ligands16. Binding energies of protein-ligand interactions play a crucial role in evaluating the potential of a molecule as a drug candidate19. The binding affinities between the protein and ligands were calculated and compared with the standard drug Diclofenac20,21. The compound with the lowest binding energy was identified as the most promising inhibitor.

After loading the proteins and ligands, the output path was set. For docking, the standard docking parameters were used. Population size = 200, generations = 70 and number of solutions=2. Mod_ leg is a programme used to generate ligand list depending on user-selected ligands. Mod_ ga is used for the docking/ screening module. After the docking process, the best poses can be obtained and visualized using Discovery studio visualizer 2024. The interaction data obtained includes summarized energy and individual energy terms. Fitness is the total energy of a predicted pose in the binding site. The total energy, van der Waals interactions (vdW), Hydrogen bonding (H-Bond), and Electrostatic interactions (Elec) can also be obtained from the software. The Empirical scoring function of iGEMDOCK is estimated according to the equation;

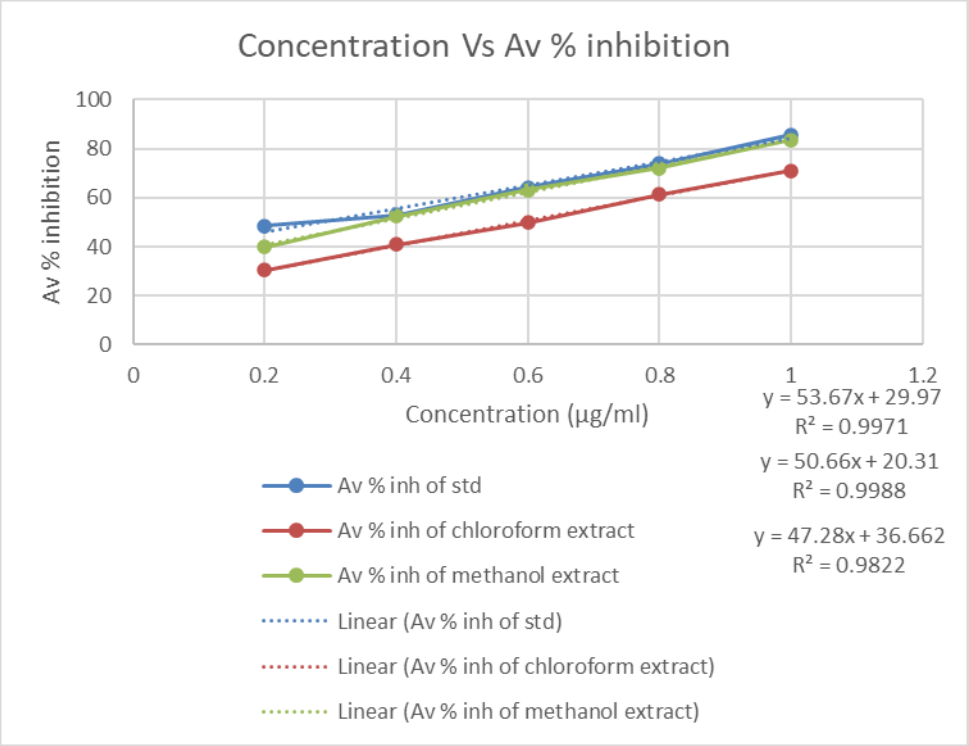
Fitness = vdW + HBond + Elec

III. Result And Discussion

From the graph given below, the percentage inhibition of standard, methanolic and chloroform extract can be evaluated.

Table No 1 Percentage Inhibition of 2% BSA by standard, chloroform and methanolic extracts

Concentration (µg/ml)	Absorption (standard)	Percentage inhibition (standard)	Absorption (Chloroform extract)	Percentage inhibition (chloroform extract)	Absorption (methanol extract)	Percentage inhibition (methanol extract)
0.2	0.511	48.59	0.691	30.48	0.599	39.74
0.4	0.467	53.02	0.587	40.95	0.474	52.31
0.6	0.355	64.29	0.499	49.8	0.366	63.18
0.8	0.261	73.74	0.384	61.37	0.277	72.13
1	0.144	85.51	0.289	70.93	0.164	83.5



It is found that the IC50 value of standard is the lowest, followed by methanolic and chloroform extracts. The lowest value for IC50 in the case of methanolic extract may be attributed to the increased phytochemical content in it, as it is evident from the image of the extracts given in the experimental section**.** Moreover, the contents are completely miscible in the case of methanolic extract.

Table No 2 IC50 values of standard, chloroform and methanolic extracts

Sl No.	Sample	IC50
1	Chloroform extract	**0.586064±0.021µg/ml**
2	Methanol extract	**0.373207±0.001µg/ml**
3	Standard	**0.282107±0.004µg/ml**

Docking

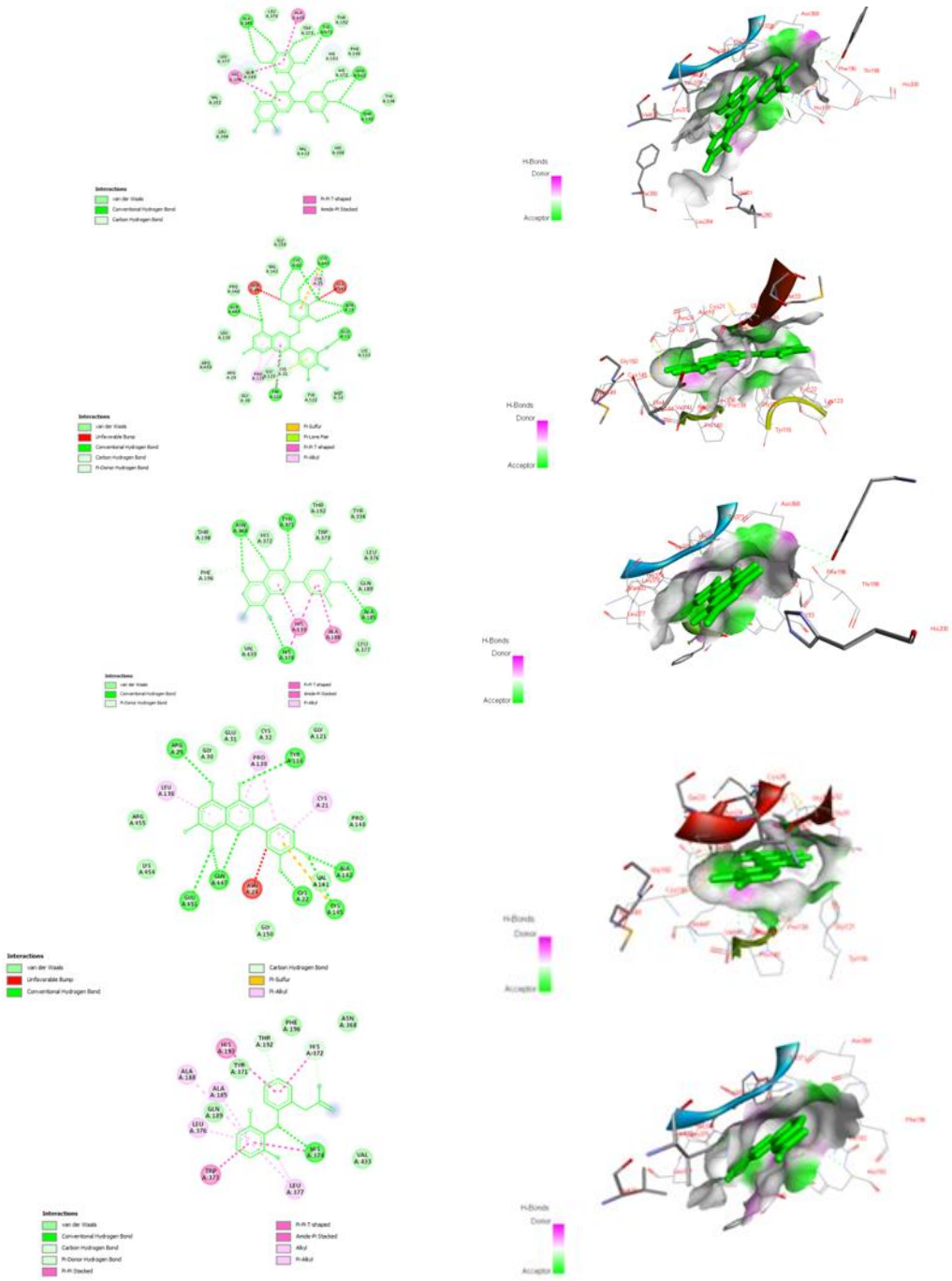
The phytoconstituent Hibiscitrin exhibited hydrogen bond interactions with residues ASN A:368, THR A:198, TYR A:371, and ALA A:185, along with π–π interactions involving ALA A:188 and HIS A:374. These interactions contributed to a total binding energy of –143.59 kcal/mol.Molecular docking images revealed that Delphinidin formed hydrogen bonds with GLN A:447, CYS A:22, TYR A:116, CYS A:145, ASN A:19, and GLU A:31. Additionally, π–π interactions were observed with PRO A:139 and CYS A:21, although two unfavorable interactions were detected with ASN A:24 and ALA A:142. The total binding energy for Delphinidin was –141.09 kcal/mol.Hibiscetin showed conventional hydrogen bonding with ASN A:368, TYR A:371, HIS A:374, and ALA A:185, along with π–π stacking interactions with histidine and alanine residues. These interactions resulted in a total binding energy of –117.36 kcal/mol.Another phytochemical, Gossypetin, demonstrated hydrogen bonding with TYR A:116, ARG A:29, GLU A:451, GLN A:447, CYS A:22, ALA A:142, and CYS A:145. However, an unfavorable interaction was observed with ASN A:24. The overall binding energy for Gossypetin was –107.14 kcal/mol.

For the standard drug Diclofenac, hydrogen bonding and van der Waals interactions were identified with ASP A:594. HIS A:535 and GLY A:374 were involved in hydrogen bonding, while ASN A:368, PHE A:196, TYR A:371, and GLN A:189 contributed through van der Waals interactions. The binding energy for Diclofenac was calculated to be –100.02 kcal/mol.

Overall, the primary interactions observed across all compounds were hydrogen bonds and van der Waals interactive forces. Among the tested ligands, Hibiscitrin showed the strongest binding affinity, while the standard drug Diclofenac had the weakest. The visual representations of these interactions are provided below and detailed docking results are summarized in **Table 3**

Table 3 Docking scores of phytochemicals from *hibiscus sabdariffa*

Sl No.	Compound	Energy	VDW	H Bond	Elec
1	COX-Hibscitrin-0pdb	-143.59	-105.31	-38.27	0
2	COX-delphinidin-3-glucoside-1. pdb	-141.09	-104.33	-36.76	0
3	COX-Hibscetin-0.pdb	-117.36	-93.26	-24.1	0
4	COX-Gossipetin-1.pdb	-107.14	-81.31	-25.83	0
5	COX-diclofenac-0pdb	-100.02	-91.16	-4.82	-4.04



IV. Conclusion

The *insilico* and *invitro* anti-inflammatory study of the *hibiscus sabdariffa* is carried out in a low polar and highly polar solvent. The plant is reported to have diverse biological activity. This is reiterated by the anti-inflammatory activity study conducted in our lab. Both the extracts under study showed significant activity. The result shows that the highly polar methanolic extract has got a high anti inflammatory activity than the low polar

chloroform. The utility of a halogenated solvent cannot be considered as a promising route for the extraction purpose, since it showed a lower bioactivity. The results of protein denaturation assay showed that the methanolic extract is found to be more active so this can be a future ingredient of Ayurvedic drug.

The *insilico* results revealed that Hibiscitrin exhibited the highest binding affinity with a binding energy of –143.59 kcal/mol. All other tested phytochemicals also showed more negative binding energy values compared to the standard drug Diclofenac, which had a binding energy of –100.02 kcal/mol. These findings suggest that all the phytochemical compounds demonstrate strong activity against the COX protein, a key enzyme involved in inflammation, indicating their potential as effective anti-inflammatory agents

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