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Synergistic Anti-rheumatoid Activity: Green Extraction, Homology Modelling and Molecular Docking of *Boswellia serrata* and *Hygrophila auriculata*

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Abstract

Background: *Boswellia serrata* and *Hygrophila auriculata* are essentially medicinal plants with significant anti-inflammatory properties. While *B. serrata* is rich in boswellic acids that inhibit the activity of inflammatory mediators, *H. auriculata* is rich in flavonoids and alkaloids recognised for their anti-arthritic effects. Therefore, the therapeutic application of the combined extracts may manifest additional benefits by exerting a synergistic effect toward inflammation problems like rheumatoid arthritis. **Aim:** The study aims to evaluate the activity of protein denaturation using *B. serrata* and *H. auriculata* extraction samples in an *in vitro* anti-inflammatory experiment. **Methodology:** The test extract, at different concentrations, was incubated with Bovine Serum Albumin (BSA) under controlled experimental conditions and subjected to the determination of absorbance at 660 nm to evaluate the anti-inflammatory property. Aspirin was used as the reference standard drug. In addition, *in-silico* studies such as molecular docking were performed to check for interactions with key inflammatory proteins. **Results:** The combination of the extracts from *B. serrata* and *H. auriculata* substantially inhibited the protein denaturation ability. The IC-50 values showed enhanced efficacy compared to the single extract, indicating synergy. Molecular docking analysis revealed higher binding affinities to the inflammatory target proteins, showing that it could serve as an anti-arthritic agent. **Conclusion:** The synergistic combination of *B. serrata* and *H. auriculata* extracts enhances their anti-inflammatory activity, thereby increasing the potential use of this combination for the treatment of rheumatoid arthritis. This also emphasises the importance of plant bioactive compounds in the development of potential natural anti-arthritic therapies.

Major Findings: The extracts of *B. serrata* and *H. auriculata* together showed remarkable inhibition of protein denaturation, and this was interpreted as a synergistic anti-inflammatory effect. The GC-MS profiling revealed the presence of bioactive compounds, including caryophyllene oxide, which was found to have a strong binding affinity for inflammatory target proteins in the molecular docking studies. All these findings together suggest the plant combination as a natural management strategy for rheumatoid arthritis through the way of therapeutic application.

Keywords: *B. serrata, H. auriculata*, Molecular Docking, Rheumatoid Arthritis, Synergistic Activity

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1. Introduction

Rheumatoid Arthritis (RA) is a worldwide, chronic inflammatory autoimmune disease, with a prevalence of about 1% of the world population and 0.75% in India[1,2]. Inflammation of synovial joints is a characteristic of RA, causing pain, stiffness, and possible destruction of joints. A complex interplay of genetic and environmental factors results in synovitis and joint degradation[3]. It is characterised by symmetric polyarthritis, commonly affecting initially small joints of the hands, wrists, and feet[4]. Cytokines, particularly interleukin-1β and interleukin-6, are mediators of destruction in the synovium and cartilage[5]. Extra-articular manifestations may involve the skin, lungs, and heart. Current treatments for RA, which include NSAIDs and DMARDs, are associated with adverse side effects; thus, alternative therapy has gained some attention. Salient Features of RA - symptoms: The most common manifestations include morning stiffness, joint pain and fatigue, loss of mobility. Pathophysiology: Cell-mediated immune response of the disease involves active participation by CD4+ T cells and B cells in inflammation[6]. Current treatments for RA, which include NSAIDs and DMARDs, are associated with adverse side effects; thus, alternative therapy has gained some attention. Herbal remedies possess a few advantages as compared to conventional therapies, such as fewer side effects and greater accessibility.

Hygrophila auriculata (Figure 1a) is found widely across tropical and subtropical regions and has been reported for its medicinal properties. The plant contains phytochemicals in abundance, which includes phytosterols, flavonoids, alkaloids, and fatty acids. It exhibits good pharmacological activity with hepatoprotective, antinociceptive, anti-inflammatory activities, antioxidants, hypoglycaemic, and antimicrobial effects[7]. Traditionally, different parts of *H. auriculata* were used for inflammation, jaundice, urinary infections, and diabetes. Having a widespread distribution in Asia, it acts as a great inflammatory and antioxidant agent. Being loaded with various classes of phytochemicals such as phytosterols, flavonoids, and alkaloids, the plant gives a clue about its wide range of pharmacological activities, like hepatoprotective, antinociceptive, and hypoglycaemic activity. The present study shines a light on the possibilities of *H. auriculata* as a source of bioactive compounds that may be used to treat inflammatory disorders and other diseases.

Boswellia serrata (Figure 1b), the mother of all Indian frankincense, grows in dry deciduous forests in India. It has long been used in *Ayurveda, Unani*, and *Siddha* systems of medicine[8,9]. The genus *Boswellia*, *Boswelliaserrata* Roxb. ex Colebr., is considered indigenous to India. The genus is said to manifest a wide range of pharmacological effects and hence is used in various traditional systems of medicine. The mucilaginous oleoresin (tree gum and resin) harvested mainly from its bark constitutes the medicinal portion of this plant. The gum consists of volatile oil, water-soluble gum (polysaccharides), lipophilic terpenes, and insoluble matter[10]. The oleo-gum-resin of *B. serrata* is considered to have the majority of medicinal properties, and boswellic acids and essential oils are the major constituents[11]. A great number of reports are available concerning the pharmacological activities of *B. serrata*, including anti-inflammatory, analgesic, anticancer, and antimicrobial activities[12,13]. *Boswellia* has been used in a variety of disorders, including asthma, arthritis, and skin diseases. As an inflammation inhibitor for anti-RA therapy, *B. serrata* Extract (BSE) shows great promise. BSE has been proven to decrease inflammation parameters, reduce pro-inflammatory cytokines, and modulate antioxidant mechanisms in RA animal models.

1.1 Rationale for Plant Selection and Combination

The choice of each plant extract that was used in this research was influenced by its traditional use in treating inflammatory diseases and rheumatologic disorders, as well as by the literature evidence that backed its anti-inflammatory and immunomodulatory powers. These plants have long been used in ethnomedicine to alleviate the main symptoms of rheumatoid arthritis, such as pain, swelling, and stiffness. The presence of active constituents like polyphenols, flavonoids, terpenoids, and alkaloids in the plants has been reported in previous studies. Many of these active constituents are known to interfere with the signalling, expression, and other pathways responsible for inflammation by including NF-κB signalling, cytokine expression, oxidative stress, and cartilage degradation.

The plants have been shown individually to possess anti-arthritic and anti-inflammatory effects, but their combined application is not widely studied. The use of multi-component botanical therapies from a pharmacological perspective may provide synergistic benefits, the first of which is the interaction of compounds from the different extracts that leads to increased bioavailability. Other benefits may include targeting complementary molecular pathways and reducing the toxicity that is linked with high-dose monotherapy. Such synergy is in line with traditional poly-herbal formulations relying on "compound-to-compound cooperation" rather than single-agent therapy.

Hence, the present study aimed to determine if these extracts when combined would exhibit increased biological activity than individual administration, thus offering scientific justification for their traditional co-use and paving the way for the potential development of phytotherapeutic combinations for the management of rheumatoid arthritis.

2. Materials and Methods

2.1 Plant Collection, Identification and Authentication

2.1.1 Hygrophila auriculata

The plant *H. auriculata* was bought from the medicinal plant supplier Swati Naturals, Dindugal. The plant authentication was obtained from the Tamil Nadu Agricultural University (TNAU), Tamil Nadu, Coimbatore. After authentication, the collected whole plant parts were properly dried in shade for 2-3weeks (Figure 1c). They were pulverised using a blender, sieved using a sieve shaker of mesh size 12, and used for further studies.

2.1.2 Boswellia serrata

The dried gum resin of *B. serrata* (Figure 1d) was bought from a traditional plant drug store near Townhall, Coimbatore. The resins were crushed to coarse powder (Figure 1e), and they were passed through a sieve of mesh size 12 (Figure 1f) and used for further studies.



Figure 1a.



Figure 1b.



Figure 1c.



Figure 1d.

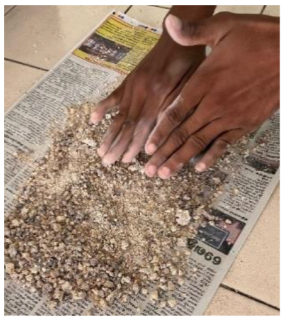


Figure 1e.



Figure 1f.

Figure 1. Fresh and dried plant materials and processing steps: **(a).** Fresh plant of *Hygrophila auriculata*; **(b).** Dried plant of *Hygrophila auriculata*; **(c).** Fresh plant of *Boswellia serrata*; **(d).** Dried gum resin of *Boswellia serrata*; **(e).** Powdered gum resin of *Boswellia serrata*; **(f).** Sieving process using mesh No. 12.

2.2 Green Extraction

The green extraction of bioactive substances using ethanol is stacking up to become a sustainable method utilised for modulation from different natural sources. This approach stands to retain all merits of ethanol in acting as a solvent while lessening the impact on flora and fauna, hence its preferability in terms of green chemistry. Ethanol extraction reduces reliance on harmful solvents like hexane, promoting eco-friendly practices in oil recovery from palm oil waste[14,15]. Ethanol extraction of phytochemicals from *H. auriculata* is one such promising green method to enhance the recovery of valuable phytochemicals. Ethanol, being polar, dissolves a wide range of phytochemicals, making it suitable for the extraction of compounds with health benefits. The following sections describe the advantages of this method and the results discovered.

2.2.1 Ethanol as a Green Solvent

It has been considered eco-friendly and safer compared to other organic solvents, thus implying green chemistry principles. It has also been proven to render better extraction rates of bioactive compounds such as β-sitosterol-β- D-glucoside, which acts against inflammation[16].

2.2.2 Health Benefits of Extracts

The ethanolic extract of *H. auriculata* has exhibited protective effects against male infertility in animal models by restoring sperm parameters and testosterone levels[17]. Furthermore, the extract has been used in the green synthesis of silver nanoparticles, shedding light on its potential and versatility in nanotechnology[18].

2.2.3 Optimisation of Extraction Conditions

Research suggests that an optimised ethanol-water composition can greatly improve the extraction of phenolic compounds, which are responsible for antioxidant activity[19].

While ethanol extraction offers several advantages, variations in extraction efficiency with varying extraction conditions and plant materials need to be taken into account. Hence, more studies may need to be undertaken to set process standards, enabling industrial-level application.

2.2.4 Preparation of Plant Extract

The whole plant of *H. auriculata* was converted into a coarse powder, of which 50 g were taken and macerated with 200 ml of ethanol for 3 days. Then the extract was filtered through filter paper and kept in a conical flask.

The dried gum resin of *B. serrata* was converted into a coarse powder, of which 50 g were taken and macerated with 200 ml of ethanol for 3 days. Then the extract was filtered through filter paper and kept in a conical flask.

2.2.5 Classical Extraction by the Percolation Method

It is a traditional technique for extracting materials for conventional medicines. It is simple and flexible for all sorts of materials. It is more suitable in the case of the extraction of heat-sensitive components[20,21]. Factors affecting the process of percolation are the composition of the solvent, time of impregnation, the speed under which percolation occurs, and solvent consumption. Such extraction methods are important for drug discovery and development to yield samples for qualitative and quantitative studies of natural products[22,23].

2.3 Evaluation of Phytochemical Composition

2.3.1 Preliminary Phytochemical Screening

The phytochemical standardised screening method was carried out on the ethanolic extract of *H. auriculata* for the purpose of detecting the major class of secondary metabolites. The dried plant material was subjected to ethanolic extraction, and the resulting extract was filtered and concentrated. The concentrated dried extract was re-dissolved in ethanol and subjected to qualitative testing.

Tests for alkaloids, flavonoids (Shinoda test), phenols and tannins (Ferric Chloride test), saponins (Foam test), steroids (Liebermann Burchard test), terpenoids (Salkowski test), and Total Phenolic Content (TPC) test were performed using standard procedures, and their findings are discussed in (Table 1).

2.3.2 Gas Chromatography-Mass Spectrometry (GC-MS)

Gas Chromatography-Mass Spectrometry (GC-MS) is a sophisticated analytical technique that

merges the actions of gas chromatography and mass spectrometry to help identify and quantify substances in larger mixtures. In pharmaceutical development, it is used to develop and validate methods in pharmaceutical research for accurately measuring active ingredients[24].

Gas chromatography-mass spectrometry used the Perkin Elmer Clarus SQ8C instrumentation, which has a high-performance system. It gives an intuitive response for sensitivity and reliability in analytical applications. The substances are separated with a DB-5 MS capillary column, which is a standard nonpolar column widely used for different applications, such as those derived using this dimension from the products given, due to its selectivity for volatile and semi-volatile organic compounds. This column is of the length of 30 meters ID of 0.25 mm; the film thickness is 0.25 µm; thus, it provides an excellent balance between resolution efficiency and analysis time on the sample. Helium (He) is the carrier gas for the analyses because of its inert properties, diffusion properties, and ability to give constancy of flow to allow the proper separation of peaks and repeatability. Injection volume was 1 µl for extremely sensitive detection with no wastage. Sample ID is IPSG HA-1 2910I, for which reference standards can be traced. Within the assembly, settings take up to a condition of chromatographic separation with very fine details and sensitive detection by mass spectrometry, making it very suitable for qualitative and quantitative studies on heterogeneous mixtures.

Retention time marked the peaks in the GC-MS chromatogram (Figure 2) of the ethanol extract of *H. auriculata*. Their retention time and their amount of presence can be seen listed in (Table 2). Through qualitative analysis, 43 compounds in the ethanolic extract were indicated.

2.3.3 In-vitro Test Analysis

2.3.3.1 Protein Denaturation using Bovine Serum Albumin (BSA)

The reaction mixture contained ethanolic extract of *B. serrata* and *H. auriculata* at different concentrations and 1% of aqueous solution of Bovine Serum Albumin (BSA). To prepare 1% BSA solution, 0.5ml of Bovine serum albumin and 50 mL of W/V cold distilled water were taken. The mixture is stirred continuously to obtain a clear component.

2.3.3.2 Assay using Bovine Serum Albumin

Bovine Serum Albumin (BSA) is commonly used in proteinase inhibitory tests. Although it interacts with many proteins and inhibitors, BSA can serve as a proteinase substrate in the assay. Such assays are meant to study how different substances inhibit proteinase activity, as various studies have demonstrated.

2.3.3.3 BSA as a Protein Substrate in Protein Assays

Frequently, BSA is used as a substrate in proteinase assays to analyse how inhibitors can negatively affect certain substances. For example, in the inhibition of *Candida albicans* Sap2 proteinase activity, BSA hydrolysis was used to check inhibition by antibodies; however, the specific antibodies showed no significant inhibition[25]. Similarly, the BSA substrate was used to demonstrate inhibition of elastase and trypsin by lens proteins[26].

2.3.3.4 Inhibition by Some BSA Preparations

On the other hand, some commercial BSA preparations have evidently inhibited some reactions, like inhibiting erythrocyte agglutination in serological tests. Interpreting assay results may become difficult if inhibitors are mediated by the BSA preparation; thus, one must exercise great care in the selection and screening of the BSA batch for use in an experiment[27].

2.3.3.5 BSA and Protein Complex

Being able to form complexes with other proteins, such as α1-antitrypsin, BSA loses its capacity to bind to other constituents and inhibit functions. This interaction is essential in antimicrobial peptide activity inhibition, where the BSA/α1-antitrypsin complex inhibits peptide activity[28]. While BSA is useful in proteinase assays, its interactions with other proteins and its capacity to inhibit must be considered. Inhibitors in BSA preparations or complex formation by BSA with other proteins could affect the outcomes of the assays, thus placing more caution upon experimental designs and interpretation.

Sample mixture- A 0.5-ml total mixture is prepared by combining 0.2 ml of 1 % BSA with 2 ml of ethanolic extract of *B. serrata* at varying concentrations and 2.8ml of phosphate buffer (pH 7.4). The same is prepared using the ethanolic extract of *H. auriculata*.

Standard mixture: The full mixture of 0.5ml is assembled from 0.2ml of 1% BSA, 2ml of standard aqueous drug aspirin, and 2.8ml of phosphate buffer (pH-7.4).

Control mixture: The full-volume mixture of 0.5ml is composed of 0.2ml of 1% BSA, 2.8ml of phosphate buffer (pH 7.4), and 2ml of triple-distilled water.

2.3.3.6 Proteinase Inhibitory Action

The reaction mixture (5ml) containing 0.2% of 1% BSA, 2.8ml of phosphate buffer, and 2ml of ethanolic extract of *H. auriculata* (Table 3), *B. serrata* (Table 4), and combined extract - containing 50:50 ratio- *i.e.*, 1ml of *B. serrata* extract and 1ml of *H. auriculata* (Table 5), and standard aspirin mixture was incubated at 37° C for 30 minutes. After 30 minutes, the pH of all the reaction mixtures was noted using a pH meter, and checked whether the pH was 7.4 and the reaction mixture was adjusted using 0.1% conc. HCl to maintain the same pH (pH=7.4). Heat the reaction mixtures in a water bath at 70° C for 15 minutes. After cooling, the absorbance was measured at 660nm using a UV Spectrometer (using triple-distilled water as a blank)[29].

2.3.3.7 Formula for Calculating the Percentage of Inhibition

The percentage of inhibition of protein denaturation was calculated using the formula below:

Percentage Inhibition = (Absorbance of control - Absorbance of sample) x 100 / Absorbance of control

2.4 Homology Modelling Using Swiss Model

Homology modelling is a computational method of calculating the three-dimensional (3D) structure of proteins based on known structures of homologous proteins (Figure 3). This method is extremely important in several fields, particularly in drug design and vaccine development, due to its ability to rapidly and efficiently generate accurate models. The following sections will highlight the methodology and implications of homology modelling.

2.4.1 Homology Modelling Methodology

Template selection: This begins with the identification of a homologous protein with a known structure that will serve as a template for the modelling[30]. Alignment and modelling: Modeller and I-TASSER align the target sequence with the template, upon which a 3D model is generated from the alignment[31]. Validation: The models so derived get validated through methods such as Ramachandran plots (Figure 4) to verify the structural fidelity[32].

2.4.2 Impact of Homology Modelling

Accelerating drug discovery: Homology modelling cuts further time and resources out of drug development by supplying crucial structural information at the outset of the process[33].

2.4.1 Swiss-Model

Swiss-Model, the very first automated server for homology modelling, has always grown and continues to grow in order to promote homology modelling for users lacking any specialised computational expertise. Now, the server can model both homo- and heteromeric complexes and infer from amino acid sequence information stoichiometry and overall structure. Recently, the modelling Swiss-Model saw enhancements with the introductory of the ProMod3 modelling engine alongside QMEANDisCo for the local model quality estimation. It has been heavily employed in the sphere of scientific computing, even though homology modelling tools were comparatively studied on a receptor protein lying within the human genome. These tools help bridge the gap between experimentally determined structures and the known sequences of proteins[33]. This process includes retrieving protein sequences from resources such as UniProt or NCBI, aligning the sequences, and modelling based on the use of a template structure. The quality of models produced, however, can be assessed in several ways. One such manner is to check Ramachandran's plots showing the distribution of amino acid residues along core and disallowed domains. Good quality models are said to have more than 70% of the residues in the core regions, whereas some studies have noted figures of about 97-98%. The reliability of the models can also be confirmed by other statistics, such as MolProbity scores and QMEAN values[34,35].

2.5 Molecular Docking using AutoDock Vina

Molecular docking, an essential methodology for drug discovery, predicts ligand-protein interactions. AutoDock Vina, combined with UCSF Chimera, is among the highest-ranked very fast and accurate docking software available. Molecular docking

utilising AutoDock Vina has become a common practice in drug discovery for predicting ligand-receptor interactions. Steps involved include target protein and ligand preparation, docking simulation, and analysis of binding affinities and interaction patterns[36].

2.5.1 Preparation of Target and Ligand

Target protein: The protein structure is obtained from a database such as the Protein Data Bank (PDB), and the structure is prepared by removing water molecules and adding hydrogen atoms.

2.5.2 Protein Selection

Having been considered to have an anti-inflammatory action, the target protein 1XJD (Figure 5) was selected for a molecular docking study. The structure of the protein was retrieved from the RCSB Protein Data Bank (RCSB PDB). It is said to be of the Transferase class and of Homo sapiens origin, and is expressed by *Escherichia coli* BL21(DE3). Specific mutations are present in the protein, which has been solved by X-ray diffraction to a resolution of 2.00 Å. Other quality stats are R-value free of 0.216, R-value work of 0.201, and an R-value observed of 0.201. As said by the depositor, this factor strongly validates that the structure is immensely suitable for *in silico* interaction study involving selected phytocompounds.

Ligand preparation: In general, ligands are optimised for geometry and converted to a docking-docked format, and PyRx is one such software used[37].

2.5.3 Docking Setup

Docking simulation: Docking is conducted by AutoDock Vina through predicting the binding conformations of the ligands (known ligand - Aspirin) (Figure 6 -9) to the target protein while calculating binding energies (ΔG) concerning the strength of the binding interactions[38].

2.5.4 Running AutoDock Vina Program

AutoDock Vina is an application meant to be run *via* the command line on Windows. The receptor and ligand files should be browsed in PDBQT format. These locations are then fed into the vina.exe, followed by the name of the design document[39]

Validation: Docking results of unknown ligands (*e.g.*, Caryophyllene oxide) (Figure 10-12) are compared to those of known ligands to give reliable results[40].

2.5.5 Analysis of Results

Binding affinity: The binding energy values tell how stable the ligand-receptor complex is, with lower binding energy values implying stronger interactions (Table 6)[41]. Interaction patterns: This basically tells us the nature of binding interactions, something like hydrogen bonds or hydrophobic contacts, inferred with a deeper meaning that is important for the understanding of the binding mechanism[42].

Recent improvements in Vina algorithms have seen hybrid parallelisation methods being developed and the integration of machine-learning scoring functions, thereby enhancing its capabilities. AutoDock Vina consequently serves as an attractive platform for drug development by virtue of its performance in the design of novel antimicrobial peptides[43]. The highly intuitive environment attracted more and more researchers who do not have much bioinformatics background to use it; simultaneously, the diffusion of the software in the research mentioned above has been fostered[44]. These upgrades ultimately underscore the eminent role of AutoDock Vina in contemporary computational drug discovery and development.

3. Results

3.1 Preliminary Phytoconstituent Screening

The ethanolic extract of *H. auriculata* showed the presence of various classes of secondary metabolites, which are usually linked to pharmacological activities.

3.2 Gas Chromatography-Mass Spectrometry (GC-MS)

In the GC-MS chromatogram of the ethanol extract of *H. auriculata*, peaks were marked with retention time. Their Retention Time (RT) and amount of their presence, as indicated by listed in Table 1. The qualitative analysis of the extract showed the presence of 43 compounds in the ethanolic extract, out of which 12 phytoconstituents having the largest peak height and peak area were subjected to further studies *i.e.* molecular docking.

3.3 *In Vitro* Analysis of Anti-inflammatory Activity

Table 1. Preliminary phytochemical screening of the ethanolic extract of *H. auriculata*

Phytochemical Parameter	Test Method	Observation	Result
Alkaloids	Mayer's / Dragendorff's	Cream/orange precipitate	Present (+)
Flavonoids	Shinoda test	Pink/red colour	Present (+)
Phenolics / Tannins	Ferric chloride test	Blue-black colour	Present (+)
Saponins	Foam test	Stable persistent foam	Present (+)
Steroids	Liebermann-Burchard test	Green colour	Present (+)
Terpenoids	Salkowski test	Reddish-brown colour	Present (+)
Total Phenolic Content (TPC)	Folin-Ciocalteu method	High concentration of phenolic compounds	Present (+)

3.4 Homology Modelling Analysis

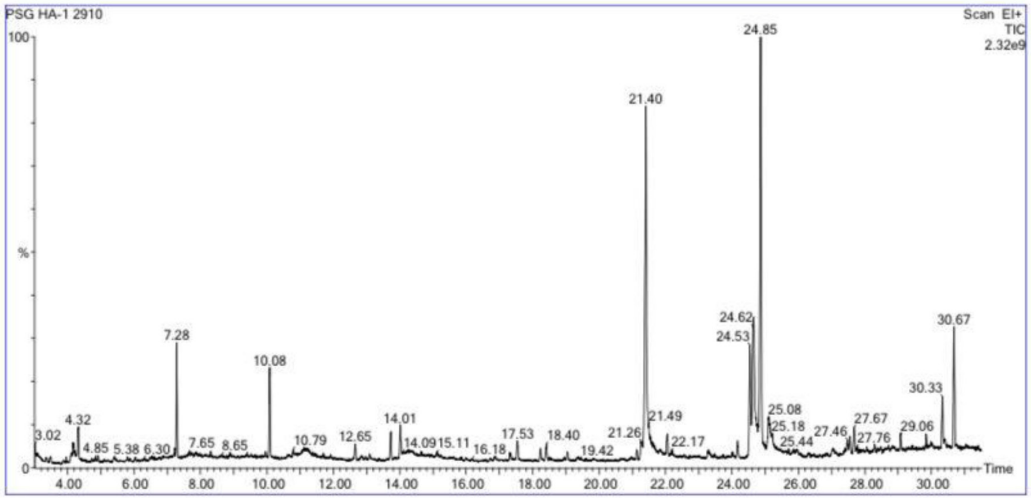


Figure 2. GC-MS chromatogram of the ethanolic extract of *H. auriculata* showing the detected phytoconstituents.

Table 2. Phytochemical constituents selected from the ethanolic extract of *H. auriculata* for biological and docking evaluation

S. No.	Phyto-constituents	RT	Height	Peak Area	Pharmacological Activity
1	2,5-Dimethylphenylacetic Acid	4.164	110,427,448	7,355,904.5	Anti-inflammatory activity
2	Azetonitrile	4.324	191,841,072	10,056,026	Not well-defined activity
3	Octanenitrile	4.324	191,841,072	10,056,026	Limited direct activity
4	Cyclohexane (1-methylpropyl)	4.324	191,841,072	10,056,026	No intrinsic activity
5	Hexanenitrile, 5-methyl	7.280	625,797,568	16,757,030.0	No specific activity
6	6-Bromohexanoic Acid	10.081	489,114,208	14,651,948	Anti-bacterial and anti-fungal properties
7	Ethanol 2,2-diethoxy	17.534	107,134,424	4,921,519.0	Specific activity depends on derivatisation
8	Oxirane, octyl-	21.401	1,896,691,712	118,009,320.0	Serves as precursor
9	6-Tetra Decanone	21.401	1,896,691,712	118,009,320.0	Anti-inflammatory, anti-fungal, anti-microbial properties
10	Caryophyllene oxide	24.852	2,160,505,600	101,201,656.0	Anti-cancer, anti-inflammatory, anti-fungal properties
11	Diaziquone	25.192	92,199,472	6,545,030.0	Anti-cancer activity
12	5-Formylsalicylic Acid	30.674	656,125,120	27,397,308.0	Anti-inflammatory, anti-oxidant properties

Table 3. Percentage inhibition exhibited by the ethanolic extract of *H. auriculata* in the *in-vitro* anti-inflammatory assay

S. No.	Concentration	Absorbance	% Inhibition
1	50	0.453	11
2	100	0.414	18.66
3	200	0.396	22.20
4	400	0.355	30.25
5	600	0.332	34.77
6	800	0.214	57.9
7	1000	0.196	61.4

Table 5. Comparative percentage inhibition obtained for the combined extract treatment, demonstrating synergistic enhancement of biological activity

S. No.	Concentration	Absorbance	% Inhibition
1	50	0.371	27.11
2	100	0.315	38.11
3	200	0.295	42.04
4	400	0.193	62.08
5	600	0.092	81.92
6	800	0.08	84.28
7	1000	0.055	89.19

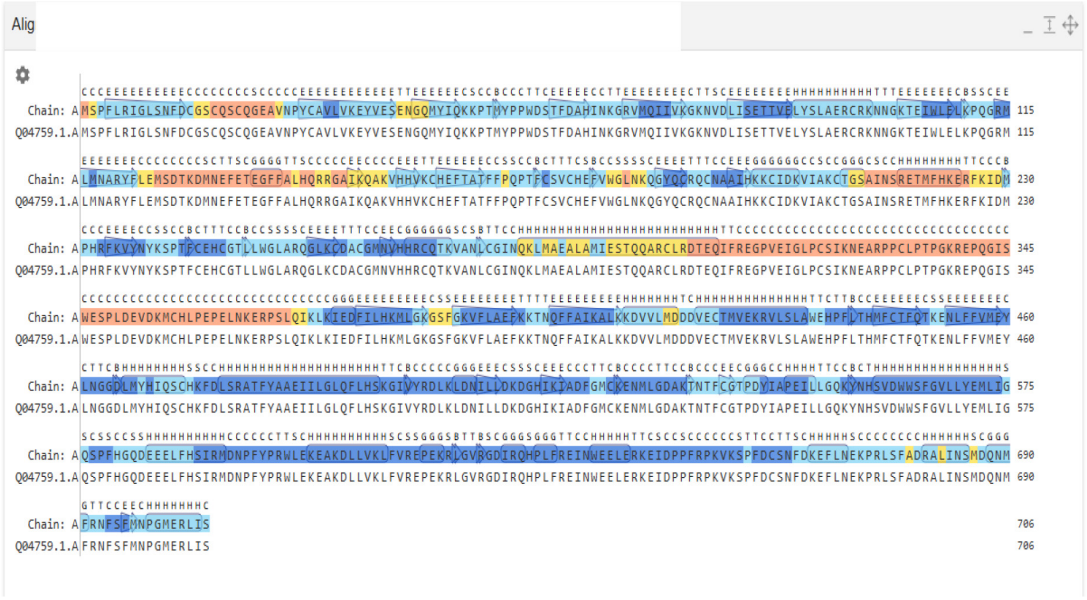


Figure 3. Homology-based multiple sequence alignment of the target protein and its closest structural homologues, highlighting conserved residues.

Table 4. Percentage inhibition exhibited by the extract of *B. serrata* in the *in-vitro* anti-inflammatory assay

S. No.	Concentration	Absorbance	% Inhibition
1	50	0.450	11.59
2	100	0.429	15.71
3	200	0.417	18.07
4	400	0.363	28.68
5	600	0.251	50.62
6	800	0.186	63.40
7	1000	0.154	69.74

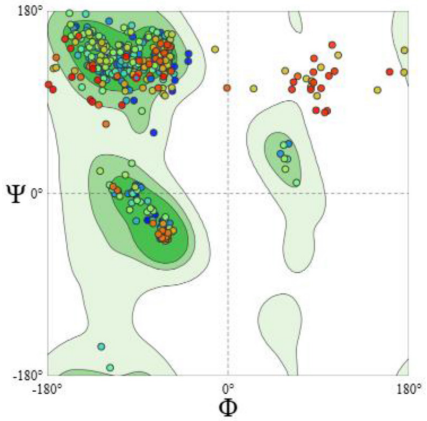


Figure 4. Ramachandran plot of the predicted protein structure showing the distribution of backbone dihedral angles (φ and ψ) to assess stereochemical quality.

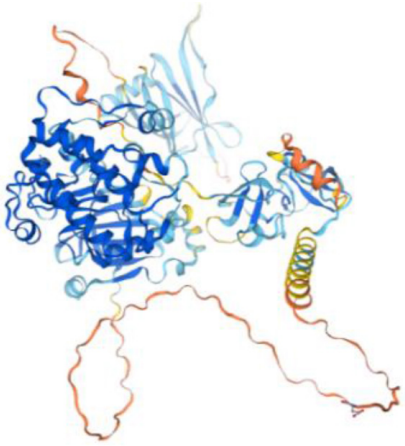


Figure 5. Predicted 3D structural model of the target protein showing secondary-structure organisation.

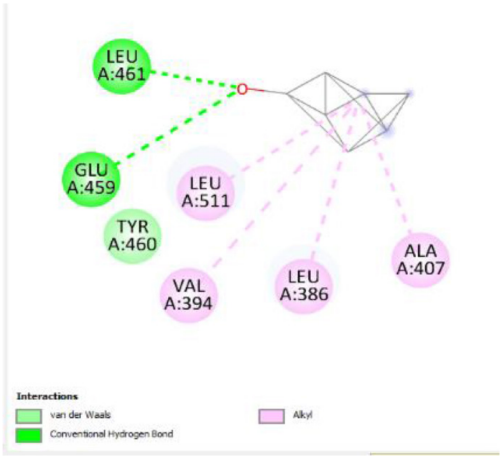


Figure 6. 2D interaction map illustrating the binding of aspirin to amino-acid residues in the target. .protein.

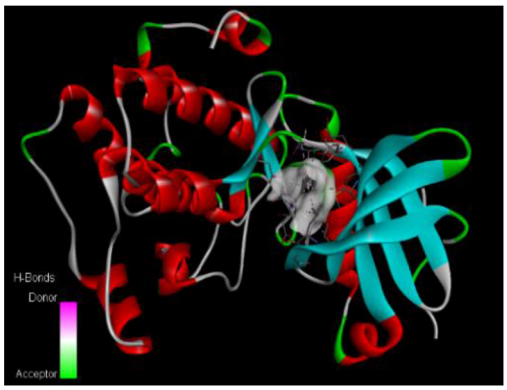


Figure 7. 3D molecular docking visualisation of aspirin bound to the target protein, highlighting hydrogen-bond donor and acceptor interactions within the binding pocket.



Figure 8. Hydrogen-bond interaction profile of aspirin docked with the target protein.

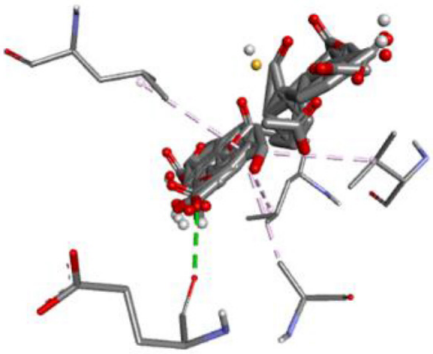


Figure 9. Interaction profile of aspirin within the binding pocket of the target protein, showing key hydrogen-bond contacts.

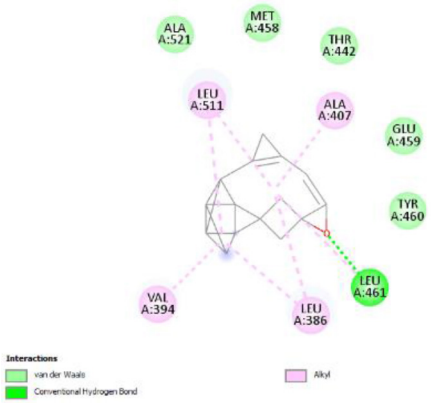


Figure 10. 2D interaction map illustrating the binding of caryophyllene to amino-acid residues in the target protein.

3.5 Molecular Docking Analysis

Docking score analysis for the Standard (Aspirin) and Sample (*H. auriculata*).

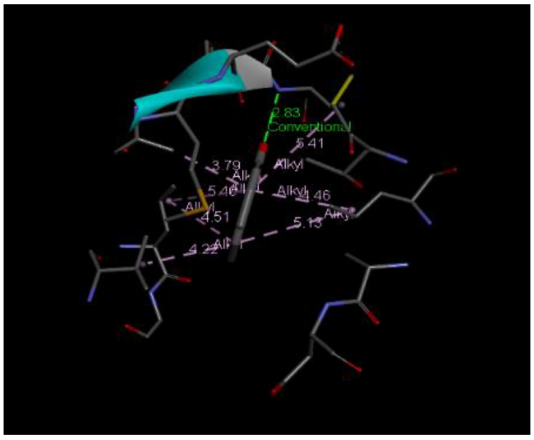


Figure 11. 2D interaction map of Caryophyllene with the target receptor.

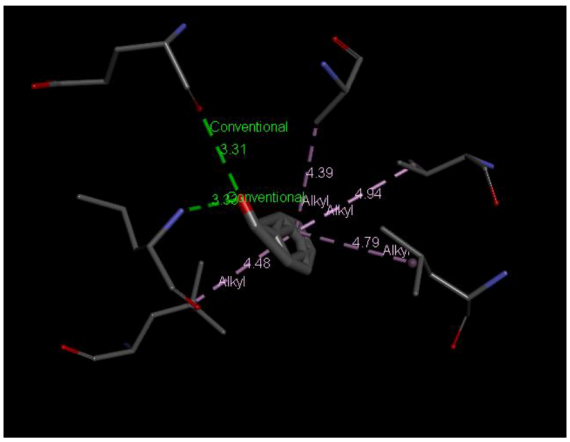


Figure 12. Molecular interaction of Caryophyllene oxide with the target receptor.

Table 6. Molecular docking interactions of key phytochemicals from the ethanolic extract of *H. auriculata* with the selected rheumatoid arthritis target protein

Mol. No.	Target Ligands Binds with Protein – (1xjd) Anti inflammatory Activity	Docking Score	Amino Acid Docking Site Center Interactions (x, y, z)	(x, y, z)	
Control Aspirin					
1	Aspirin	-6.4	Glu (a:459), Leu (a:461), Val (a:394), Leu (a:511), Leu (a:386), Ala (407)	57.77,10.3,3.11	-7.5087, -12.887, 0.5650
***H. auriculata* Samples**					
1	Caryophyllene oxide	-9.8	Ala (a:401), Leu (a:511), Val (a:394), Leu (a:386)	57.7,10.3,2.60	-7.5087, -12.887, 0.5650
2	Diaziquone	-8.7	Leu (a:547)	55.7,8.58,3.06	-7.5087, -12.887, 0.5650
3	5- Formylsalicylic acid	-6.5	Lev (a:461)	57.0,10.6,2.434	-7.5087, -12.887, 0.5650
4	2,5-Dimethyl phenylacetic acid	-6.4	Val (a:394), Lys (a:409), Ala (a:521), Met (a:458), Asp (a:522)	52.2,9.702,0.75	-7.5087, -12.887, 0.5650
5	6-Tetra decanone	-5.9	Zeu (a:511), Ala (a:407), Leu (a:386), Val (a:396)	56.24,9.339,2.027	-7.5087, -12.887, 0.5650
6	Cyclo hexane (1- methyl propyl)	-5.9	Leu (a:511), Leu (a:461), Ala (a:407), Leu (a:386)	58.2,10.432,3.437	-7.5087, -12.887, 0.5650
7	Azetonitrile	-5.7	Leu (a:461), Leu (a:511), Leu (a:386), Ala (a:407)	58.58,10.971,3.23	-7.5087, -12.887, 0.5650
8	Oxirane, octyl	-5.5	Leu (a:511), Met (a:308)	54.5,9.796,1.254	-7.5087, -12.887, 0.5650
9	Octanitrile	-4.9	Lys (a:4090), Vac (a:394), Ala (a:521), Gly (a:389), Met (a:458)	51.8,8.80,0.102	-7.5087, -12.887, 0.5650
10	Hexanenitrile 5- methyl	-4.8	Leu (a:386), Val (a:397), Met (a:458), Leu (a:511), Ala (a:407)	56.3,9.62,1.99	-7.5087, -12.887, 0.5650
11	6-Bromo hexanoic acid	-4.5	Arg (a:503), Tpo (a:538), Val (a:501), Glu (a:528), Cys (a;526)	53.096,9.975,0.678	-7.5087, -12.887, 0.5650
12	Ethanol 2,2 - diethoxy	-3.8	Ala (a:521), Asp (a:522), Met (a:458), Lys (a:409), Val (a:394)	52.3,9.62,0.70	-7.5087, -12.887, 0.5650

4. Discussion

In vitro anti-inflammatory analyses of extracts derived from *H. auriculata* and *B. serrata* showed a dose-dependent inhibition of protein denaturation; *H. auriculata* had 61.4% and *B. serrata* had 69.74% inhibition at 1000 µg/mL. The synergy showed supra-additive inhibition (89.19% at 1000 µg/mL), implying complementarity with respect to phytochemical composition and mechanistic action. Green extraction of *H. auriculata* was carried out for environmental sustainability and maximum recovery of phytocompounds. GC-MS studies reported bioactives: Phytol, hexadecanoic acid, and caryophyllene oxide, all with anti-inflammatory and antioxidant properties. Furthermore, molecular docking studies (AutoDock Vina) corroborated strong binding affinities of phytol and caryophyllene oxide with inflammation targets COX-2 and TNF-α. Swiss-Model homology modelling was used aiming at an accurate protein structure for docking. This integrated study, comprising *in vitro* assay, green extraction, phytochemical profiling, and *in silico* docking, stands for *H. auriculata* as a promising candidate for anti-inflammatory drug discovery and supports the synergism.

5. Conclusion

Evidence of the synergistic anti-inflammatory effect from *B. serrata* in combination with *H. auriculata* against rheumatoid arthritis has emerged from protein denaturation inhibition activity studies, the presence of various bioactive constituents as revealed by GC-MS analysis, and strong ligand-protein interactions as depicted by molecular docking. By combining green extraction methods with *in vitro-in silico* studies, this study not only validates the traditional claims of these plants but also brings to light their considerable potential as sustainable plant-based therapeutics. These results underscore the significance of phytochemical synergy in drug discovery and open up possibilities for further preclinical and clinical investigations toward the development of safe, effective, and eco-friendly anti-arthritic formulations.

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Author Contribution

Saranya Devi C. S. contributed to the research design, performed the Swiss ADME analysis, and was primarily responsible for developing and writing the overall manuscript. Anudeepa J. and Dr. Sangeetha M. provided continuous scientific guidance, supervision, and critical review of the research work. Mohanram S. and Harikrishnan S. contributed to the experimental research work, particularly the *in-vitro* assessments, and assisted in formatting and organising the manuscript. All authors have read and agreed to the published version of the manuscript.