

Identification and molecular docking analysis of active ingredients with medicinal properties from edible *Baccaurea sapida*

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Abstract:

Underutilized plant species has started changing the conception of plants by expanding the use well beyond from foods and fibers to rich source of medicinally important secondary metabolites. Bioactive compounds from natural sources are gaining importance as potential drug candidates towards many inflammatory conditions like Rheumatoid Arthritis (RA). The focus of the present study has been centred to reveal the anti-inflammatory potential of an underutilized fruits of *B. sapida*. Further efforts towards its medicinal significance may provide relieve from symptoms of RA by reducing the side effects that are observed in available medications. Total 10 compounds in fruit crude methanol extract were identified and quantified by LC-MS/MS analysis followed by the agar well diffusion method for their anti microbial activity. Among all studied micro organism *S. aureus* was found to surmount the inflammation in RA through domain B of surface protein A (Staphylococcal surface protein A). Identified compounds (having anti-inflammatory properties) were scrutinized for their toxicity and quantitative structure-activity relationship (QSAR) using laser toxicity and Molinspiration servers respectively. Further, docking studies have been carried out between domain B and studied compounds using AutoDock. Out of 6 anti-inflammtory compounds, quercetin has been identified as the most potent compound in reference to its inhibitory constant (47.01) and binding energy (-5.90 kcal/mol) to bacterial protein. Our data suggest that methanol extract of *B. sapida* fruit posses medicinally significant anti-inflammatory compounds and thus justifies the use of this fruit as folklore medicine for preventing inflammation related diseases.

Keywords: Phytochemicals, Antimicrobial, Anti-inflammatory, LC-MS/MS, Molecular Docking, QSAR.

Background:

Baccaurea sapida (*syn. Baccaurea ramiflora*), commonly known as Kusum, belongs to the Euphorbiaceae family that grows in the eastern sub-Himalayan tract. This fruit has been put into the category of underutilized fruit among 675 wild, edible fruits [1] and harvested during the months of May to July. Underutilized fruits, vegetables and grains are non-commodity crops which are quite popular among farmers but have been neglected by the users for a variety of agronomic, genetic, economic, social and cultural factors [2]. The bark, roots and wood are harvested for medicinal uses and is also used to treat arthritis, abscesses, injuries and skin diseases [3]. The pulp of *B. sapida* is

a good source of vitamin C and the fruit juice is used to treat constipation and is used to make wine and stewed [4]. Recent study has reported anti-inflammatory action of *B. ramiflorai* leaf through rosmarinic acid that inhibits prostaglandin biosynthesis [5]. A significant increase in the production of tropical exotic fruit has been observed in market due to the presence of bioactive compounds and attractive sensory properties [6]. Therefore, lighting up the medicinal properties of such underutilized fruits can be a good attempt to enhance the utilization and for health benefits in inflammatory conditions such as RA. Basically, RA is a heterogenic, auto-immune disease of joints which ultimately leads to the loss of function

[7]. Available treatments for RA are disease modifying anti rheumatic drugs (DMARDs) and non-steroidal anti inflammatory drugs (NSAIDs) but these drugs only control the symptoms such as pain and stiffness [8]. In spite of this, these drugs have some side effects such as gastrointestinal problems, elevated risk of damage to the retina of the eyes [9], diarrhea [10]. Therefore, investigation continues for alternative products

and natural phytochemicals from plants origin used as traditional medicines. Studies have reported that consumption of fruits and vegetables is related with a decreased risk of cancer, stroke, cardiovascular disease, and various factors associated with ageing [11-15].

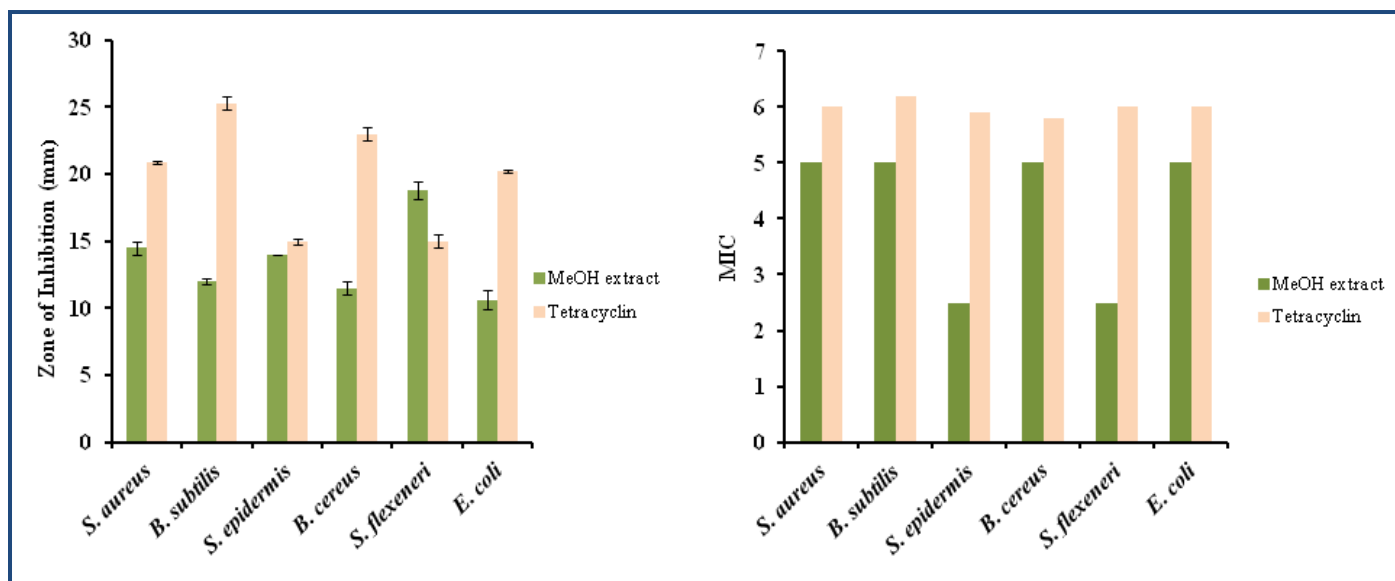


Figure 1: Antimicrobial activity of *B. sapida* fruit MeOH extract & standard tetracycline (Lupsi MeOH extract MIC is in mg/ml and of tetracycline $\mu\text{g/ml}$)

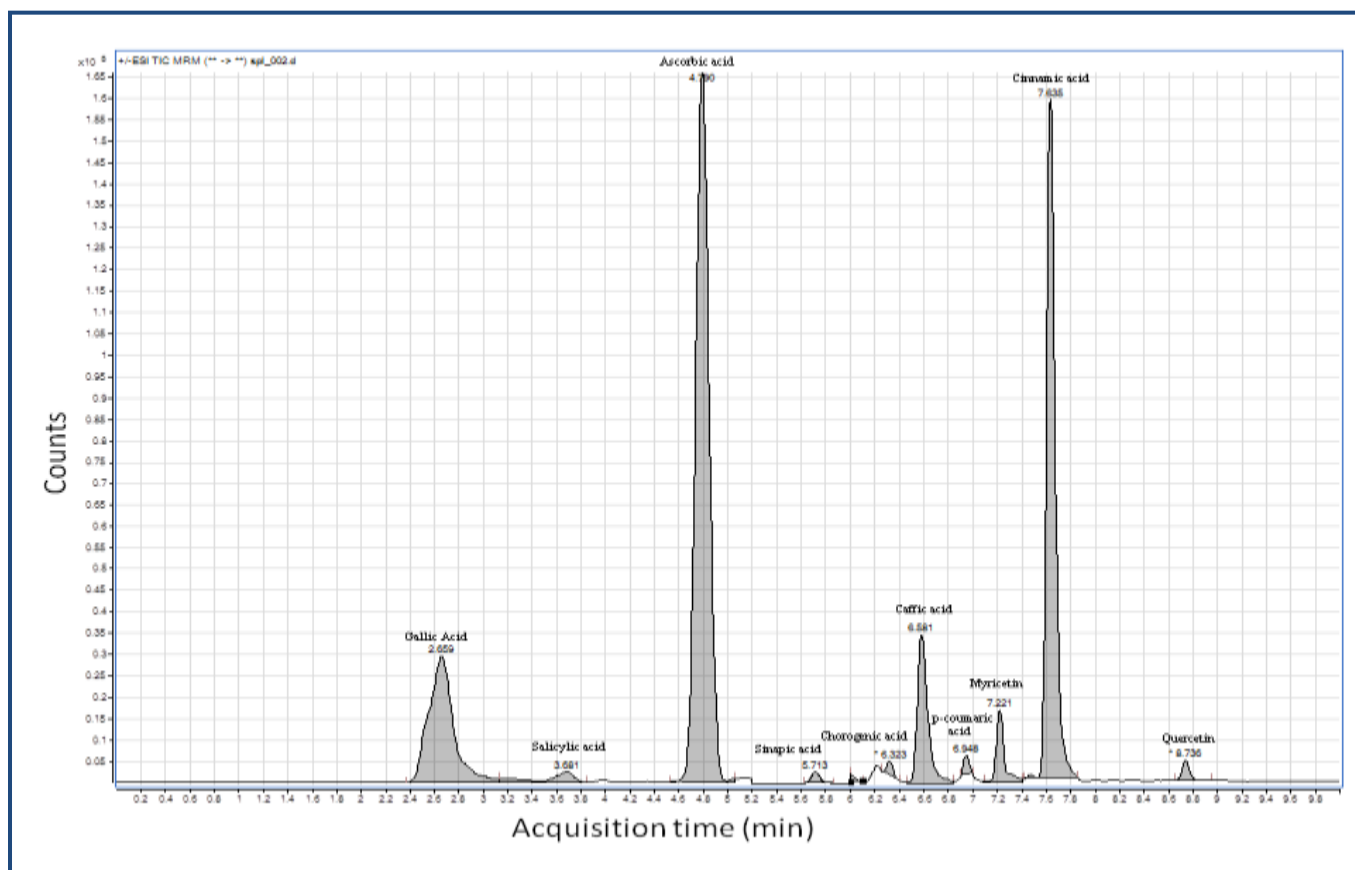


Figure 2: LC-MS/MS chromatogram of *B. sapida* (MeOH extract). Each peak represent a compound identified based on retention time against 11 run standards. 10 compounds have shown their presence. Ferulic acid was not found in the extract.

Thus, in the present communication we have characterized medicinal importance, phytochemical constituents and studied antimicrobial potential of *B. sapida* fruits. During the characterization, we have quantified and identified 10 compounds having antioxidant and anti-inflammatory properties. Salicylic acid, Sinapic acid, Quercetin, Myricetin, p-coumaric acid and Chlorogenic acid are selected for further study on the basis of their anti-inflammatory properties. Bacteria (against which anti microbial activity was observed) were analysed for the presence of surface protein having role in pathogenesis of RA. Among all studied bacteria *S. aureus* was found as potent instigator of RA via its domain B of surface protein A. Surface proteins of bacteria are found to be associated with pathogenesis of various diseases including RA [16]. Staphylococcal protein A of *Staphylococcus aureus* played an important role in the progression of RA [17-20]. This protein helps *S. aureus* in survival and virulence by adhering it to host cell that ultimately leads to various arthritic symptoms [21]. Inhibition of protein A function will inhibit bacterial host interaction, hence may suppress the symptoms of the disease. Therefore, analysed six compounds were docked against the protein A to check their potentiality to inhibit this protein.

However, there is no scientific justification of traditional use of *B. sapida* in the treatment of RA. Hence, present work was under taken to evaluate the anti-inflammatory activity of *B. sapida*.

Methodology:

Plant materials

The fruits of *B. sapida* were collected from local market of Kalimpong, West Bengal, India during May-July and authenticated at by Dr. H.B. Singh at National Institute of Science Communication and Information Resources, Council of Scientific and Industrial Research, Delhi, India.

Extraction

Fresh fruit sample (100g) was crushed partially and solvent extracted with Methanol (MeOH). The filtrate were filtered through Whatman filter paper and evaporated at low temperature. After evaporation of organic solvents, the extract yields were calculated and the extract was stored at 4 °C till further analyses.

Quantification of polyphenols by LC-MS/MS

The polyphenols were analyzed by the chromatographic system consisting of an Agilent 1100 series HPLC instrument equipped with 6460 triple quad MS detector [22]. Analytical separations of the extracts were carried out on a C18 column (4.6 mm×100 mm×5 μm, Agilent Technology) at a flow rate of 0.8 /min, with a two solvent mobile phase (eluent A=10 mM ammonium acetate and 1 % acetic acid in water; eluent B= 1 % acetic acid in methanol). The gradient elution was carried out as follows: 0-3 min, 15-50 % A; 3-5.5 min, 50-90 % A; 5.5-9 min, 90 % A; 9-9.5 min, 90-15 % A; 9.5-10 min, 15 % A. The sample injection volume was 20 μL. The fragmentation was done in ESI-MS/MS (Agilent Jet stream) in negative ionization mode. Data was acquired and quantified by Agilent triple quad LC-MS Mass Hunter work station based on external standardization by employing calibration curves in the range of 1-50 ng/ based on the peak area calculated from selected ion

chromatograms of the corresponding [M-H]⁻ ion. Results were expressed in μg per 100 mg extract.

Antimicrobial assay

Antimicrobial activities of the extracts were tested by agar well diffusion method [23] against Gram-positive bacteria and Gram-negative bacteria (Figure 1). Minimum inhibitory concentrations (MICs) were determined through a standard two-fold micro-dilution technique. Tests were performed in sterile flat-bottom 96-well microplates (Difco Laboratories, Detroit, USA) by maintaining a constant volume (200μL/tube) for serial dilutions of extracts. The MICs were expressed in mg/ml extract and were defined as the lowest extract concentration for which the optical density of a well was null. Tetracycline was used as positive control.

Docking studies

Preparing target molecule

To explore the interaction of identified bioactive compounds, NMR spectroscopic structure of domain B of Staphylococcal protein A (PDB ID: 1BDC) was obtained from the Protein Data Bank (<http://www.rcsb.org/pdb>) and energy was minimized using Swiss PDB viewer (SPDBV).

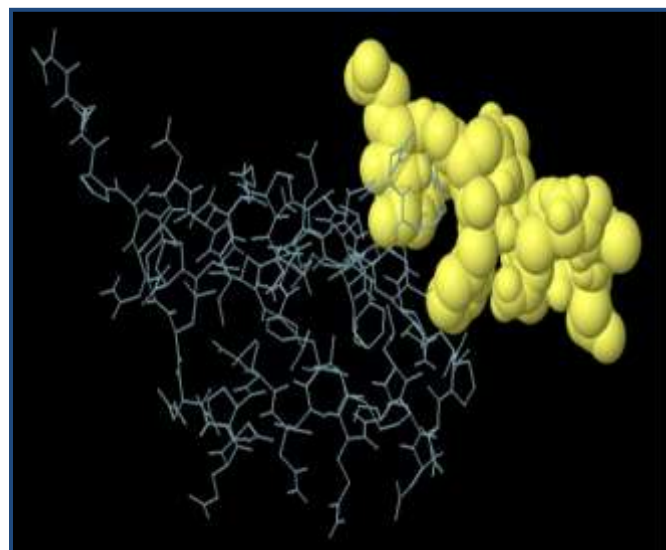


Figure 3: Largest epitope (represented in yellow colour) present in the domain B of protein

Ligand characterization

Total six bioactive compounds namely quercetin, myricetin, salicylic acid, sinapic acid, chlorogenic acid and p-coumaric acid have been identified in the present studies for their anti-inflammatory properties. These compounds were downloaded from PUBCHEM (<https://pubchem.ncbi.nlm.nih.gov/>). PADEL web server was used to reveal the 2D descriptors describing the pharmacophore of the obtained ligands. The Chemistry Development Kit is utilized to calculate these descriptors and fingerprints [24]. Lethal toxicity of these compounds was scrutinized by Lazar toxicity prediction server (<http://lazar.in-silico.de/predict>) on both cell based models and animal models involving Rat, Mouse and Hamster [25]. Molinspiration web server (<http://www.molinspiration.com/cgi-bin/properties>) builds local QSAR (quantitative structure-activity relationship) models for every ligand to be predicted.

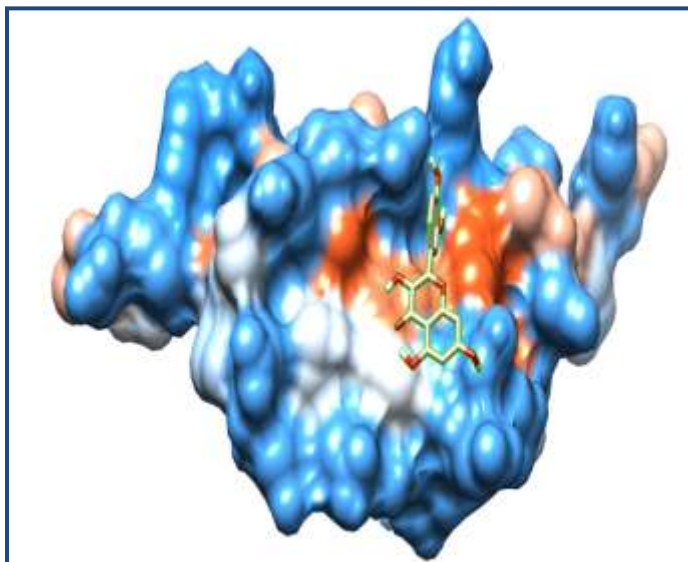


Figure 4: Docked view of Quercetin with domain B of protein. A Quercetin docked with protein A with minimum binding energy and inhibitory constant indicating a potent inhibitor of this protein.

Molecular Docking

Grid and docking parameter files were prepared using ADT and molecular docking was performed with AutoDock 4.2.1. In order to analyze the effect of ligand association, all the water molecules and the hetero atoms have been removed from the target protein. All the hydrogen atoms were added to the protein as it is required for the electrostatics and then non polar hydrogen atoms were merged together. Gasteiger charges were assigned to ligands and save them into PDBQT file format. 3D grid box comprising the interacting motif was constructed with 122x82x70 Å dimension and 0.386 Å spacing. Blind docking has been carried out fifty times to predict the highly interacting residues of protein in 10 different poses per run. Best binding pose was determined by the consistent minimum binding energy and inhibitory constant per run through lamarkian algorithm. Ellipro server (<http://tools.immuneepitope.org/ellipro/>) was utilized for the surface epitopes prediction.

Results & discussion:

Polyphenols by LC-MS/MS

The concentration of identified polyphenols, phenolic acids, flavonoids and flavonols was quantified by LC-MS/MS by using external calibration method **Table 1** (see supplementary material), **Figure 2**. Ten compounds were identified out of 11 standards run. The results showed Cinnamic acid (3.77 µg/100 g) as a predominant compound followed by identified flavonols; Quercetin and myricetin. Quercetin is reported as an effective bronchodilator and helps to reduce the release of histamine and other allergic or inflammatory chemicals in the body [26]. It has antioxidant and anti-inflammatory effects which might help in the reduction of prostate inflammation [27]. A Finnish study correlated high myricetin consumption with lower risk of prostate cancer. Phenolic acids such as gallic acid and salicylic acid were also identified in the extract. While gallic acid is known to have anti-fungal and antiviral properties and antioxidant activity. Salicylic acid is known for its ability to ease aches and pains and lower body temperature in case of fever [28]. The obtained Chorogetic acid

is reported for its antioxidant and anti-hypertensive potential. Current lifestyle adjustment for management of cardiovascular diseases and dietary consumption of Chorogetic acids sounds promising for providing a non-pharmacological approach for prevention of high blood pressure and its treatment [29]. p-Coumaric acid identified in the fruit possesses antioxidant properties and decreases the risk of stomach cancer by reducing the formation of carcinogenic nitrosamines [30]. Ferulic acid was not identified among run standards.

Antimicrobial activity

The antibacterial activities of MeOH extract of *B. sapida* is summarized in **Figure 1**. The extract (MeOH) show appreciable antibacterial activity against gram positive and gram negative bacteria. The MIC of extract against all tested bacteria ranged between 2.5–5 mg ml⁻¹. The zone of inhibition for *Shigella flexeneri* was obtained as 18.8±0.7 mm, which is higher among all tested bacteria. In our previous study [31], GC-MS results (MeOH extract) revealed the presence of most common fatty acids and other phytochemicals which are essentially used to regulate various functions like immune response, blood pressure, lipid levels and inflammation response to injury [32]. The unsaturated fatty acids - palmitic acid, oleic acid and lauric acid in *Baccaurea sapida*, exhibit antimicrobial properties [33–34]. 2-Furan carboxylic acid has been reported for its use as a preservative and also acts as a bactericide and fungicide. LC-MS/MS screening showed the presence Sinapic and cinnamic acid which may be contributing to these antimicrobial properties [35].

Docking results

Three dimensional (3D) structure of recombinant B domain of Staphylococcal protein A (PDB ID: 1BDC) was obtained from Protein Data Bank (<http://www.rcsb.org/pdb>). This protein played influential role in *S. aureus* during the pathogenesis of human infections and attachment to the cell wall envelope [36]. Protein A is considered to be the ligand of immunoglobulin and it is distributed over the surface of the bacterium [37]. Sortase enzyme is resolved by peptidoglycan substrate during the cell wall anchoring that result into amide bond formation between surface protein and bacterial cell wall envelope [38]. Recombinant B domain of Staphylococcal protein A have the function to bind with Fc region of immuno-globulin G (IgG). In the present study, identified ligands were analysed for their toxicity on the basis of experimentally determined carcinogenicity of the compounds and compares them based on the 2D descriptor, stereo chemistry and pharmacophores. The lazer toxicity analysis server revealed that these biologically active anti-inflammatory compounds may not be carcinogenic to the animal models such as hamster and mouse. The QSAR study of the ligands is detailed in **Table 2** (see supplementary material).

Protein ligand interaction study has been performed through AutoDock4 (**Figure 3**). Inhibitory constants and binding energies of these ligands are summarized in **Table 3** (see supplementary material). Docking studies indicated that quercetin and myricetin may be possible inhibitors of protein A but comparative analysis implicates quercetin as the most potent compound. It has minimum binding energy as well as inhibitory constant in comparison to myricetin and other

compounds. Also the QSAR analysis of quercetin revealed that the molecular weight and total surface polarity is considerable enough to prevent passage of ligand through the membrane barrier. This non-permeable ligand will only interfere with the domain B of protein A during the chronic Staphylococcal infection but cannot traverse the membrane to inhibit the similar kind of human proteins. Surface epitope analysis revealed binding of quercetin with antigenic epitope (TADNKFNKEQ) which may hamper its interactions with IgG (**Figure 4**). Further, it may inhibit the immune interaction by blocking the large surface epitope in spite of interfering the whole protein A. Reduced activity of this protein may lead the pathogenic bacteria to be less virulent and viable. The quercetin may be used to create the drug model with anti-inflammatory effect through the inhibitory effects on domain B of protein A. Thus, drug targeting this domain will directly interfere with the interaction of protein A with IgG so it may be used as a lead compound for more efficient future drug design through inhibition of Staphylococcal protein A.

Conclusions:

On the basis of present analysis, the fruit has been found to be a rich source of bioactive compounds and offers opportunities to develop value added products, food applications and can be used for relieving the symptoms of inflammation as in RA. Comparative docking analysis revealed quercetin as most potent anti-inflammatory compound with minimum binding energy and inhibitory constant along with no toxicity and carcinogenicity. Quercetin can inhibit bacterial surface protein Staphylococcal protein A by interfering with its surface antigenic epitope. Other than gaining higher commercial value for local underutilized fruits, the data seems beneficial to promote this locally distributed fruit and encourages its growth at a larger scale. Our study justifies the use of this fruit as folk medicine for preventing inflammation and associated disorders.

Competing interest:

The authors declare that they have no competing interests.

Authors' contributions:

SM collected the samples and prepared plant extract and carried out LC-MS/MS, antimicrobial activity. AS contributed in docking studies. SB & RKG participated in the design of the study, manuscript preparation and all authors approve the final manuscript.

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Supplementary material:

Table 1: Compounds identified via LC-MS-MS of methanolic extract of *B. sapida* fruit

Name	RT (min)	<i>B. sapida</i> (µg/100 mg extract)	MRM transition	Property*
Ascorbic acid	4.790	5.79	175/87	Antioxidant activity
Caffic acid	6.581	1.17	179/135	Antioxidant, anti- inflammatory & immunomodulatory activity
Chorogenic acid	6.323	0.45	353.3/191.3	Antioxidant activity
Cinnamic acid	7.635	3.77	147/103	Antioxidant & antimicrobial activity
Gallic acid	2.659	1.87	169/125	anti-fungal, antiviral properties & antioxidant activity
Myricetin	7.221	0.089	317/151	Anti-cancer
p-coumaric acid	6.948	0.112	163/119	Antioxidant activity
Quercetin	8.736	0.372	301/179	Bronchodilator & anti- inflammatory
Salicylic acid	3.681	0.657	137.1/93.2	Anti- inflammatory, ease pain
Sinapic acid	5.713	0.045	223-208	Antioxidant, antimicrobial, anti-inflammatory, anticancer & anti-anxiety activity

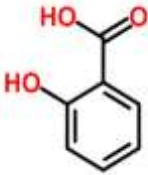
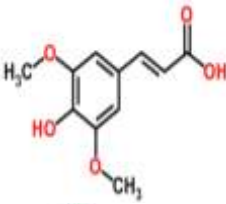
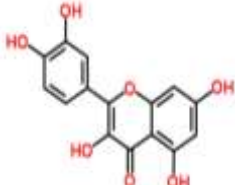
*Reference: 19-23

Table 2: QSAR analysis of identified anti-inflammatory compounds

Ligand	LogP	MW*	TPSA**	Volume
Salicylic acid	1.87	138.122	57.527	119.063
Sinapic acid	1.265	224.212	75.995	197.571
Chlorogenic acid	-0.453	354.311	164.744	296.267
Quercetin	1.683	302.238	131.351	240.048
p-coumaric acid	1.43	164.16	57.527	146.479
Myricetin	1.392	318.237	151.579	248.102

*Molecular weight; **Total polar surface area

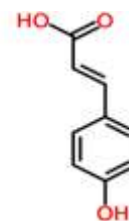
Table 3: Docking analysis of protein ligand association

Ligand	Binding Energy (kcal/mol)	Inhibitory Constant (µM)	Ligand Structure
Salicylic acid	-4.81	299.67	
Sinapic acid	-4.9	235.0	
Quercetin	-5.90	47.01	

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p-coumaric acid -4.9 257.35



Myricetin -5.67 69.92

